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A Novel Transcription Factor in Arabidopsis thaliana Abiotic Stress Response

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A Novel Transcription Factor in *Arabidopsis thaliana* Abiotic Stress Response

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Table of Abbreviations

Abbreviation	Designation
ABA	Abscisic acid
ABRC	Arabidopsis Biological Research Center
BASTA	Phosphinotricin containing media
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Tool
F2	Second stage off spring generation
GAL4BD	DNA Binding Domain of GAL4
GFP	Green Fluorescence Protein
GUS	β- glucoronidase reporter
HDAC	Histone Deacetylase
min	Minutes
PCR	Polymerase Chain Reaction
s	Seconds
SMART	Simple Modular Architecture Research Tool
TAIR	The Arabidopsis Information Resource
T-DNA	Transferred DNA of the Tumor Inducing (Ti) plasmid
35S CaMV	Promoter of 35S gene of the Cauliflower Mossaic Virus

Table of Nomenclature

Nomenclature	Designation
SLK2	Genes are written in upper case italics
SLK2	Proteins are written in upper case
slk2	Mutants are written in lower case italics

Abstract

Plants respond to environmental stress by altering their gene expression. Under stress conditions some genes are activated and some genes are repressed. Even though a lot of work has been done to understand mechanisms of gene activation under abiotic stress very little information is available on how stress responsive genes are kept repressed under normal growth conditions. Recent work has revealed that plants use transcriptional repression as common mechanism of gene repression. Transcriptional repression is achieved by recruitment co-repressor complexes to the target genes. Recent studies have revealed that the co-repressor LUH complexes with SLK1 and SLK2 to silence *Arabidopsis thaliana* stress responsive genes. However, the transcription factors involved in the recruitment of this complex to its target genes are not known. In this study, we identified SLK2INT1 (AT3G58630), as a novel transcription factor that is involved in silencing of select *Arabidopsis thaliana* stress responsive genes by recruiting the LUH-SLK2 complex.

Keywords: Activation, Repression, Co-repressors, Silencing

1. Introduction

1.1 Abiotic stress, gene expression regulation

All living organisms are susceptible to environmental stress. Plants are far less mobile compared to animals, they are unable to migrate to new favorable terrains within a single generation. Therefore, a plant's ability to perceive and to respond to these environmental stresses is very important for its survival. Plants generate their responses to abiotic stress by altering gene expression, which involves both up and down regulation of selected genes (1-3). The most common abiotic stress conditions include salt, osmotic and cold stress, which together change the expression level of about 30% of all transcribed genes of the model plant *Arabidopsis thaliana* (1). Some genes which are upregulated in abiotic stress code for transcription factors, while others are involved the in direct response (4). Some stress responses are mediated by Abscisic acid (ABA), a major plant hormone involved in dehydration response, while some responses are triggered in an ABA- independent manner (5).

In our study *Arabidopsis thaliana* is was used as a model plant because it has many favorable attributes which makes it easy to work with. These include the small size, the relatively small genome (125Mb), availability of the whole genome sequence, the short generation time (6 weeks), ease of manipulation and cultivation and the availability of wide range of knockout mutants.

1.2 Transcriptional repression as a mechanism of epigenetic gene silencing

Cis-acting elements and transcription activators involved in gene regulation during stress for both abscisic acid (ABA) dependent and independent pathways have been well studied (4, 6-8). However, mechanisms by which these stress responsive genes are negatively controlled

under normal growth conditions are not well understood. Recent work has identified transcriptional repression as a major mechanism utilized by plants in gene silencing, (9). It has been shown that gene repression plays a critical role in developmental processes of both plants and animals (9-11). Transcriptional repression can be either long-range, where the repression activity spreads even to enhancers located far away from the repressor binding site or short-range, where only activator binding sites in close proximity are affected (12).

Genes under transcriptional repression and activation are said to be epigenetically controlled, where the level of transcription depends on the accessibility of the protein coding sequence to the transcription machinery rather than the information on the DNA itself (13, 14). DNA methylation and organization of DNA into highly compact heterochromatin structure are marks of epigenetic gene silencing. For genes under epigenetic silencing, the chromatin structure is dynamically controlled by different kinds of protein complexes (9).

1.3 Gro/Tup1 super family of Co-repressors in transcriptional repression

A group of co-repressors called "Gro/Tup1 super family" plays a vital role as a part of these complexes by recruiting chromatin modifying enzymes. This family includes Groucho (Gro) from *Drosophila*, Tup1 from yeast (*Saccharomyces cerevisiae*) and transducing-like-enhancer-of-split (TLE) from humans. A common structural feature of this class of co-repressors is a highly conserved C-terminal multiple WD-40 repeat domain involved in protein-protein interactions (10, 15, 16). Except for the yeast homologs, all the other members also have an N-terminal Q-rich domain used for homo-tetramerization (fig.1). These proteins lack DNA binding domains and they interact with DNA binding repressors to be recruited to different target genes (10).

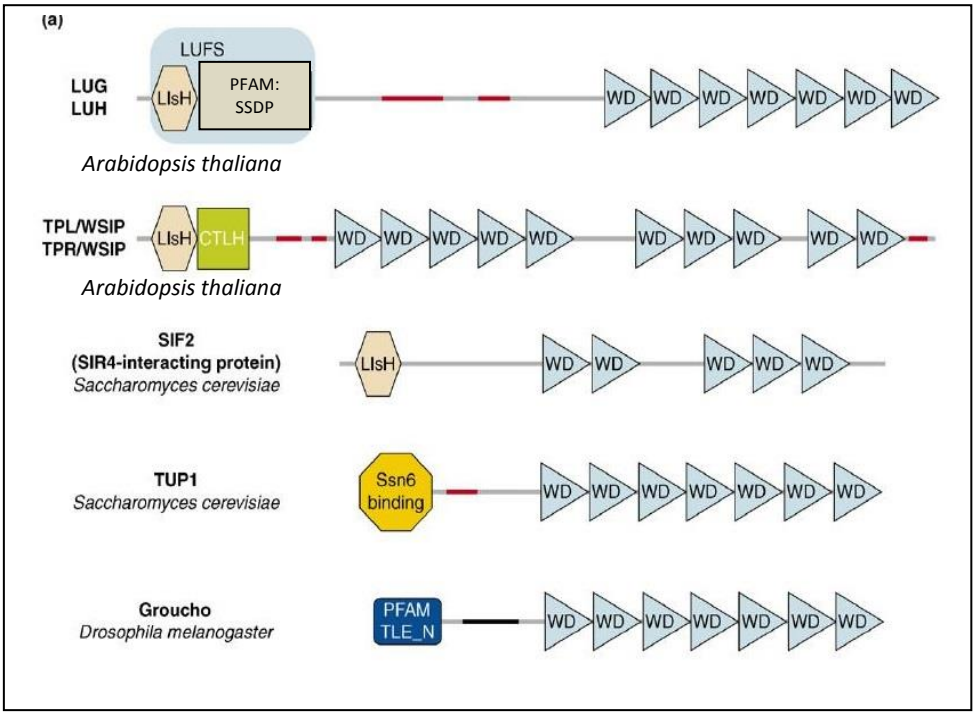


Fig. 1 Domain organization of Gro/Tup1 super family co-repressors
Comparison of domain organization between some members of the Gro/Tup1 super family of co-repressors. Glutamine (Q) rich regions are in red and proline (P) rich regions are in black (15).

Even though the yeast Tup1 does not contain a region homologous to the Q rich-domain it can still form a homo-tetramer and shows functional similarities to Groucho (10,16). Tup1 forms a complex with the adaptor protein Ssn6 (also known as Cyc8) to actively repress its set of target genes, which comprise 3% of the *Saccharomyces cerevisiae* genome (16,17). Different transcription factors bind and interact with Ssn6 to recruit the Tup1 to the promoters to mount a highly efficient repression which down regulates gene expression, sometimes more than a thousand fold. In addition to this remarkable level of repression, Tup1-Ssn6 complex is also distinguished for its ability to mitigate a wide variety of transcription activators and for the diversity of pathways in which its target genes participate (17) (fig. 2).

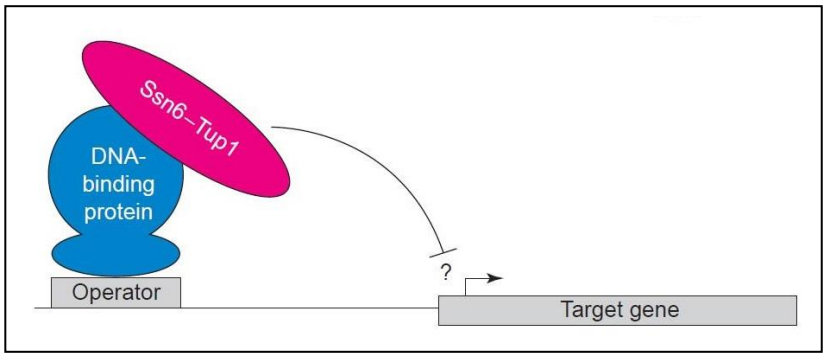


Fig. 2 Mechanism of TUP1 repression

Ssn6-Tup1 complex binds to its various targets via many different transcription factors to promote repression (17).

1.4 Gro/Tup1 family Co-repressors in *Arabidopsis*

The *Arabidopsis* genome contains 13 proteins which belong to the Gro/Tup1 family even though only few of them have been studied (15). These include LEUNIG (LUG), LEUNIG-HOMOLOG (LUH), TOPLESS (TPL), TOPLESS-RELATED (TPR) and WUSCHEL-INTERACTING PROTEINS (WSIPs). Based on the evolutionary history these homologs can be further divided to two groups, TPL/TPR/WSIP and LUG/LUH (fig. 3). In addition to the Gro/Tup1 common features, these *Arabidopsis* co-repressors also contain an N-terminal dimerization motif named LisH (lissencephaly homology) domain. Interestingly this domain together with WD repeat domains is also present in yeast SIF2p (sucrase-isomaltase foot print), an integral component of the Set3 complex (SET3C) which utilizes histone deacetylase activity for gene repression (15,18,19).

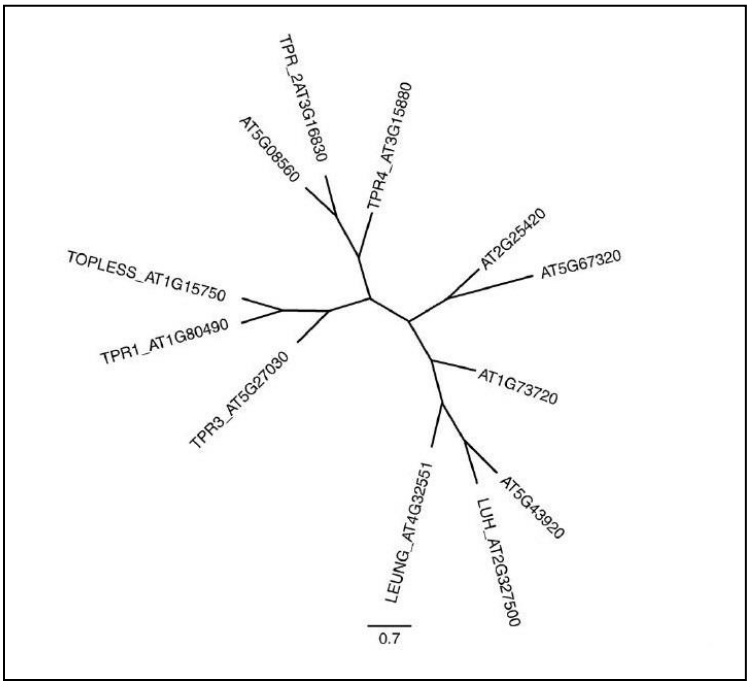


Fig. 3 Phylogenic tree showing the predicted relationship between members of the TUP1/Gro co-repressor super family (15)

1.5 LUG forms a complex with SEUSS

LUG and LUH also contains an N-terminal LUFS domain (named after LUG, LUH, Flo8 and SSDP) which is important for protein-protein interactions (15, 16). LUG binds to the adaptor protein SEUSS (SEU) via this LUFS domain. SEU contains two Q-rich domains and a conserved central dimerization domain which shows sequence similarity to the dimerization domain of LIM domain-binding (Ldb) co-regulators from *Drosophila* and mouse (20, 21). SEU does not have any repressor activity and acts as an adaptor between LUG and various transcription factors resembling the role of yeast Ssn6 in the Tup1-Ssn6 complex (20).

The role of LUG-SEU co-repressor complex in *Arabidopsis* flower development has been well-studied. The flowers are organized in to four different whorls, named 1 to 4. Inner whorls make

stamens and carpels (whorl 1 and 2) while the outer whorls transform into petals and sepals (whorl 3 and 4). This differentiation in flower organ development is mediated by *AGAMOUS* (*AG*), a floral homeotic gene only expressed in the inner two whorls (22-24). It was found that transcription factors specific to the outer two whorls recruit the LUG-SEU co-repressor complex to the second intron of *AG* to promote active repression (fig.4)(25).

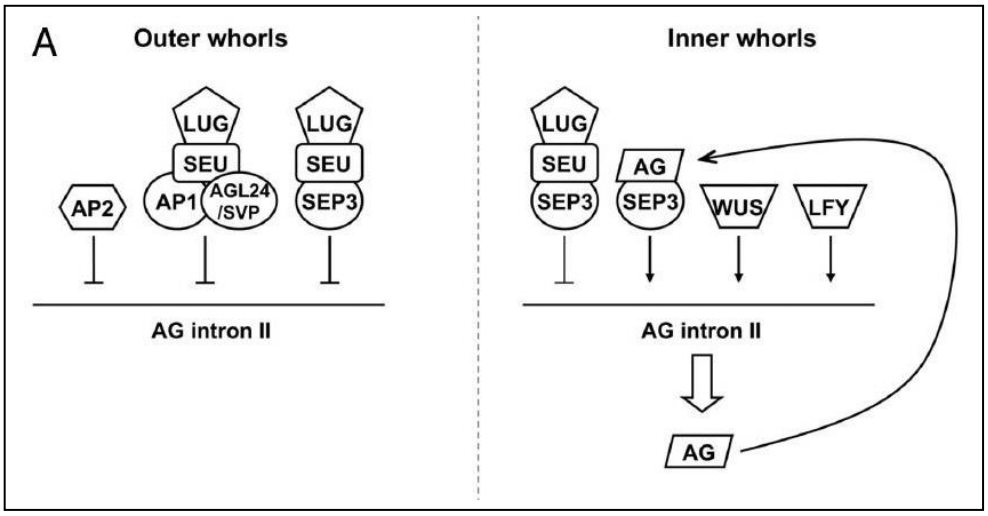


Fig.4 Mechanism of LUG mediated repression of *AGAMOUS* in of outer whorls Arabidopsis flower (16)

1.6 Repression Mechanisms of LUG

Some work has been performed to understand the mechanism of LUG repression. In a repression assay with luciferase as the reporter, Trichostatin A (TSA) an inhibitor specific for HDACs was able to alleviate LUG repression suggesting that one or more HDACs are utilized by LUG to promote gene silencing (20). Out of many HDACs present in *Arabidopsis*, the mutant of *hda19* has a similar phenotype to *lug* mutant suggesting that LUG genetically interact with HDA19 (26, 27). This assumption was further supported by the finding that LUG interacts with HDA19 in vitro (28). Interestingly, in yeast LUG activity was impaired in mutants lacking

yMED14, a component of the mediator complex (28). This finding proposed that another HDAC-independent mechanism for LUG repression, where LUG directly interacts with the transcription machinery. The same study also revealed that *Arabidopsis* CDK8, another component of the mediator complex, interacts with both LUG and SEU in yeast two hybrid assays and in-vitro repression assays.

1.7 LUH is a homolog of LUG

LUH sequence shares overall 44% similarity with LUG (29). The N-terminal LUFS domain, containing 88 amino acids, shares 80% sequence similarity between the two proteins indicating a high level of conservation (fig.5). The C-terminal seven WD repeat domains from the two proteins show 58% sequence similarity and another common domain located immediately N-terminal to the WD repeats shows 57% sequence similarity. As suggested by this high sequence similarity between the two co-repressors, LUH was found to function at least partially redundantly with LUG (30). LUH has been shown to play minor roles in floral organ identity and in abaxial organ identity in leaves redundantly with LUG (30,31).

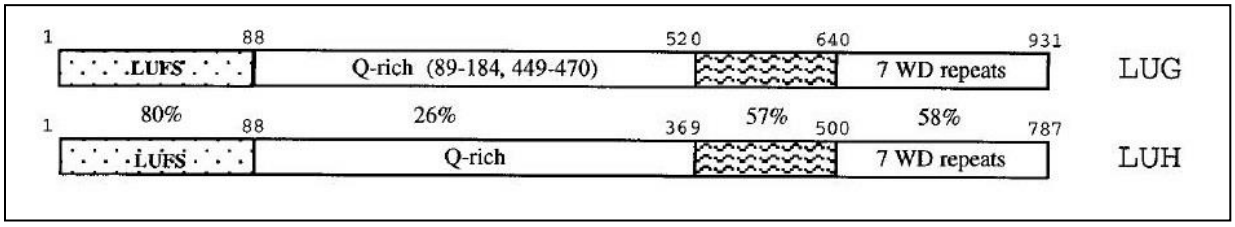


Fig. 5 Schematic representation of domain organization of LUH and LUG

Amino acid positions are given in numbers and the percentage of identity between the two proteins are given in percentage values. The LUFS domains, 7 WD repeats and the region preceding the WD repeats are highly conserved between LUG and LUH (29)

In *Arabidopsis* leaf both LUG and LUH interact with YABBY domain transcription factors (FILAMENTOUS FLOWER and YABBY3) involved in regulating abaxial cell and organ identity (31). In another study, *luh-1* single mutants showed a reduction in germination rate and shorter root lengths compared to the wild type plants (30). However the *luh-1*mutants did not show any defect in flower organs. The double mutants between LUG and LUH (*lug-3*/*lug-3*;*luh-1*/+) exhibited more severe defects in flowers compared to *lug-3* single mutants, supporting the notion that LUH functions redundantly with LUG. Since LUH was found to interact with SEU in yeast (30) it is possible that LUH binds to SEU to replace LUG in *lug* mutants. However the expression of LUH driven by 35S promoter in *lug-16* mutants did not rescue its phonotype, suggesting that these two co-repressors exhibit divergent functions and the redundancy between them is only partial (30).

Several recent investigations have identified regulation of mucilage release from *Arabidopsis* seed coat as a major role of LUH (32-34). Upon imbibition, *Arabidopsis* seed coat excretes mucilage, which mainly consist of rhamnogalacturonan I. In *luh*, mucilage modified 2 (*mum2*) mutants the structure of rhamnogalacturonan I is altered causing a serious defect in mucilage excretion. MUM2 codes for a β-galactosidase involved in modifying mucilage. Considering the fact that over expression of either MUM2 or LUG restores the *luh* mutants to normal phenotype (32), it was proposed that both LUH and LUG works redundantly to activate MUM2 expression indirectly by down-regulating a negative regulator of MUM2 (fig.6).

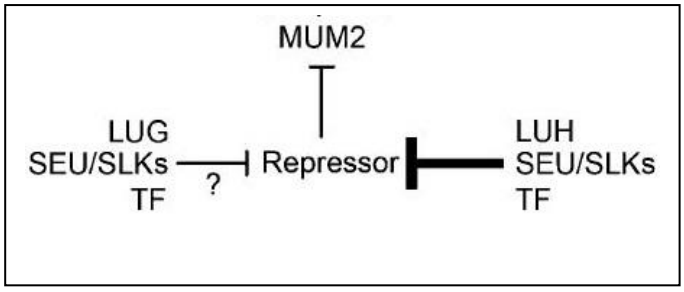


Fig.6 Repression of MUM2 by LUH and LUG

LUH promotes *MUM2* expression indirectly by repressing a negative regulator of *MUM2*. LUG may function redundantly with LUH in *MUM2* activation (32).

1.8 LUH in *Arabidopsis* abiotic stress response

The regulation of mucilage excretion from seed coat was the only major role reported for LUH, until a very recent investigation from our lab revealed that LUH is involved in abiotic stress response (35). In that report mutants of LUH (*luh-4*) were found to exhibit a strong salt and osmotic stress resistant phenotype. That work proposed a new model for LUH mediated repression of stress responsive genes. In this model SEUSS LIKE1 (SLK1) / SEUSS LIKE2 (SLK2), homologs of SEUSS, bind to LUH to form a co-repressor complex. This complex is then recruited to the target genes by an unknown transcription factor which interacts with SLK1 / SLK2. LUH then binds and recruits Histone Deacetylases (HDACs) which removes acetyl groups from histones of the downstream genes to promote silencing.

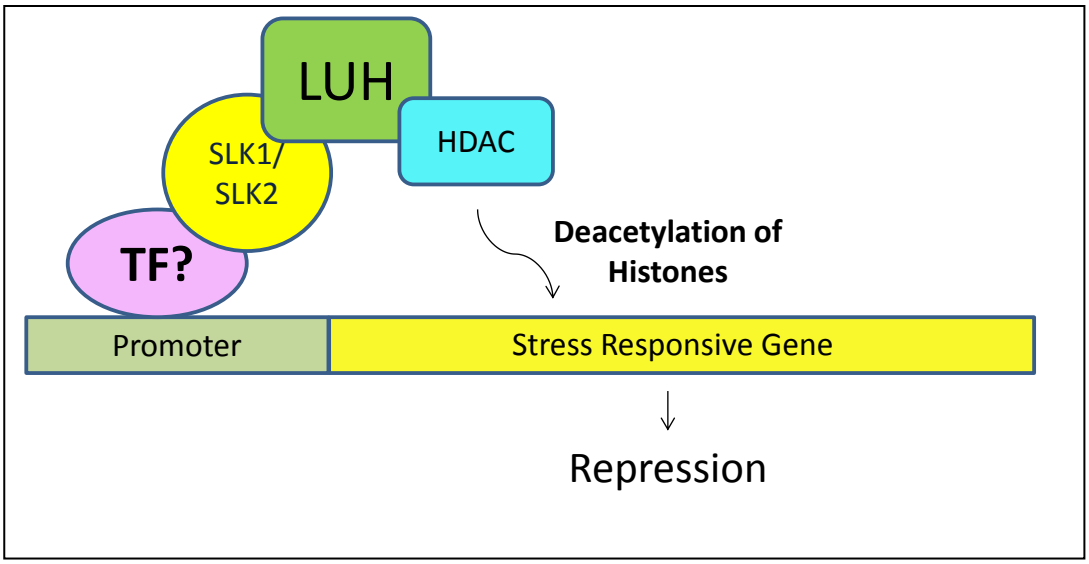


Fig.7 Proposed Model for LUH-SLK co-repressor complex mediated repression of stress responsive genes in *Arabidopsis* (35).

1.9 Specific aim of this work

In the model for repression of stress responsive genes proposed by Shrestha et al, LUH interacts with the adaptor protein SLK1 or SLK2 (only one at a time) to form a co-repressor complex which recruits a HDAC to repress target genes. However the transcription factors which bind to the regulatory regions of these stress responsive genes to recruit this co-repressor complex are unknown. The specific aim of this study is to identify those transcription factor(s) and their specific targets in order to get insight into this repression model.

2. Materials and Methods

2.1 Yeast Two Hybrid assay for library screening

2.1.1 Making plasmid constructs and yeast bait strains

The cDNA clones for SLK1 (G66746), SLK2 (G10219) and LUH (G12254) were obtained from the Arabidopsis Biological Research Center (ABRC) and were used as entry vectors in gateway cloning (LR clonase kit).

Three separate cloning reactions were performed in which 50-100 ng of each entry vectors were mixed with 100 ng of pGBD2 vector (destination). To each reaction setup water was added to bring the volume to 4µl and 1 µl of X5 LR clonase mix (total volume 5 µl) was added to each of them. These reaction mixes were then incubated at room temperature for 3 hours.

Recombinant plasmids were transformed in to DH5α competent cells using chemical transformation and cells were plated on LB agar (1% bacto tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, pH=7.5) kanamycin (50µg/ml) plates which were then incubated at 37^0 C overnight. Colony PCR was performed on the resulting colonies with GAL4BD Forward primer and Gene specific reverse primers to identify colonies carrying the correct recombinant plasmids (GAL4BD fused with SLK1, SLK2 and LUH). Positive colonies from the colony PCR were inoculated into 4 ml of PMB liquid medium (as described by Danquah *et al*. 2007) containing kanamycin (50µg/ml) and incubated at 37^0 C overnight (with shaking at 220 rpm) and the plasmids were extracted using the spin column mini prep kit (from Qiagen). Bait cell strains for the Yeast Two Hybrid assay were prepared by transforming recombinant plasmids (GAL4BD fused with SLK1, SLK2 and LUH) into Y2H gold yeast cells using the yeast transformation protocol.

2.1.2 Yeast transformation

An isolated colony of Y2H Gold yeast cells was inoculated in to 9 ml of YPDA media (2% bacto peptone, 1% yeast extract, 2%dextrose and 0.003% adenine hemisulfate) and incubated at 30^0 C overnight (with shaking). Cultured cells were centrifuged at 2540 g for 15 min. The supernatant was discarded and the cell pellet was washed by resuspending in 15 mL of sterile water and centrifuging again at 2540 g for 10 minutes. The washing step was repeated two more times and the and the pellet was washed once more with 10 mL of 100 mM lithium acetate to increase competency. To the washed cell pellets 480µl of 50% PEG (Polyethylene Glycol), and 72 µl 1 M lithium acetate and 100 µl water was added and the contents were mixed by pipetting. For each transformation, 150 µl of washed yeast cells were aliquoted to micro-centrifuge tubes. To each micro-centrifuge tube 2 µl of 10 mg/mL salmon sperm DNA (which was prepared by boiling it at 100^0 C for 5 minutes and cooling rapidly on ice) and 8µl of the respective plasmid DNA was added and the contents were mixed by vortexing for few seconds. The tubes were incubated at room temperature for 30 minutes and then heat shocked by incubating at 42^0 C for 30 minutes. After the heat shock, the tubes were centrifuged at 13000 rpm for 20 seconds. Supernatants were discarded and cells were washed with 500 µl of water. Finally the cell pellets were resuspended in 100 µl of water and plated on –trp drop out plates (containing all the essential amino acids except tryptophan).

2.1.3 Mating yeast cells

The yeast cells expressing bait proteins SLK1, SLK2 or LUH fused to GAL4BD (bait strains) were mated with the *Arabidopsis* mate and plate library cells (which express all the Arabidopsis proteins fused to GAL4AD) to obtain hybridized cells (contains the bait and prey

constructs). A colony from the bait strain plate was inoculated into 50 mL of –TRP synthetic dropout liquid media in a 250 mL flask, which was then incubated at 30^0 C with shaking at 220 rpm till the optical density (at 600 nm wave length) of the culture reached 0.8. The concentration of the culture was estimated by counting cells in a hemacytometer under a light microscope. The yeast cells were then centrifuged for 5 minutes at 2540 g, the supernatant was discarded and the pellet was resuspended in 5mL of –TRP synthetic dropout liquid media. In a 2L sterile flask 1.5 X 10^9 bait cells were mixed with 45 mL of 2x YPDA (with 50µg/ml kanamycin) and 4mL of the library cells. These cells were incubated at 30^0 C with shaking at 40 rpm for 24 hours to allow mating. Mating of bait (BD) and library (AD) cells was confirmed by checking for zygotes under light microscope. Cells were centrifuged at 6000g for 10 minutes the pellet was resuspended in 4 mL of sterile water. These cells were then plated on –TLHA agar media plates (to select colonies which express *Arabidopsi*s proteins that interact with our bait proteins) and incubated at 30^0 C for 5 days.

2.1.4 Isolation of yeast DNA to rescue prey plasmids

Isolated colonies from Y2H plates were inoculated into –TL liquid media (4 mL for each) and incubated at 30^0 C with shaking at 220 rpm for 1- 2 days. Cells were transferred to micro centrifuge tubes, spun at 13000 rpm for 20 seconds and the supernatant was discarded. The pellets were resuspended in 200 µl of zymolyase solution and incubated at 37^0 C with shaking at 220 rpm for 4 hours. Zymolyase was inactivated by incubating at 75^0 C for 15 minutes, 200 µl of lysis solution was added to each tube and the contents were mixed by inversion. To each tube 400 µl of 3M potassium acetate was added and mixed before adding 150 µl of chloroform. Tubes were centrifuged at 13000 rpm at 4^0 C for 20 minutes and the supernatants were

transferred to new tubes. To each tube 0.7 volumes of isopropanol was added, mixed by inversion and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 13,000 rpm for 15 minutes, the supernatant was discarded and the pellets were washed with 300 µl of 70% ethanol. Pellets (yeast DNA) were then air dried and resuspended in 30µl of elution buffer (10 mM Tris-HCl, pH=8.5) containing RNase A (20 µg/ml).

2.1.5 PCR amplification, sequencing and identification of interacting genes (prey proteins)

PCR was performed to amplify unknown genes fused to GAL4AD with Gal4AD Forward and Gal4AD Reverse primers. The total volume of each PCR was set to 40 µl and 6 µl of template DNA (yeast DNA) was used. For the PCR initial denaturation was carried at 95^0C for 3 minutes followed by 35 cycles of 94^0C 20 seconds denaturation, 59^0C 1 minute annealing and 72^0C 3 minutes extension. Final extension was carried at 72^0C for 10 minutes. A portion of 5 µl of each PCR product was run on 0.8% agarose gel to check for successful DNA amplification. Remainders of the PCR products were purified by passing them through DNA clean up spin columns (Quiagen).

Purified PCR products were then sequenced using the Gal4AD Forward as the sequencing primer and the sequences were used to BLAST (Basic Local Alignment Tool) against the Arabidopsis gene data-base from The Arabidopsis Information Resource (TAIR) website (https://www.arabidopsis.org/Blast/index.jsp) to identify respective *Arabidopsis* genes.

2.2 Confirmation of AT3G58630 (SLK2INT1) and SLK2 interactions

2.2.1 Yeast Two Hybrid assay

A yeast two hybrid assay was performed confirm the yeast two hybrid interaction between SLK2 and AT3G58630 (here after this is referred to as SLK2INT1). Gateway cloning was performed to create both the bait and prey plasmid constructs (proteins fused with GAL4AD and GAL4BD) which were then transformed into yeast cells (strain PJ694A) using yeast transformation protocol described above (with the exception of both plasmids were transformed together instead of only one). Cells containing both plasmids were selected on –TL (synthetic drop out media without Tryptophan and Leucine) plates. The protein interactions were determined by the activation of downstream reporter α-galactosidase in an enzyme assay.

2.2.2 Alpha galactosidase assay

Yeast colonies were inoculated into 200 µl of -TL dropout media in microtiter plates and were incubated at 30^0C with shaking at 220 rpm for 1-2 days. The cell concentrations of cultures were determined spectrophotometrically in a 96 well multi-plate reader (Wallac Victor^2 1420). Plates were then centrifuged at 4000 rpm for 10 minutes, the supernatants were separated. For each assay 80 µl of the supernatant (solubilized proteins) was mixed with 25 µl of the o-nitrophenyl-α-D-galactopyranoside (α-ONPG) substrate in 100 mM phosphate buffer (pH7.5) and incubated at 30^0 C until a yellow color was observed. The reaction was stopped by adding 75 µl of 1M sodium carbonate and the absorbance at 405 nm was read in a 96 well multi-plate reader.

2.3 Yeast Three Hybrid Assay for the ternary complex

A yeast three hybrid assay was performed to determine if SLK2 acts as an adapter to bring both LUH and SLK2INT1 together to form a three protein complex. All three proteins; SLK2INT1 fused to GAL4BD, LUH fused to GAL4AD and SLK2 (cloned in p426 plasmid) were expressed together in the same yeast cells. Plasmid constructs were transformed into yeast cells sequentially using the yeast transformation protocol (See materials and methods 2.1.2) and the expression of the downstream reporter gene β-galactosidase was measured as described below.

2.3.1 Beta Galactosidase assay

Yeast colonies were inoculated into 4 mL of dropout media (-TL) and were incubated at 30^0C with shaking at 220 rpm for 1-2 days. Tubes were centrifuged at 4000 rpm for 10 minutes, the supernatants were discarded and 150 µl of 2% Y'per reagent was added to each tube and transferred to microtiter plates. Plates were agitated at room temperature for 20 minutes and centrifuged at 4^0C for 10 minutes. For each assay 80 µl of the supernatant (solubilized proteins) was mixed with 25 µl of the o-nitrophenyl-β-D-galactopyranoside (β-ONPG) substrate in Z-buffer in a new microtiter plate and were incubated at incubated at 30^0C until a yellow color developed (only positive samples gave a color). The reaction was stopped by adding 75 µl of 1M sodium carbonate and the absorbance at 405 nm was measured with a 96 well plate reader. The total protein concentration for each sample was determined with Bicinchoninic Acid (BCA) assay.

2.3.2 BCA assay for total protein determination

BCA working solution was prepared by mixing reagent A (1% BCA, 2%Na_2CO_3, 0.95% $NaHCO_3$, 0.4% NaOH, 0.16% sodium tatarate, pH=11.25) and reagent B (4% Copper sulfate) in 50:1 ratio. In a microtiter plate 50µl of each protein sample was mixed with 150 µl of BCA working solution, incubated at 37^0C for 30 minutes and the absorbance was measured at 570 nm.

2.4. Isolation of mutant plants

2.4.1 Genotyping of *Arabidopsis* plants

Seeds for *slk2int1* mutant plants were obtained from Arabidopsis Biological Research Center (ABRC). DNA was isolated from three to four week old plants as described below. PCR was used to identify their genotypes with respect to the relevant gene. Two sets of PCRs were carried out for each DNA sample with gene LP and gene RP primers (amplifies the wild type gene) in one set and Lba1 and gene RP primers (amplifies the mutant copy of the gene) in the other set. PCR products were analyzed on agarose gel and homozygous mutant plants were identified. Identified mutant plants were allowed to grow (approximately 6 to 8 weeks) and seeds were harvested.

2.4.2 Extraction plant DNA

Three to four week old plants were marked and one medium size leaf or two small leaves from each plant were harvested into micro centrifuged tubes. Leaves were homogenized in 500µl of plant DNA isolation buffer and the tubes were incubated at 65^0C for 30 minutes mixing every 5 minutes. The contents of each tube were mixed with 500 µl of chloroform and

centrifuged at 13000 rpm for 15 minutes. The supernatants were transferred to fresh tubes, mixed with 350 µl of isopropanol, incubated at room temperature for 10 minutes and centrifuged for 15 minutes. The pellets (which contains DNA) were washed with 70% ethanol, air dried and resuspended in 40µl of elution buffer (10 mM TrisHCl) containing RNase A (20 µg/ml).

2.5 Abiotic stress assay for plants

Seeds from mutant and wild type (Col-0) plants (approximately 50 µl) were sterilized by mixing with 1 ml of bleach (solution of 50% Clorox bleach and 0.2% Tween-20) for 10 minutes and washed three times with sterile water. Seeds were then incubated at 4^0C for 2 days (this is a cold shock to synchronize seed germination) and plated on ½ strength MS (Murashige & Skoog) containing 0.7% agar plates. Plates were incubated at 21^0 C in florescence chambers for (6-7) days and the plants were transferred to stress plates containing ½ strength MS, 0.8% agar and either 0.3M mannitol (for osmotic stress) or 125mM NaCl (for salt stress). Stress plates were incubated for another 6-7 days under same conditions before the root length and the fresh weight of each plant was measured.

2.6 Generating transgenic plants

2.6.1 Generating transgenic plants with *SLK2INT1* promoter and reporter GUS fusion.

The slk2int1 promoter was cloned to PCR8/ GW/TOPO plasmid then transferred to pMDC164 plasmid by gateway cloning. The recombinant plasmid was then transformed to agrobacteria, which was then cultured, harvested and resuspended in 5% sucrose (to bring OD_{600}

to 2.0) containing 0.2 µl/ml silvett. The agrobacterium cell suspension was used to dip *Arabidopsis* inflorescences from flowering stage Col-0 (wild type) plants. The dipping process was repeated and plants were kept covered in dark for 24 hours. Agrobacterium treated plants were then grown uncovered under normal day /night periodic light conditions and seeds were harvested. Transgenic plants were selected by plating the seeds on ½ MS growth media with Hygromycin (50 mg/l) and the selection was repeated for another generation (F2 seeds).

2.6.2 Generating transgenic plants for complementation assay

The slk2int1 full length gene was cloned to PCR8/ GW/TOPO plasmid then transferred to pEG303 plasmid by gateway cloning. The recombinant plasmid was then transformed to *slk2int1* mutant plants by agrobacterium mediated transformation protocol described earlier. Transgenic plants were selected with BASTA (10 mg/l phosphinotricin)

2.6.3 Staining of transgenic plants for GUS expression

Healthy plants were selected and vacuum infiltrated in GUS staining buffer (0.2% Triton X-100, 2mM Ferrocyanide, 2mM Ferricyanide and 2mM X-GLUC Phosphate buffer 50 mM, pH=7.2) for 10 minutes or until bubbles appear in solution. Plants were then incubated in the same solution at 37^0C overnight and destained in 70% ethanol for two days, transferring plants to fresh ethanol every twelve hours. Stained plants were observed under light microscope (Leica $MZ7_5$) equipped with a digital camera (EC3).

2.7 Identification of SLK2INT1 Target genes

2.7.1 Plant RNA isolation

Tissues from 21 days old plants (0.5-1g for each sample) were harvested and frozen immediately in liquid nitrogen. Tissues were ground while maintaining cold conditions by adding liquid nitrogen. Trizol reagent was added to the ground tissues (12ml per 1.0 g of tissues) and the liquid suspension was transferred to centrifuge tubes. Tubes were added with 1-Bromo-3-Chloropropane (200 µl of BCP for each 1ml of trizol), mixed vigorously, incubated and centrifuged. RNA was precipitated with isopropanol, washed with 75% ethanol and resuspended in Diethyl-pyrocarbonate **(**DEPC) treated water. RNA was treated with DNase, purified in a column and then used in cDNA synthesis.

2.7.2 Quantitative RT-PCR

The expression levels of selected genes were measured by using total RNA and gene specific primers (listed in appendix) in qRT-PCR (cycle conditions; 95^0 C 1 min, 55^0 C 30 s, 72^0 C 35 s for 30 cycles) for Col-0, *slk2* mutants and *slk2int1*mutants. As an internal control the expression of *ACTIN2* gene was also measured.

2.8 GFP localization assay for SLK2INT1

To determine the cellular location of SLK2INT1 a localization assay was performed. A CaMV 35S driven SLK2INT1-GFP fusion was made by amplifying cDNA with gene specific primers and cloning into the Bam H1 site of the pXDG vector. The protoplasts were transfected with the recombinant plasmids and incubated in the dark at room temperature for 16 hours. The protoplasts were then incubated with 1µg/ml 4, 6-diamidino-2-phenylindole(DAPI). Protoplasts

were then examined for DAPI and GFP localization under a fluorescent microscope (Nikon Exclipse E800). The images were processed with imagej program.

2.9 Repression assay for the ternary complex

To generate 35S CaMV:: LUH , 35S CaMV::SLK2 and 35S CaMV:: SLK2INT1, the respective cDNA were PCR amplified and inserted at the BamH1 site by In-Fusion HD Cloning Plus in the pXSN vector. The protoplast transfection and reporter gene assay was performed as described as *Sridhar* et al 2005(25).

3. Results

3.1 Yeast two hybrid assay for protein-protein interactions

3.1.1 Proteins which interact with SLK2 adaptor protein

To identify the transcription factors involved in recruiting adaptor protein SLK2 to its target genes a library of Arabidopsis genes in yeast two hybrid vectors were screened. SLK2 was used as the bait fused to the GAL4-BD (the DNA binding domain of the GAL4 transcription factor). From all of interacting proteins only the ones localized to the nucleus were selected for further investigation.

Table 1 List of proteins which interact with SLK2 in yeast

Gene(ATG#)	Description
AT2G21170	Encodes a plastidic triose phosphate isomerase (pdTPI). Mutants with reduced pdTPI levels have difficulty transitioning from heterotrophic to autotrophic growth
AT2G39720	**Encodes a putative RING-H2 finger protein RHC2a. / zinc ion binding**
AT5G24420	Encodes a cytosolic 6-phosphogluconolactonase (PGL) thought to be involved in the oxidative pentose-phosphate pathway
AT2G41040	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein, involved in response to karrikin. located in chloroplast, plastoglobule
AT5G14640	Shaggy-like kinase 13 (SK13), has protein serine/threonine kinase activity, ATP binding, involved in response to salt stress, hyperosmotic response
AT1G70760	A subunit of the chloroplast NAD(P)H dehydrogenase complex, involved in PSI cyclic electron transport
AT5G14470	GHMP kinase family protein with kinase activity and phosphotransferase activity.
AT3G59970	Methylenetetrahydrofolate reductase
AT2G40890	Coumarate 3-hydroxylase (C3H), a P450-dependent monooxygenase
AT3G01650	Encodes RGLG1 (RING domain ligase 1), a RING domain ubiquitin E3 ligase that negatively regulates the drought stress response by mediating ERF53 transcriptional activity.
AT4G35450	Involved in targeting of chloroplast outer membrane proteins to the chloroplast.
AT1G03880	Protein is tyrosine-phosphorylated and its phosphorylation state is modulated in response to ABA in Arabidopsis thaliana seeds
AT2G29630	Encodes a protein involved in thiamin biosynthesis

AT5G47620	RNA-binding (RRM/RBD/RNP motifs) family protein, functions in RNA binding, nucleotide binding, nucleic acid binding; biological_process unknown
AT2G40890	Coumarin biosynthetic process, flavonoid biosynthetic process, lignin biosynthetic process, oxidation-reduction process, phenylpropanoid biosynthetic process, positive regulation of flavonoid biosynthetic process, response to wounding
AT4G36400	Encodes a (D)-2-hydroxyglutarate dehydrogenase.
AT1G71480	Nuclear transport factor 2 (NTF2) family protein, functions in protein transporter activity, involved in protein import into nucleus, located in chloroplast thylakoid membrane, intracellular, nucleus, chloroplast
AT5G47000	Peroxidase superfamily protein, functions in peroxidase activity, xylan 1,4-beta-xylosidase activity, involved in oxidation reduction, response to oxidative stress
AT5G09640	Serine carboxypeptidase-like (SCPL) protein
AT2G26080	Glycine decarboxylase P-protein 2 (GLDP2) which has glycine dehydrogenase (decarboxylating) activity
AT3G47000	Glycosyl hydrolase family protein, has hydrolase activity, hydrolyzing O-glycosyl compounds, involved in carbohydrate metabolic process
AT1G52750	Alpha/beta-hydrolases superfamily protein, contains InterPro domain/s: Epoxide hydrolase-like
AT2G04570	GDSL-like Lipase/Acylhydrolase superfamily protein, has hydrolase activity
AT2G21385	unknown protein./ biological_process, carotenoid biosynthetic process, isopentenyl diphosphate biosynthetic process, mevalonate-independent pathway/ chloroplast, chloroplast envelope, chloroplast stroma, nucleus
AT2G30505	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family, located in nucleus of guard cells
AT4G30810	Scpl29, serine carboxypeptidase-like 29
AT4G26780	AR192, MGE2, mitochondrial GRPE 2, located in chloroplast and mitochondria
AT1G64510	Translation elongation factor EF1B, ribosomal protein S6 family protein, chloroplast, chloroplast thylakoid membrane, ribosome, thylakoid
AT1G56500	A thylakoid membrane protein with thioredoxin-like and beta-propeller domains
AT1G54580	Acyl carrier protein 2(ACP2), involved in fatty acid biosynthetic process
AT4G21660	A proline-rich spliceosome-associated (PSP) family protein, involved in mRNA processing, located in nucleus
AT2G42300	**A basic helix-loop-helix (bHLH) DNA-binding superfamily protein, has sequence-specific DNA binding transcription factor activity, located in nucleus**
AT4G27430	A positive regulator of light-regulated genes. Novel nuclear protein which requires light for its high level expression
AT3G16400	A nitrile-specifier protein NSP1 responsible for constitutive and herbivore-induced simple nitrile formation in rosette leaves
AT3G21570	An unknown protein located in nucleus

AT5G54580	A RNA-binding (RRM/RBD/RNP motifs) family protein, located in mitochondrion
AT1G06760	**A winged-helix DNA-binding transcription factor family protein has DNA binding ability, involved in nucleosome assembly, located in nucleus**
AT3G58630	**Sequence-specific DNA binding transcription factor, located in nucleus**
AT1G72030	Acyl-CoA N-acyltransferases (NAT) superfamily protein
AT3G51260	20S proteosomal alpha subunits. Interacts with SnRK, SKP1/ASK1 during proteasomal binding of an SCF ubiquitin ligase / chloroplast, cytoplasm, cytosol, cytosolic ribosome, nucleus, phragmoplast, proteasome core complex, proteasome core complex, alpha-subunit complex, spindle, vacuolar membrane, vacuole
AT4G30270	A protein similar to endo xyloglucan transferase in sequence. It is also very similar to BRU1 in soybean, which is involved in brassinosteroid response, located in Golgi apparatus, apoplast, cell wall, cytoplasm, extracellular region, plant-type cell wall, plasma membrane

3.2 Phenotypic analysis for mutants of selected SLK2 interactors

3.2.1 Phenotypes of *at2g39720, at2g42300* and *at1g06760* mutants

From all the proteins found to interact with SLK2, a set of interesting proteins that could be involved in recruiting LUH-SLK2 co-repressor complex to its targets were selected for further study. Since *slk2* mutant showed increased tolerance towards salt and osmotic stress (35), mutation of any transcription factor involved in recruiting SLK2 to the stress responsive genes should show a similar phenotype. Therefore *at2g39720*, *at2g42300* and *at1g06760* mutants were subjected to salt and osmotic stress treatment. It was observed that in the control plate, the salt stress plate (125 mM NaCl) and the osmotic stress plate (0.3 M mannitol) the mutants plants phenotypes did not vary significantly from the wild type plants (fig. 8) suggesting that these proteins are unlikely to be involved in the repression of the stress responsive genes.

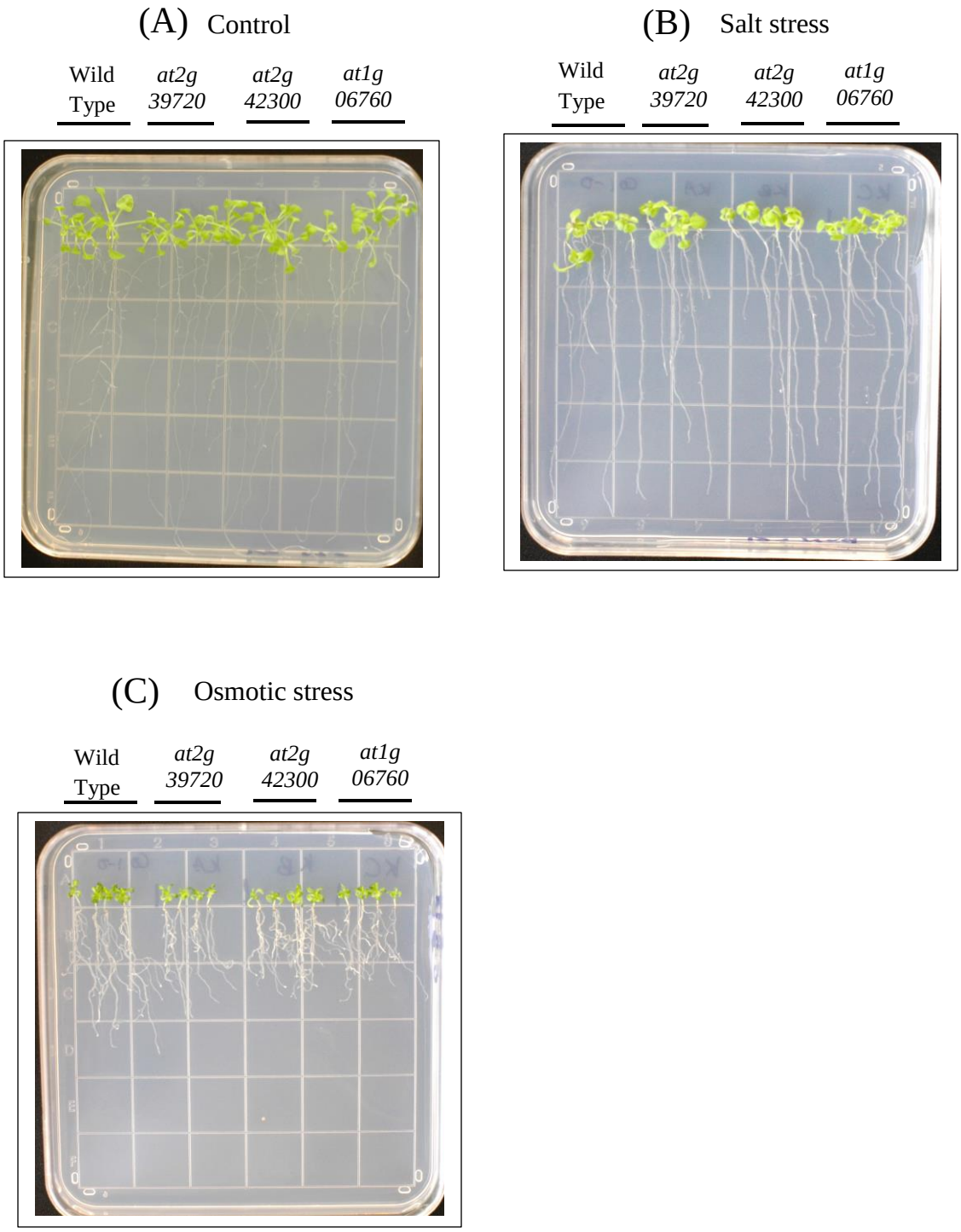


Fig. 8 Phenotypic comparison of Col-0 (Wild type), *at2g39720*, *at2g42300* and *at1g06760* plants

Arabidopsis seeds were grown in ½ MS media for seven days and transferred to ½ MS media containing 125 mM NaCl and 300mM Mannitol plates for salt and osmotic stress respectively. The control plate contained only ½ MS media. **A**. ½ MS control plate **B**. 125 mM NaCl in ½ MS **C**. 300mM Mannitol in ½ MS

3.2.2 Phenotype of *slk2int1* mutant

Another interesting interactor of SLK2 revealed by the yeast two hybrid screen, is AT3G58630 (SLK2INT1). Therefore, *slk2int1* mutants were also subjected to same stress conditions to determine if they showed resistance to abiotic stress. Interestingly, it was observed that the *slk2int1* mutant also has a phenotype similar to the *slk2* mutant (fig.9).

The root length of each plant was measured to assess its health in control and stress induced plates. In the ½ MS control plate the wild type Col-0 plants, *slk2* mutants and *slk2int1* mutants have similar root lengths (fig.10 a). In both salt stress (125 mM NaCl) and osmotic stress (300mM mannitol) plate's *slk2* mutants and *slk2int1* mutants have significantly longer roots compared to Col-0 plants (see fig. 10 b and c).

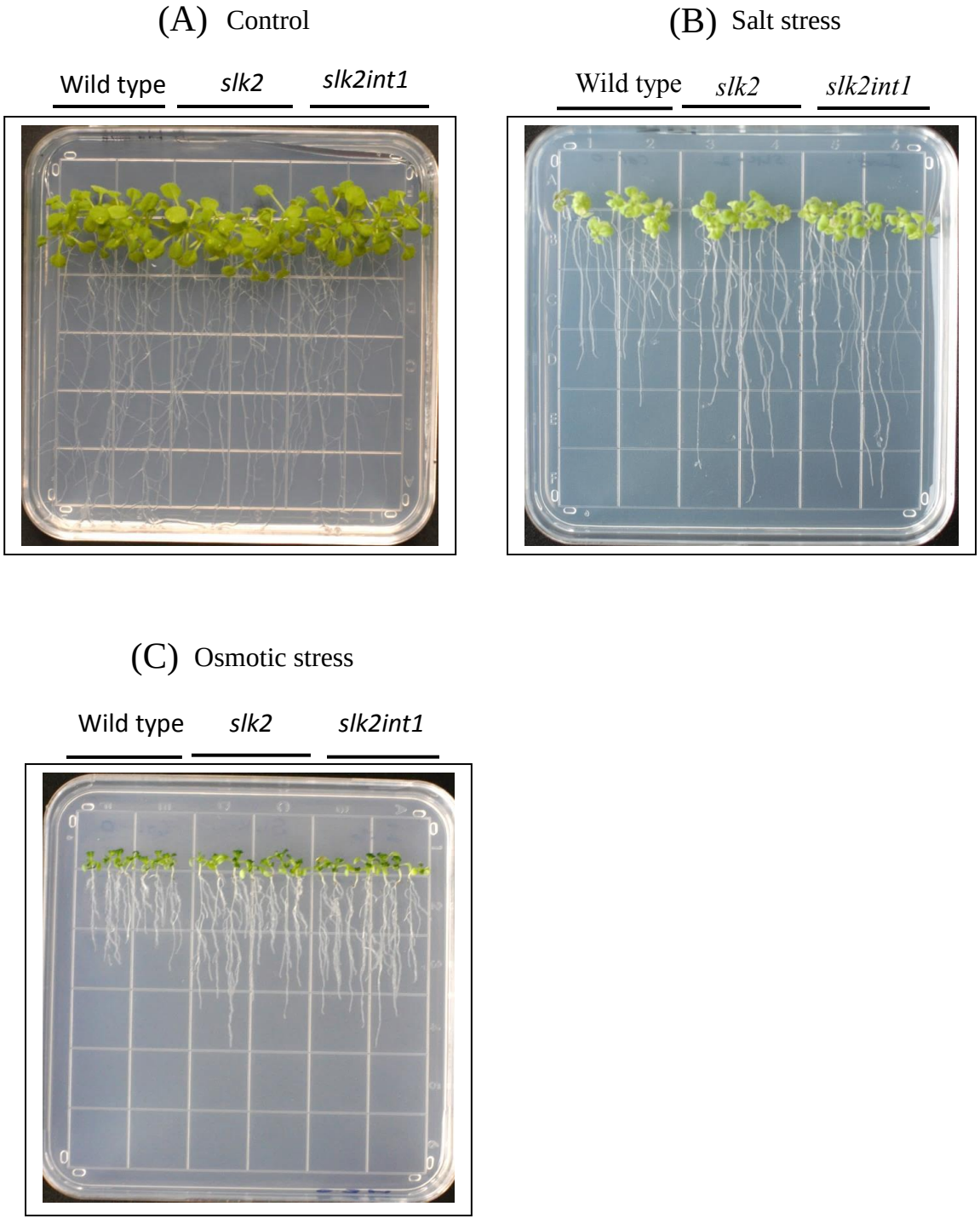


Fig. 9 The comparison of Col-0 (Wild type), *slk2* and *slk2int1* phenotypes

Col-0, *slk2* and *slk2int1* seeds were grown in ½ MS media for seven days and transferred to ½ MS media containing 125 mM NaCl and 300mM Mannitol plates for salt and osmotic stress respectively. The control plate contained only ½ MS media. **A**. ½ MS control plate **B**. 125 mM Nacl in ½ MS **C**. 300mM mannitol in ½ MS

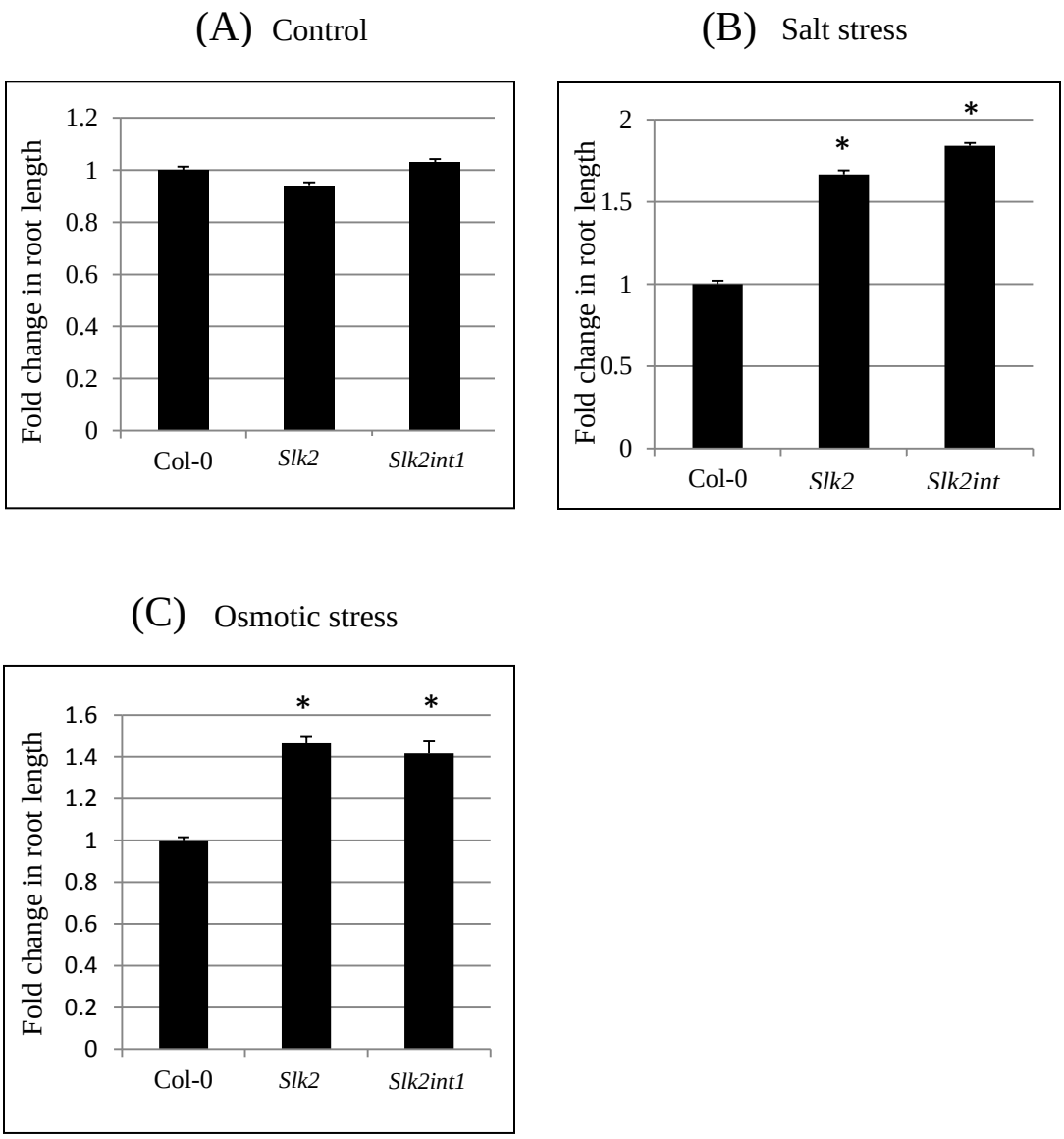


Fig. 10 The root length analysis of Col-0 (Wild type), *slk2* and *slk2int1* mutant plants in stress plates

Arabidopsis seeds were grown in ½ MS media for seven days and transferred to ½ MS media containing 125 mM NaCl and 300mM Mannitol plates for salt and osmotic stress respectively. Root lengths were measured after 6-14 days. **A**. ½ MS control plate **B**. 125 mM NaCl in ½ MS **C**. 300mM mannitol in ½ MS. Asterisks indicate values that are significantly different from the wild type Col-0 plants (*P <0.05 Student's t test, n=3)

3.3 Phenotypic analysis for the *slk2int1* complement

The *slk2int1* mutants obtained from the Arabidopsis Biological Research Center (ABRC), were created by inserting T-DNA into gene (AT3G58630). To confirm the salt and osmotic stress resistances of these plants are due to the loss of function of the AT3G58630 gene, a complement was made by reintroducing the gene with its own promoter to the *slk2int1* plants. As expected the complement plants were more sensitive towards salt and osmotic stress similar to control plants (fig. 11 and 12).

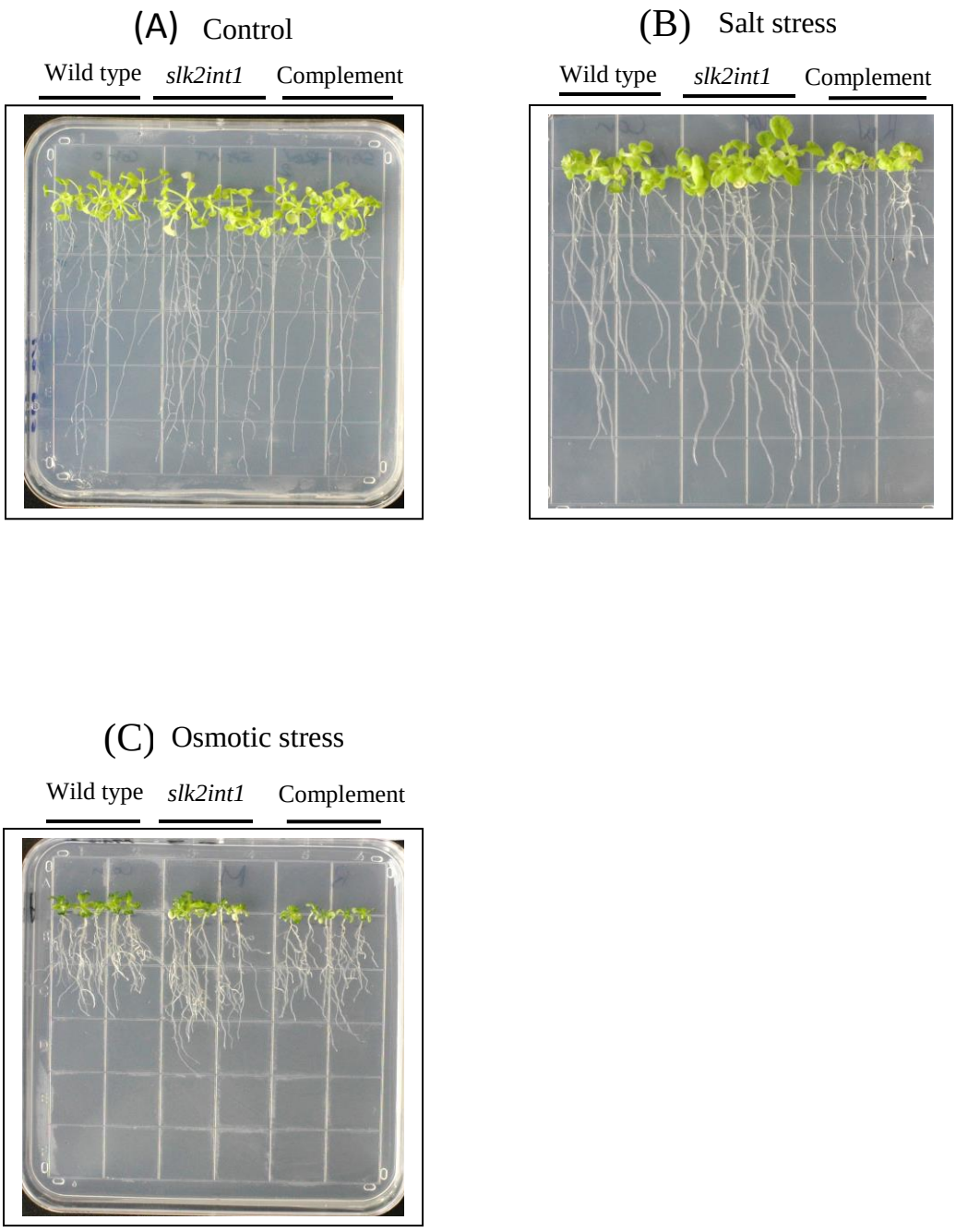


Fig. 11 The comparison of Col-0 (Wild type), *slk2int1* and complement phenotypes

Arabidopsis seeds were grown in ½ MS media for seven days and transferred to ½ MS media containing 125 mM NaCl and 300mM mannitol plates for salt and osmotic stress respectively. The control plate contained only ½ MS media. **A**. ½ MS control plate **B**. 125 mM NaCl in ½ MS **C**. 300mM mannitol in ½ MS

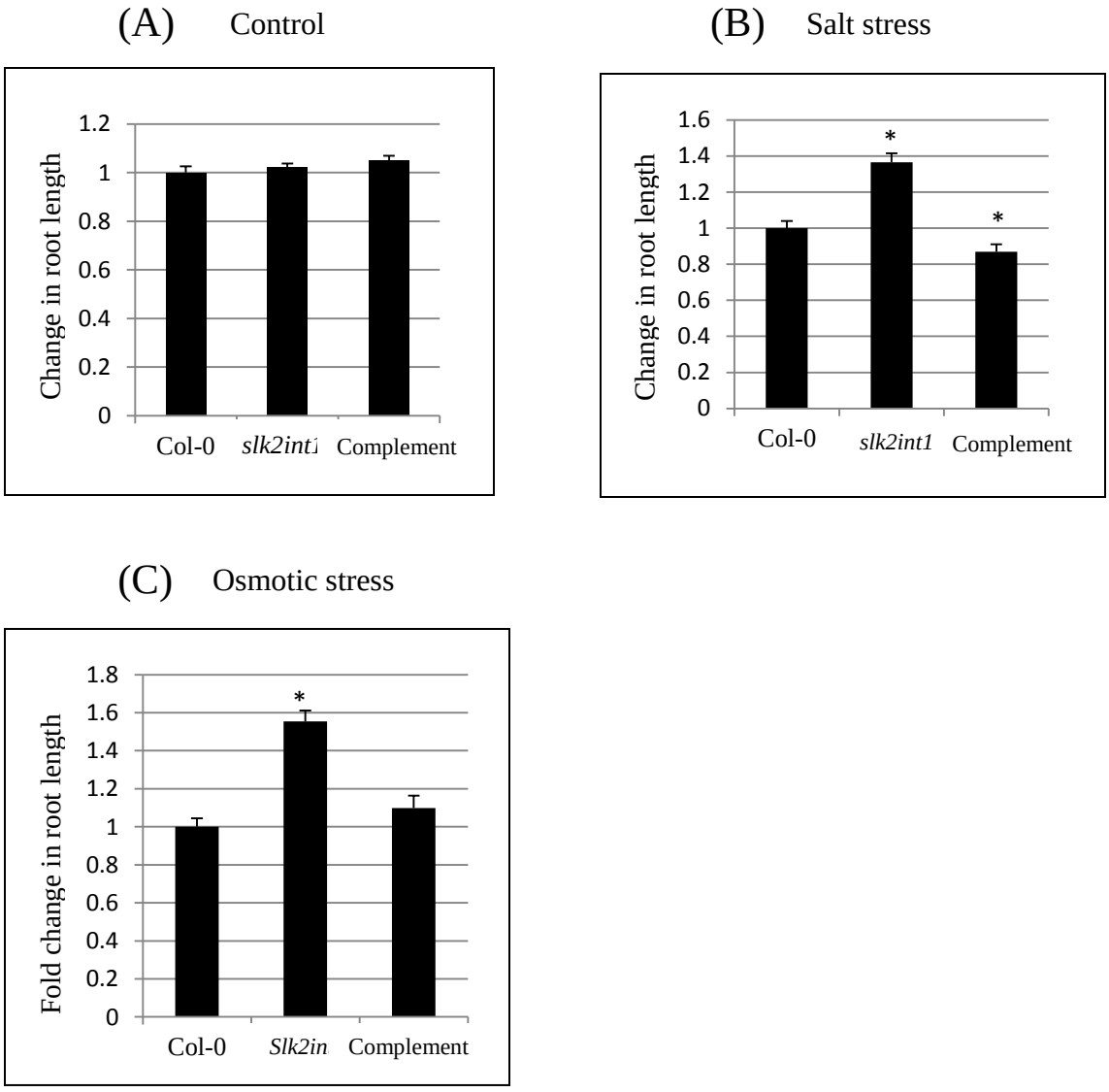


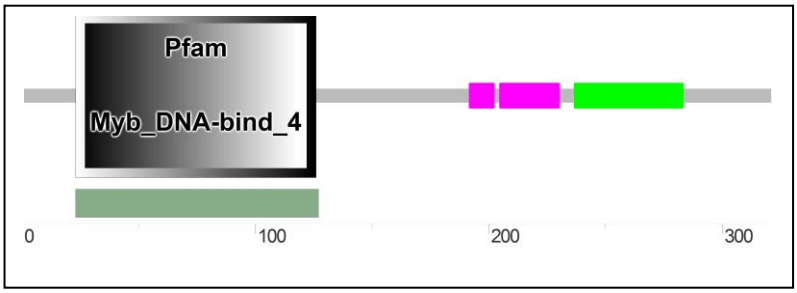
Fig. 12 The root length analysis of Col-0 (Wild type), *slk2int1* and mutant revertant plants in stress plates

Arabidopsis seeds were grown in ½ MS media for seven days and transferred to ½ MS media containing 125 mM NaCl and 300mM mannitol plates for salt and osmotic stress respectively. Root lengths were measured after 6-14 days. **A**. ½ MS control plate **B**. 125 mM NaCl in ½ MS **C**. 300mM mannitol in ½ MS. Asterisks indicate values that are significantly different from the wild type Col-0 plants (*P <0.05 in Student's t-test, n=3).

3.4 Analysis of domain architecture and structure prediction of SLK2INT1

Since SLK2INT1 (AT3G58630) is a transcription factor, it was interesting to find out the protein domains it contains. Therefore the protein sequence was used in a query to BLAST (Basic Local Alignment Search Tool) against the protein database from SMART (Simple Modular Architecture Research Tool) web resource (http://smart.embl.de/) to determine the presence of known domains.

A



B

Name	Start	End	E-value
Pfam:Myb_DNA-bind_4	23	126	$6.4e^{-27}$

Fig. 13 The domain architecture of SLK2INT1

A) Schematic representation of domain organization of SLK2INT1 based on hits against SMART general database. **B**) Matching domain found in SLK2INT1, its location and the error value.

The domain search with SMART suggested that SLK2INT1 contains a MYB binding domain at the N-terminal (fig. 13 A). Since the resolved crystal structure is not available for this protein, the amino acid sequence was used in the SWISS-MODEL (36-38) to predict the protein

structure based on homology modelling. The predicted domain structure contained three α- helices (fig. 14) which is a common feature of DNA binding MYB domain.

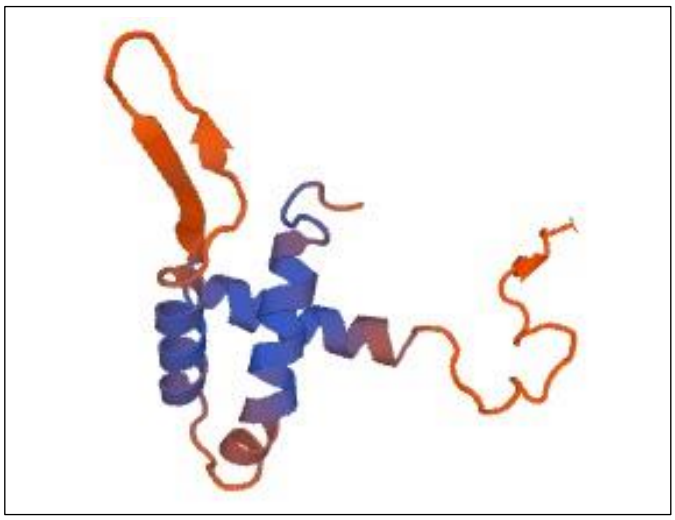


Fig. 14 Predicted structure of SLK2INT1

The structure of the MYB DNA binding domain of SLK2INT1 predicted with homology modelling using the SWISS-MODEL (36-38). Three alpha-helices are indicated in blue.

3.5 Interaction between SLK2INT1 (AT3G58630) and SLK2 in yeast two hybrid assays

To demonstrate that SLK2INT1 can physically interact with SLK2, a yeast two hybrid assay was performed using the full length SLK2INT1 and SLK2 cDNA. As expected it was found that these two proteins physically interact with each other in yeast. Both SLK2-GAL4BD and SLK2INT1-GAL4BD fusion proteins activated the α-galactosidase reporter when expressed alone (together with GAL4 AD not fused to another protein) indicating a low level auto activation (fig. 15). Cells expressing the SLK2 protein fused to GAL4-BD domain together with SLK2INT1 fused to GAL4AD showed a significantly high α-galactosidase activity.

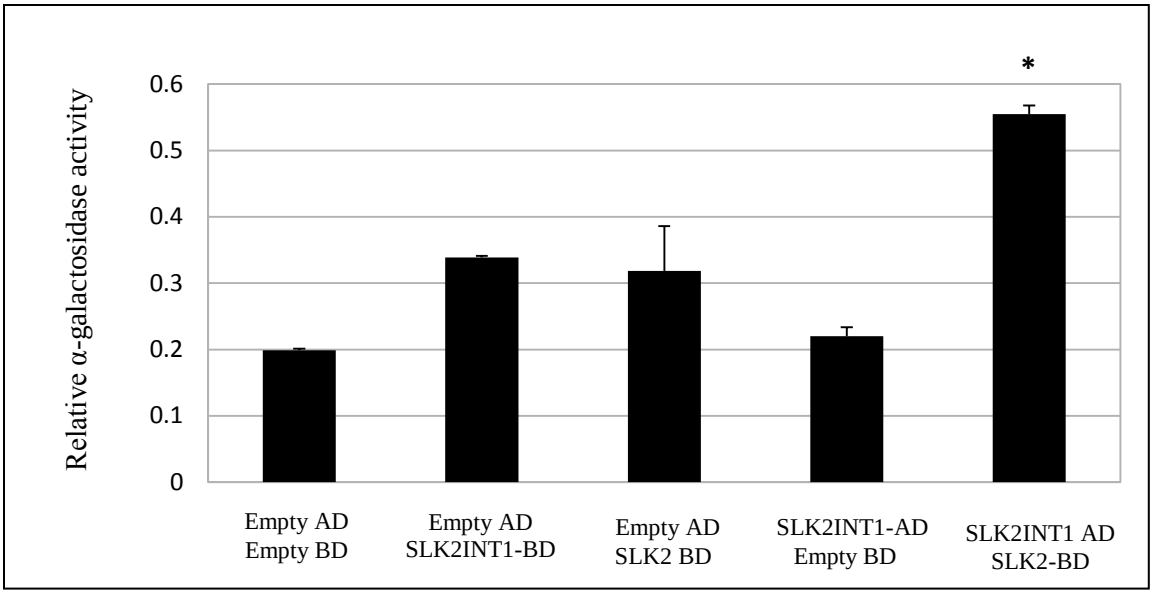


Fig. 15 Relative α-galactosidase activity in yeast two hybrid assay for SLK2 and SLK2INT1 interaction

Both SLK2 and SLK2INT1were expressed fused to GAL4AD and Gal4 BD**.** For the control experiments cells were transformed with either one or both AD and BD plasmids without a fused protein. The α-galactosidase activity was normalized with cell density of each sample. The assay for each plasmid combination was repeated three times with different colonies. Asterisks indicate values that are significantly different from all the other control experiments (*P <0.05 Student's t test, n=3)

3.6 Interaction between SLK2INT1, SLK2 and LUH in yeast three hybrid assay

Since previous studies revealed that SLK2 interacts with LUH in yeast (Shrestha et al 2014), a yeast three hybrid assay was performed to see whether SLK2 can bind to both LUH and SLK2INT1 at the same time to form a complex. When all the three proteins were expressed together the cells showed very high β-galactosidase activity (fig. 16) compared to the controls indicating that all three proteins form a complex to activate the reporter gene. To eliminate the possibility of SLK2INT1 interacting directly with LUH without binding to SLK2, a control experiment was carried out with yeast cells expressing only SLK2INT1-BD and LUH-AD

constructs. As expected an increase in β-galactosidase activity was not observed indicating SLK2 is required to bring SLK2INT1 and LUH together to form a protein complex.

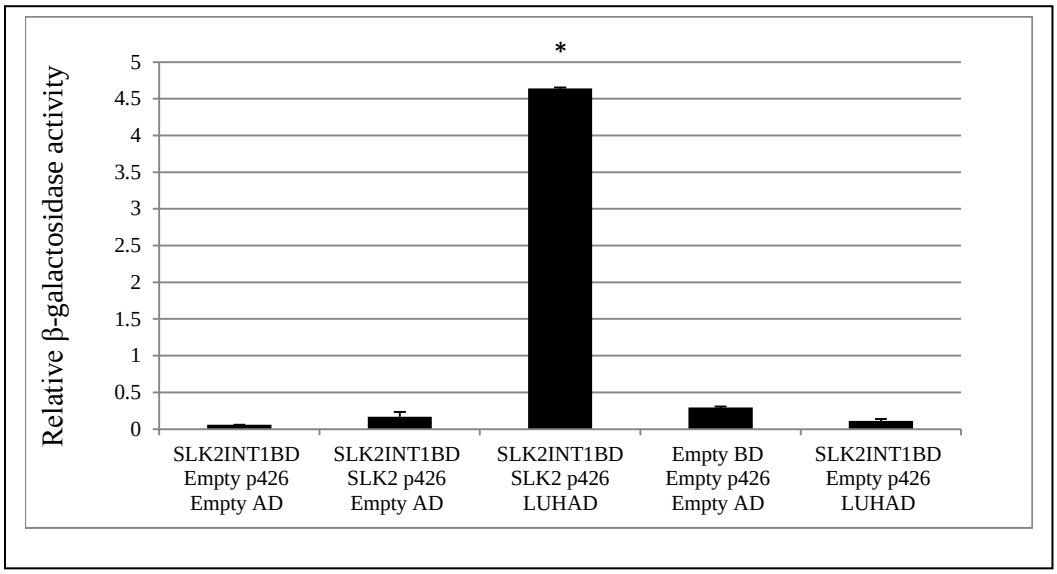


Fig. 16 Relative β-galactosidase activity in yeast three hybrid assay for SLK2 and SLK2INT1 interaction

In this assay SLK2INT1 was expressed fused to Gal4 BD, LUH was fused to Gal4AD and SLK2 was expressed as a separate protein. Cells from the test sample carried all three proteins and control cells carried one, two or none of the proteins. The β-galactosidase activity was normalized with total protein concentration determined using Bradford assay for each sample. The assay for each sample was repeated three times with different colonies. Asterisks indicate values that are significantly different from all the other control experiments (*P <0.05 Student's t-test, n=3).

3.7 Identification of SLK2INT1 target genes

To identify the possible targets of SLK2INT1, the expression levels of some selected stress responsive genes were measured. RNA from *slk2int1* mutant plants were isolated and used in quantitative reverse transcriptase PCR (qRT-PCR). For comparison Col-0 plants and *slk2* mutant plants were also used. Among the investigated genes significant increase in the RD20, COR15A, MYB2 and AMY3 genes was observed (fig. 17) indicating that they could be targets of SLK2INT1.

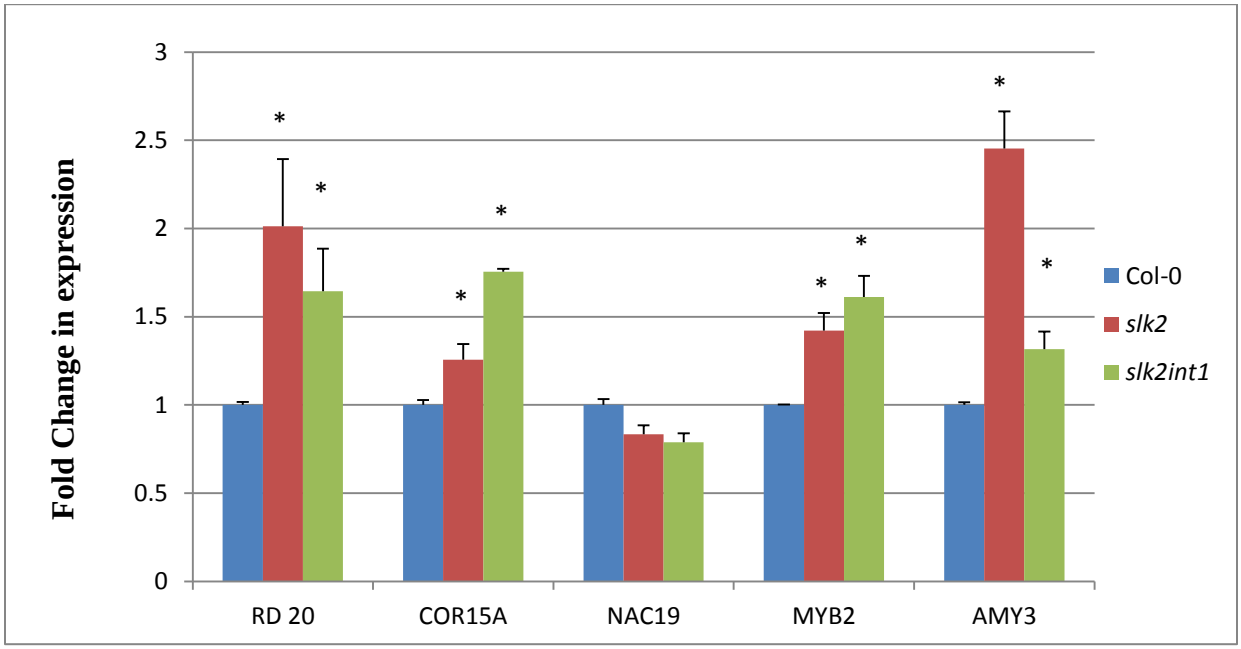


Fig. 17 Comparison of expression levels of selected stress responsive genes between Col-0 control, *slk2* mutant and the *slk2int1* mutant

The mRNA was isolated from tissues of *slk2*, *slk2int1* and Col-0 control plants as described in methods and were quantified in qRT-PCR. Asterisks indicate values that are significantly different from Col-0 controls (*P <0.05 Student's t-test).

3.8 GUS reporter assay for *SLK2INT1* expression

It was also interesting to find out if the *SLK2INT1* expression was confined to any particular type of tissues and a specific growth stage of the plant. Therefore, a GUS (β-glucoronidase) reporter assay was carried out in which *SLK2INT1* promoter region was fused with the β- glucoronidase coding sequence and transformed into wild type plants. Transformed plants were selected and stained to assay for the GUS activity. Plants transformed with the *SLK2* promoter-*GUS* construct and the *LUH* promoter–*GUS* construct were also used in the

assay for comparison. In all three constructs, the whole plant was exhibited GUS activity indicating that all three proteins are expressed ubiquitously in the plant with similar expression patterns (see fig. 18 and 19).

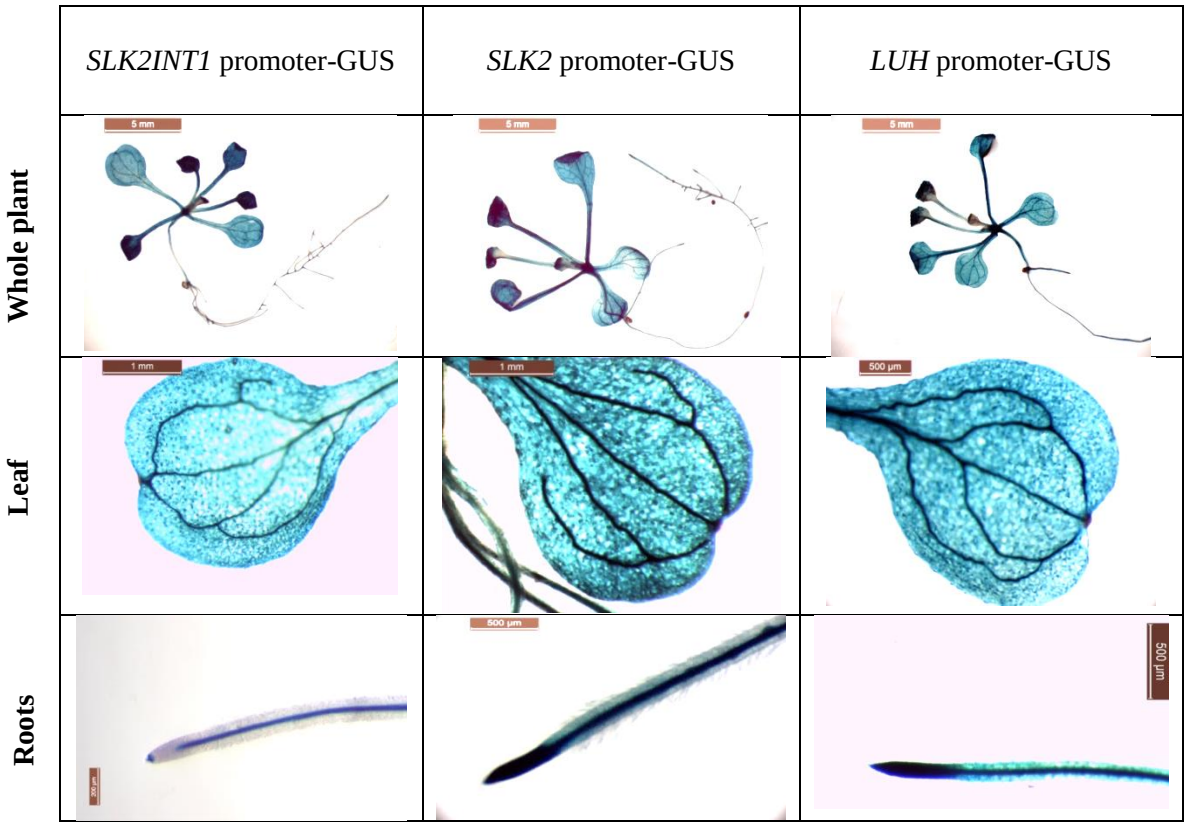


Fig. 18 Transgenic plants carrying promoter-GUS reporter constructs stained for GUS activity.

Fourteen days old young transgenic plants expressing GUS driven by gene promoters were used. Staining was carried out as described in the materials and methods and observed under light microscope attached to a camera.

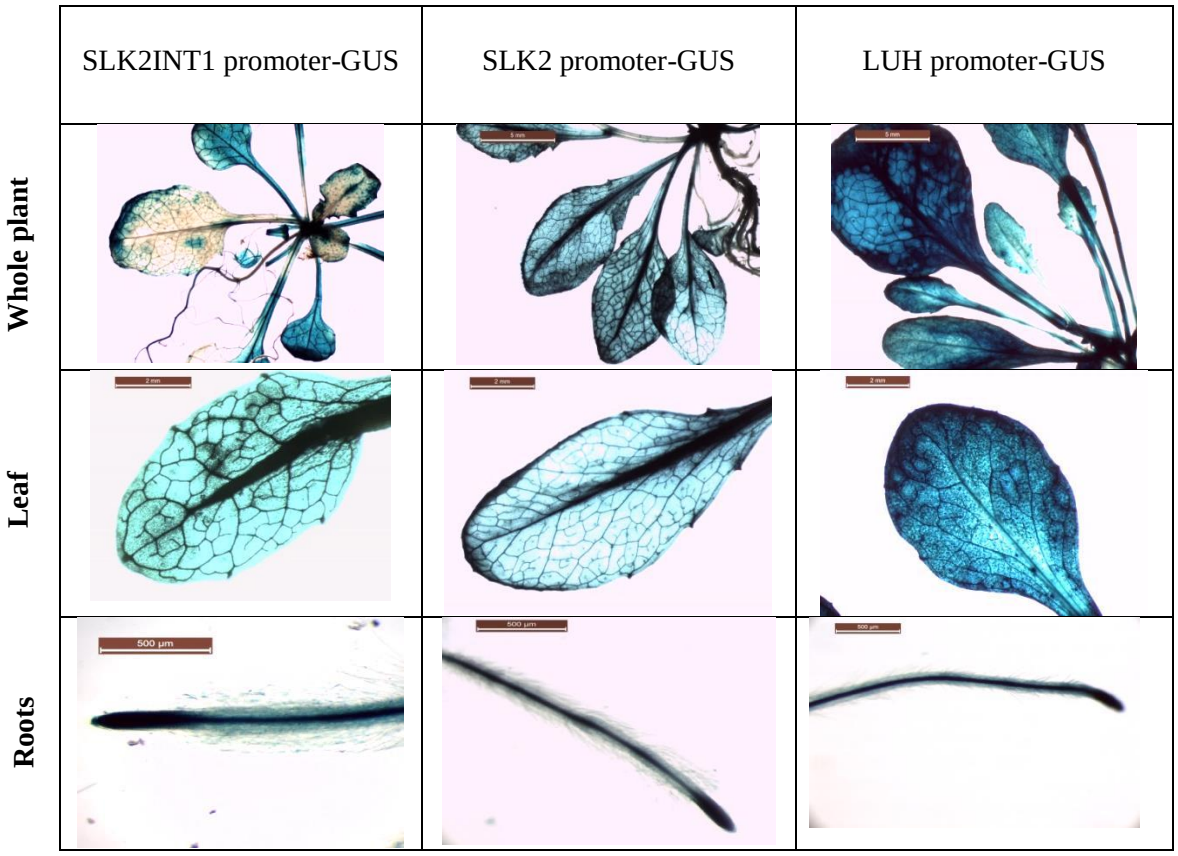


Fig. 19 Transgenic plants carrying promoter-GUS reporter constructs stained for GUS activity.

Twenty eight days old matured transgenic plants expressing GUS driven by gene promoters were used. Staining was carried out as described in the materials and methods and observed under light microscope attached to a camera.

3.9 GFP localization assay for SLK2INT1

Since the co-repressor complex is recruited to genes on chromosomal DNA for repression, the transcription factor SLK2INT1 should be localized to the nucleus. To determine this SLK2INT1 was fused to the Green Fluorescent Protein (GFP) and expressed in *Arabidopsis* protoplasts. The fusion protein was observed in fluorescent microscope and as expected it was found to be compartmentalized in the nucleus (fig. 20).

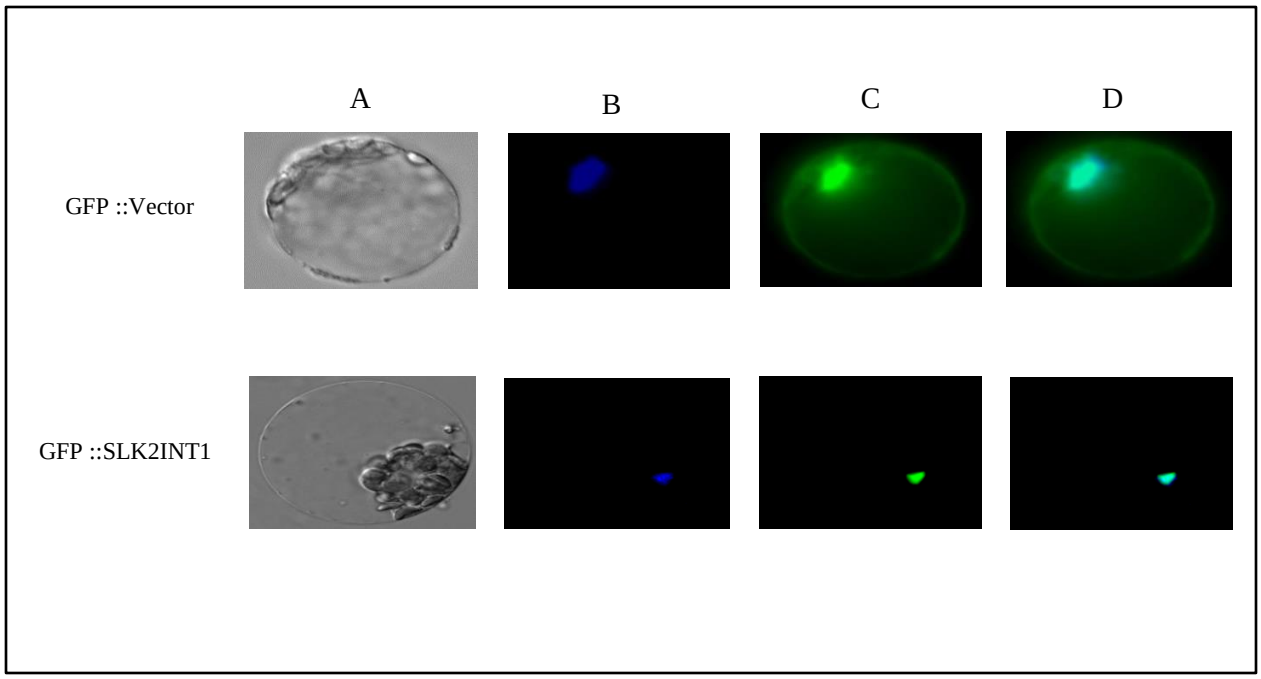


Fig. 20 Subcellular localization of SLK2INT1 fused with GFP

(A) Phase-contrast image of the protoplast. **(B)** Protoplast stained with DAPI for the visualization of the nucleus. **(C)** Protoplast image of GFP visualization**. (D)** Merged image of B and C. Shown in the top row are the protoplasts transformed with vector expressing free GFP and in the bottom row are the protoplasts transformed with vector expressing GFP fused SLK2INT1.

3.10 Repression assay for the ternary complex

To demonstrate that SLK2, SLK2INT1 and LUH form a ternary complex capable of actively repressing a target gene, a repression assay was performed. All three proteins were expressed in *Arabidopsis* protoplast containing a reporter system in which the LUCIFERASE (LUC) reporter was fused to a 5 X MYB binding sites and a TATA box. It was observed that luciferase expression drastically increased when SLK2INT1 is expressed alone and it is significantly reduced when all the three proteins were present (fig. 21).

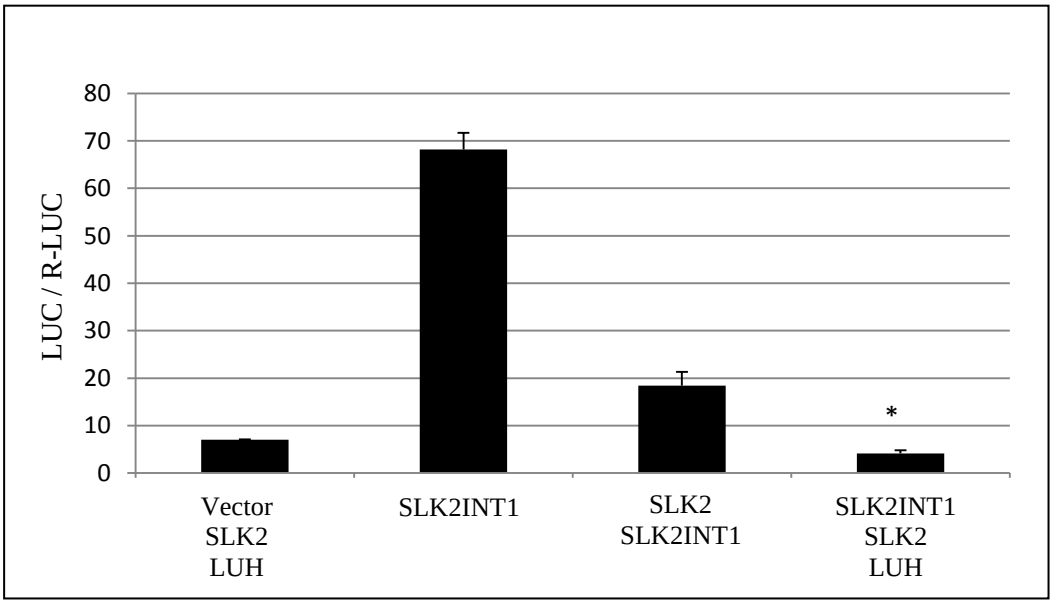


Fig. 21 Comparison of luciferase activity in *Arabidopsis* protoplast, expressing different combinations of proteins in the LUH co-repressor complex

Reporter construct containing 5X MYB binding site with luciferase and Renilla luciferase was mixed for transfection in Arabidopsis protoplasts. The ratio of LUC/RLUC was used to indicate relative reporter gene activity and control for transfection efficiency. Ten micrograms of 35S::SLK2In1 , 35S::SLK2 and 35S::LUH- DNA was used for transfection assays. Asterisks indicate values that are significantly different from assays expressing only SLK2INT1 and assays expressing only SLK2INT1 and SLK2. (*P <0.05 Student's t-test, n=3).

4. Discussion

It has been demonstrated that the LUH-SLK co-repressor complex plays a significant role in abiotic stress tolerance in *Arabidopsis* (35). Arabidopsis genome contains three SLK genes. They are SLK1, SLK2 and SLK3 (39). Our work was focused on SLK1and SLK2, because mutants of *SLK-1* and *SLK-2* exhibited increased tolerance towards salt and osmotic stress similar to mutants of *LUH-4* (35). When recruited to a control region, LUH promotes the silencing of the downstream gene by recruiting a histone deacetylase (35). However proteins of this complex do not contain DNA binding domains and the mechanism by which they are recruited to target genes remains a mystery. The specific aim of this research was to identify transcription factor(s) involved in recruiting the LUH/SLK co-repressor complex to its target genes.

Yeast two hybrid assays were used to identify all the *Arabidopsis* proteins that interact with SLK1, SLK2. An *Arabidopsis* cDNA library construct was used as the prey and yeast colonies were observed in high stringency plates indicating strong interactions between these prey proteins and baits (SLK1 and SLK2). Out of all the interactors, proteins located in the nucleus were examined in great detail because transcription factors should be localized to the nucleus. Secondly, whether those proteins had a known function in abiotic stress response was also examined.

Similarly another yeast two hybrid screen was performed to identify LUH interactors. One objective of this experiment was to identify any transcription factor which can directly bind to LUH without the need of a SLK adaptor. Another objective was to identify any protein which is involved in the removal of LUH from the cells, because the mechanism by which cells under stress remove LUH to activate stress responsive genes has not been solved yet. One of the

interesting proteins which interact with LUH, in yeast which was not further studied in this work is AT3G61050 (appendix). It is a calcium dependent lipid binding protein which has been identified as a transcriptional repressor involved in drought and salt tolerance in *Arabidopsis thaliana* (40). It is possible that this repressor binds to LUH to recruit a histone deacetylase to silence its target genes. Therefore in the future it will be important to further investigate this transcription factor.

Two of the interesting SLK1 interactors are AT2G19430 and AT5G59080. AT2G19430 is a nuclear protein found to be a negative regulator of the signaling pathway of ABA (abscisic acid), the main plant hormone involved in stress response (41). It is possible that this protein acts as a transcription repressor to recruit histone deacetylase via SLK1-LUH co-repressor complex to promote the silencing of ABA responsive genes. AT5G59080 is also an interesting SLK1 interactor because it is an unknown protein localized to the nucleus and it responds to oxidative stress (42). Even though SLK1 interactors were not further studied in this work, both AT2G19430 and AT5G59080 should be investigated to elucidate any function related to *Arabidopsis* stress response.

Out of all the SLK2 interactors AT2G39720, AT2G42300, AT1G06760 and AT3G58630 (SLK2INT1) were selected for further studies (see table 1). The protein AT2G39720 is classified as a ring-domain E3 ubiquitin ligase which also has a motif named domain of unknown function (DUF) in the c-terminal region (43). There are two more ring- domain E3 ubiquitin ligases containing the DUF motif in *Arabidopsis* ; AtRDUF1 and AtRDUF2 both are induced by drought and abscisic acid (43). For this reason it was interesting to see if AT2G39720 plays a role abiotic stress response. The protein AT1G06760 was also found to interact with SLK2 in yeast assays and was localized to the nucleus (44). It is annotated as a

winged-helix DNA binding transcription factor by TAIR (analysis reference: 501756968). Interestingly, AT1G06760 was also found to interact with SLK1 in yeast suggesting that this transcription factor might be involved in recruitment of both SLK1 and SLK2 adaptors to the target genes.

AT2G42300 is another interesting SLK2 interactor because it has been identified as a DNA binding transcription factor with an unknown function (45, 46) which is localized to the nucleus (44). The mutants of AT2G39720, AT2G42300 and AT1G06760 were obtained and their phenotypes were analyzed. Since these proteins were hypothesized to be negative regulators/repressors of stress responsive genes, mutant plants were expected to show increased tolerance towards salt and osmotic pressure (mimics drought). In contrast, the mutants had same length roots and shorter roots compared to Col-0 control plants in salt and osmotic stress induced plates respectively (fig. 8), suggesting that these proteins do not function as negative regulators on *Arabidopsis* stress responsive genes. However all these mutants showed shorter roots in osmotic stress plates, suggesting that these proteins might be positive regulators of osmotic stress. They should be further investigated to determine if they actually function as transcriptional activators of osmotic stress in *Arabidopsis thaliana*. The ring-domain E3 ubiquitin ligase AT2G39720 could be involved in the removal of SLK2 from the plant cell via ubiquitin proteasome pathway (UPP) to activate stress responsive genes.

Another interesting SLK2 interactor which should be further investigated is the late embryogenesis abundant (LEA) protein AT2G30505 (appendix). It does not appear to be a transcription factor and therefore was not further examined in this work. However LEA proteins are known to be induced under freeze, salt and drought to promote stress tolerance (47). They have been reported not only in plants, but also in some invertebrates, bacteria and cyanobacteria

(48). Therefore, it would be interesting to see if this LEA protein interacts with SLK2 to promote stress tolerance in *Arabidopsis*.

The protein AT3G58630 was also chosen for further analysis because it is a novel DNA binding transcription factor (45) localized to the nucleus (44) with an unknown biological function. This protein is reported to be expressed in many plant tissues including leaves, stem, roots, seeds and flowers (49). To see if *AT3G58630* plays a role in abiotic stress response, mutants of this gene were obtained and subjected to salt and osmotic stress together with *slk2* mutants and Col-0 control plants (fig. 9) for comparison. As expected *at3g58630* mutants and *slk2* mutants exhibited similar phenotypes with longer roots indicating increased tolerance towards salt and osmotic stress (fig. 10). This result suggests that AT3G58630 could be a negative regulator of stress response and SLK2 and AT3G58630 are likely to function in the same genetic pathway.

The *at3g58630* (*slk2int1*) mutants used for the above experiment were obtained from Arabidopsis Biological Research Center (ABRC). Since the mutation of the gene had been performed by T-DNA insertion, there is a possibility of the T-DNA being inserted to a random gene other than *SLK2INT1*. To demonstrate that the mutant's stress resistant phenotype was not due to a mutation of a random gene but of *SLK2INT1*, a complement was created by reintroducing the *SLK2INT1* gene with its own control region. The phenotype of this complement was analyzed, and it was found to be sensitive towards NaCl (salt stress) and mannitol (osmotic stress) as indicated by shorter roots which were not significantly different from that of Col-0 control plants (fig. 11and 12). The fact that the reintroduction of *SLK2INT1* was able to rescue the mutant phenotype confirms the stress resistance observed in the

*slk2int1*mutants was indeed due to the lack of function of *AT3G58630* (*SLK2INT1*) and not of any other gene mutated by random incorporation of T-DNA.

The domain analysis of AT3G58630 with SMART (Simple Modular Architecture Research Tool) web resource (http://smart.embl.de/) revealed that SLK2INT1 contains a MYB DNA binding domain from residue 23 to 126 (see fig. 13). DNA binding proteins with at least one MYB domain repeat are collectively known as MYB transcription factors. These transcription factors play diverse roles in plants including abiotic stress response and in *Arabidopsis* they comprise 9% of total transcription factors (45). The MYB repeat consists of 52 amino acids forming three α-helices with the second and the third helix forming a helix-turn-helix (HTH) structure (50). MYB TFs can include one to four of this repeat sequences. Since SLK2INT1 has only one MYB repeat, it can be classified into the MYB-related subgroup with its members containing only a single or a partial repeat (50). The crystal structure of the SLK2INT1 has not been resolved to date. Therefore the SWISS-MODEL protein structure homology-modelling server was used to build a model based on SLK2INT1's homology to the known protein structures (36-38) and as expected the model had three α-helices in the region from 22 to121 amino acids (fig. 14). This result further sugessts that SLK2INT1 is a MYB-related protein.

The yeast two hybrid assays performed with full length SLK2 and SLK2INT1 showed that these proteins physically interact with each other in the yeast system (fig. 15). Previously it was demonstrated that SLK2 also interacts with LUH to form a co-repressor complex (35). To show that SLK2INT1 can bind to this co-repressor complex via SLK2, a yeast three hybrid was performed. Results from this assay indicated that SLK2 indeed functions as an adaptor between LUH and SLK2INT1 suggesting that SLK2INT1 could function as a transcriptional repressor to

recruit the SLK2/LUH co-repressor complex to the stress responsive genes (fig. 16). A very similar mechanism from *Arabidopsis* would be SEUSS functioning as an adaptor between different transcription factors (AP1/AGL24/SEP3) and LUG to regulate the expression of *AGOMOUS* (25) in floral development. Another example would be the co-repressor TOPLESS (TPL) acting as a bridge between AP2 and HDA19, again in *Arabidopsis* in floral development (51).

In an attempt to identify targets of SLK2INT1, quantitative RT-PCR analysis of mRNA from *slk2int*1mutants, *slk2* mutants and Col-0 control plants revealed that the removal of SLK2 or SLK2INT1 elevates expression of four selected stress responsive genes (fig. 17). They are *RD20, MYB2, COR15A* and *AMY3*. All the genes under investigation had MYB binding sites in their control regions (52). *Arabidopsis* RD20 is a well-studied transcription factor involved in stomatal control and transpiration shown to be induced by dehydration, salt and ABA (53). MYB2 is also a transcription factor involved in the regulation of several genes in response of dehydration and high salinity (54, 55). The gene *COR15A* is induced by cold, drought and ABA, and its product is targeted to the chloroplast to prevent its proteins from aggregation (56, 57). The gene *AMY3* encodes an α-amylase and is a homolog of AMY1which is induced in abiotic stress (58). Our results indicate that mutation in either SLK2 or SLK2INT1 increases the expression of *RD20, MYB2, COR15A* and *AMY3* suggesting that SLK2INT1 acts as a repressor on these genes. Shrestha *et al.* reported that *RD20* and *MYB2* are expressed at elevated levels also in *slk2* and *luh* mutants. This, together with our results suggests that *RD20* and *MYB2* are repressed by SLK2INT1 via the recruitment of the LUH-SLK2 complex.

As mentioned earlier, a global gene expression analysis of *Arabidopsis* revealed that *SLK2INT1* is expressed in most of the tissues including leaves, roots and stems (49). To confirm

this *SLK2INT1*promoter region was fused to GUS reporter gene and as expected GUS activity was detected in the whole plant (see fig. 18 and 19). The universal expression of this transcription factor suggests that it could be playing a major role in the repression of *Arabidopsis* stress responsive genes in all tissues.

SLK2INT1 was annotated as a nuclear localized protein based on the machine-based protein localization predictor AtSubP (40). Even though this *Arabidopsis* specific AtSubP is claimed to predict protein localization more accurately than other general tools, confirmation with real experimental data was important. Therefore a subcellular localization assay was performed with SLK2INT1-GFP fusion protein, it was found to be localized to the nucleus (see fig. 20).

The yeast three hybrid assay indicated that SLK2, LUH and SKL2INT1 can form a complex in the yeast system. To demonstrate that this complex is formed in plant cells and that it can repress an actively transcribed gene, a repression assay was performed with *Arabidopsis* protoplasts. It was observed that when SKL2INT1 was expressed alone, the LUCIFERASE reporter activity increased drastically compared to the control (fig. 21). This suggests that SKL2INT1 can function as a transcriptional activator when SLK2 and LUH are absent. When SLK2 was introduced, a significant decrease in the reporter activity was observed. This was probably due to the masking of the activation region of SKL2INT1 by SLK2. However, when all the three proteins were expressed together the reporter activity decreased drastically, even below the level of leaky expression. This result confirms that these three proteins work together to repress gene expression in *Arabidopsis*. It is possible that SKL2INT1 works as both a transcription activator and a repressor in *Arabidopsis*. Based on these observations it can be proposed that under normal growth conditions SKL2INT1 can recruit the SLK2-LUH complex

to the stress responsive genes *RD20* and *MYB2* to promote repression. And it is possible that under salt and osmotic stress SLK2 and LUH are removed from the ternary complex by an unknown mechanism to turn SKL2INT1 into a transcriptional activator, facilitating a high level of gene expression.

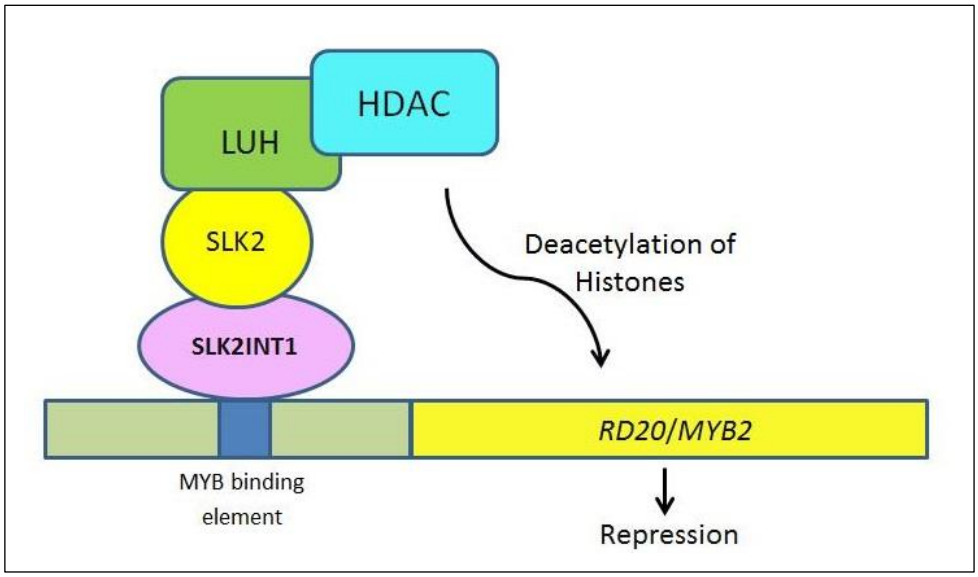


Fig. 22 Model for repression of RD20 and MYB2 genes by LUH-SLK2 complex

SLK2INT1 binds to the MYB binding sequence of the RD20 and MYB2 promoter and recruits the LUH-SLK2 complex. LUH then recruits HDAC to repress the downstream region by deacetylation of histones (adapted from Shrestha *et al*. 2014).

Conclusions

This study reports SLK2INT1 is a novel transcription factor involved in silencing of *Arabidopsis* stress responsive genes. Mutation in *SLK2INT1* enhances the salt and drought tolerance of *Arabidopsis* plants. Evidence from protein interaction assays and repression assays suggest that the mechanism of repression involves the recruitment of the LUH-SLK2 co-repressor complex and SLK2 act as an adaptor protein between LUH and SKL2INT1. Gene expression analysis from mutant plants suggests that targets of SKL2INT1 may include *RD20, MYB2*. A chromatin immunoprecipitation (CHIP) assay on tissues from a transgenic construct expressing a tagged SKL2INT1 will reveal more of its target genes and confirm existing ones. Furthermore follow up on SLK1 interactors will lead to the discovery of more transcription factors working with LUH-SLK1 co-repressor complex to repress specific genes. Also LUH interactors revealed from Yeast two hybrid screening should be followed up to identify any transcription factors that interact directly with LUH to promote repression.

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Appendix

List of Primers

Name	Description	Sequence
Gal4BD Forward	Colony PCR for bait constructs yeast two hybrid Assay	GGTCAAAGACAGTTGACTGTATC
SLK1 Reverse	Colony PCR for bait constructs in yeast two hybrid Assay	GTTGCCTCAGTCTCTGCTGCTGAAG
SLK2 Reverse	Colony PCR for bait/prey constructs in yeast two hybrid Assay	CCACCTAGCTGAACACTAGGATC
LUH4 Reverse	Colony PCR for bait constructs in yeast two hybrid Assay	GCAGCCAAAGCACTAGCATTAGAC
Gal4AD Forward	Colony PCR for prey constructs yeast two hybrid Assay and identification of prey proteins in Yeast two hybrid screening	CTATTCGATGATGAAGATACCCCACC AAACCC
Gal4AD Reverse	Identification of prey proteins in Yeast two hybrid screening	GTGAACTTGCGGGGTTTTTCAGTATC TACGATTC
SLK2p42 5GPD_F	Cloning of SLK2 to p426 yeast three hybrid vector	TAGAACTAGTGGATCATGGCTTCTTC AACTTCTGGG
SLK2p42 5GPD_R	Cloning of SLK2 to p426 yeast three hybrid vector	CGGTATCGATAAGCTTCATGACTTCC AAGAATATCCTCCC
24733CS _ LP	Genotyping AT2G39720 mutants	TTTTGCAAATATGTTAAGCAATTTG
24733CS _ RP	Genotyping AT2G39720 mutants	CAAGAGTTACGAATCGCAAGC
92968CS _LP	Genotyping AT2G42300 mutants	GGCTGAACAATAACGACCTTG
92968CS _RP	Genotyping AT2G42300 mutants	CCGTTTTGCTCTGTAGCAATC
128430C _LP	Genotyping AT1G06760 mutants	TTGAAATCCCACGTTTATTGG
128430C _RP	Genotyping AT1G06760 mutants	GGGAGTTTAAACGAGGCTTTG
TFSLK2_ LP	Genotyping AT3G58630 mutants	AGTTCGCGTTACCCATTTAGC
TFSLK2_ RP	Genotyping AT3G58630 mutants	CCTCTTGCCAGTGTTTCTGAC
Lba1	Genotyping all mutants, binds to T-DNA insertion	TGGTTCACGTAGTGGGCCATCG

Name	Description	Sequence
SLK2int1 PromoF	Cloning SLK2INT1 promoter and the full gene to PCR8/GW/TOPO vector	ACAAAGAAAAAAGAGCAAACTAC AGTTTTTGC
SLK2int1 PromoR	Cloning SLK2INT1 promoter and the full gene to PCR8/GW/TOPO vector	TATAAGGTGAACGACGGCGAGTTA GATCG
SLK2int1 _FL_R	Cloning SLK2INT1 full gene to PCR8/GW/TOPO vector	GAAGTAACTAGGGAAATCCATCAT CCCATA
SLK2int1 _GFP_F	Cloning of SLK2INT1 cDNA to pXDG vector	CGCGGGCCCGGGATCCATGGACAC CGTCAACGATTCC
SLK2int1 _GFP_R	Cloning of SLK2INT1 cDNA to pXDG vector	TAGATCCGGTGGATCCTAGAAGTA ACTAGGGAAATCC
SLK2_L UC_F	Cloning of SLK2 for the repression Assays	TACTCGAGGGGGATCATGGCTTCTT CAACTTCTGGGA
SLK2_L UC_R	Cloning of SLK2 for the repression Assays	ATTCGCTAGTGGATCTCATGACTTC CAAGAATATCCTCCC
LUH_LU C_F	Cloning of SLK2 for the repression Assays	TACTCGAGGGGGATCATGGCTCAGA
LUH_LU C_R	Cloning of SLK2 for the repression Assays	ATTCGCTAGTGGATCCTACTTCCAA
SLK2int1 _LUC_F	Cloning of SLK2 for the repression Assays	TACTCGAGGGGGATCATGGACACC GTCAACGATTCC
SLK2int1 _LUC_R	Cloning of SLK2 for the repression Assays	ATTCGCTAGTGGATCCTAGAAGTA ACTAGGGAAATCC
RD20RT-PCRF	Quantification of gene expression in qRT-PCR	CCGAAGGAAGGTATGTCCCA
RD20RT-PCRR	Quantification of gene expression in qRT-PCR	GTTTGCGAGAATTGGCCCTC
MYB2RT -PCRF	Quantification of gene expression in qRT-PCR	CAACGATTGGGGCTGTGTTG
MYB2RT -PCRR	Quantification of gene expression in qRT-PCR	TCAGGGGATTAAAACAAGAGAGGA
NAC019 RT-PCRF	Quantification of gene expression in qRT-PCR	TAACCCAAACCGCATCTCGT
NAC019 RT-PCRR	Quantification of gene expression in qRT-PCR	ACTTGCCCCGAATACCCAAA
ACT2RT-PCRF	Quantification of gene expression in qRT-PCR	GATCTCCAAGGCCGAGTATGAT
ACT2RT-PCRR	Quantification of gene expression in qRT-PCR	CCCATTCATAAAACCCCAGC
COR15R T-PCRF	Quantification of gene expression in qRT-PCR	AAGGTGACGGCAACATCCTC

COR15A RT-PCRR	Quantification of gene expression in qRT-PCR	CTCTCCTGCTTTACCCTCCG
MYB2RT-PCRF	Quantification of gene expression in qRT-PCR	CAACGATTGGGGCTGTGTTG
MYB2RT-PCRR	Quantification of gene expression in qRT-PCR	TCAGGGGATTAAAACAAGAGAGGA
AMY3RT-PCRF	Quantification of gene expression in qRT-PCR	CAGGAACAGACAGAAACTCCACTG
AMY3RT-PCRF	Quantification of gene expression in qRT-PCR	GATGTTTCCCACACCTTGTAGTC

List of proteins which interact with SLK1 in yeast

Gene (ATG#)	**Description**
AT2G17420	NADPH-dependent thioredoxin reductase, major cytosolic isoform
AT5G14970	An unknown protein; BEST Arabidopsis thaliana protein match is: unknown protein (TAIR:AT2G14910.1)
AT2G47590	Photolyase/blue light photoreceptor (PHR2)
AT5G10470	Kinesin that binds cyclin-dependent kinase, demarcates the division site in plant cells.
AT3G47060	An FtsH protease that is localized to the chloroplast
AT1G06760	A winged-helix DNA-binding transcription factor family protein; has DNA binding ability, involved in nucleosome assembly, located in nucleus,
AT1G70760	A subunit of the chloroplast NAD(P)H dehydrogenase complex, involved in PSI cyclic electron transport, Located on the thylakoid membrane, mutant has impaired NAD(P)H dehydrogenase activity
AT1G79160	An unknown protein
AT3G11670	A protein responsible for the final assembly of galactolipids in photosynthetic membranes. Provides stability to the PS I core complex
AT1G08970	Nuclear factor Y, subunit C9 cytoplasm, cytosol, intracellular, located in nucleus, has sequence-specific DNA binding transcription factor activity
AT5G41970	Metal-dependent protein hydrolase with unknown function, located in mitochondrion
AT3G14100	RNA-binding (RRM/RBD/RNP motifs) family protein, has mRNA 3'-UTR binding ability
AT2G19430	This gene is predicted to encode a protein with a DWD motif, may be involved in the formation of a CUL4-based E3 ubiquitin ligase, DNA

	repair, gene silencing by RNA, negative regulation of abscisic acid mediated signaling pathway, negative regulation of photomorphogenesis, production of ta-siRNAs involved in RNA interference, protein ubiquitination, reproductive structure development, located in cytoplasm and nucleus
AT1G21700	A member of the *Arabidopsis* SWI3 gene family. Protein physically interacts with ATSWI3B and ATSWI3A, the other two members of the SWI3 family. Homologous to yeast SWI3 & RSC8, components of the SWI/SNF and RSC chromatin remodeling complexes. Referred to as CHB3 in Zhou et al (2003)
ATCG00520	A protein required for photosystem I assembly and stability. In cyanobacteria, loss of function mutation in this gene increases PSII/PSI ratio without any influence on photoautotrophic growth.
AT2G14255	An Ankyrin repeat family protein with DHHC zinc finger domain, has zinc ion binding activity, located in Golgi apparatus and membrane
AT4G12040	An A20/AN1-like zinc finger family protein, has DNA binding and zinc ion binding activity
AT5G46630	A clathrin adaptor complexes medium subunit family protein
AT1G20225	A thioredoxin superfamily protein with unknown function
AT5G59080	An unknown protein, response to oxidative stress, located in nucleus
AT1G72510	A protein of unknown function located in nucleus

List of proteins which interact with LUH in yeast

Gene (ATG#)	**Description**
AT5G67500	A voltage-dependent anion channel
AT2G31300	A protein involved in actin filament organization, regulation of actin filament polymerization, has nucleotide binding activity
AT1G18650	A member of the X8-GPI family of proteins, localized to the plasmodesmata and is predicted to bind callose
AT1G70760	a subunit of the chloroplast NAD(P)H dehydrogenase complex, Chlororespiratory reduction 23(CRR23), Also known as NADH Dehydrogenase-like complex L, (NDHL)
AT1G58235	unknown protein located in mitochondrion
AT5G05600	A 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, has oxidoreductase activity and ion binding activity, involved in response to salt stress, response to karrikin
AT3G61050	A novel transcriptional regulator, a calcium-dependent lipid-binding protein containing a C2 domain, that binds specifically to the promoter of thalianol synthase 1 (THAS1). It can bind ceramide and is involved in drought and salt tolerance, located in endoplasmic reticulum, nuclear membrane, plasma membrane

AT4G14160	A Sec23/Sec24 protein transport family protein, has transporter activity and zinc ion binding activity, involved in intracellular protein transport, transport, ER to Golgi vesicle-mediated transport, response to salt stress located in COPII vesicle coat
AT4G28610	Similar to phosphate starvation response gene from Chlamydomonas. Weakly responsive to phosphate starvation / nucleus
AT1G06040	B-box domain protein 24(BBX24), involved in salt tolerance, golgi organization, glycolysis, hyperosmotic response, photomorphogenesis, response to abscisic acid stimulus, response to cadmium ion, response to karrikin, response to light stimulus, response to salt stress, response to temperature stimulus, water transport intracellular, located in nucleus
AT4G19700	Botrytis susceptible 1 interactor (BOI). Has E3 ubiquitin ligase activity. Interacts with and ubiquitinates BOS1 (Botrytis Susceptible 1), prevents caspase activation and attenuates cell death, located in cytoplasm, nucleus
AT2G38240	A 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, has oxidoreductase activity, involved in response to salt stress
AT3G01230	unknown protein with unknown function, located in endomembrane system
AT3G10290	A nucleotide-sugar transporter family protein of unknown function
AT3G10470	C2H2-type zinc finger family protein, a sequence-specific DNA binding transcription factor, involved in regulation of transcription
AT5G67070	RALF-LIKE 34, RALFL34/ Member of a diversely expressed predicted peptide family showing sequence similarity to tobacco Rapid Alkalinization Factor (RALF), located in apoplast, extracellular region
AT3G56460	GroES-like zinc-binding alcohol dehydrogenase family protein, has oxidoreductase activity, located in peroxisome

Vita

Achira Weerathunga was born in Colombo, Sri Lanka. He obtained his Bachelor's degree on Biochemistry and Molecular Biology from University of Colombo in 2007. In 2012 He started the Master's program in Biology at University of New Orleans and became a member of the Dr. Vaniyambadi Sridhar's research group.