Search for Novel Factors Affecting Nuclear Structure and Function in *Arabidopsis thaliana*

Honors Thesis
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Abstract

This thesis project searches for novel factors affecting nuclear structure and function in the plant model organism, *Arabidopsis thaliana.* The nuclear lamina is of critical importance to the cell as it has a multitude of roles. Its various functions include nuclear transport, involvement in signaling pathways, and chromatin organization. The structure of the nuclear lamina impacts its function, so an in depth understanding of the proteins composing the nuclear lamina is imperative. The well-studied animal nuclear lamina is composed of lamin, yet plants lack lamin orthologs. However, the plant nuclear lamina contains analogous proteins that are structurally and functionally similar. One of these proteins, CRWN4 is encoded by a member of a small family of genes required to maintain the structure of the plant nucleus. This thesis project utilizes a suppressor screen of the *crwn4-2* mutation to find genes that could play a role in the structure and function of the plant nuclear lamina. The *crwn4-2* allele is a missense mutation that reduces the abundance of the protein in the nucleus. Through this project, I identified a mutation that suppressed the *crwn4-2* allele and restored normal protein levels in the nucleus. A series of genetic experiments were conducted to characterize the suppressor mutation and lay a foundation for its molecular identification. Through these genetic crosses, it was found that the suppressor allele is inherited in a semi-dominant manner and exhibits allele-specificity. Whole Genome Sequencing is currently being undertaken to locate the allele's position within the genome and identify a candidate gene. The findings of this thesis provide evidence that genetic suppressor screens can be used to identify novel genes and interactions affecting plant nuclear architecture.

Introduction

The nuclear lamina, a complex network of proteins that maintains the structure of the nucleus, provides the necessary physical scaffold for numerous cellular processes, such as chromatin organization, transcriptional regulation and spatial coordination of nuclear pore complexes (Gruenbaum et al., 2005; Mattout et al., 2015). Furthermore, the nuclear lamina anchors connections between the nucleus and the cytoskeleton, which are essential for nuclear movement within the cell (Chang, Worman, & Gundersen, 2015; Meinke & Schirmer, 2015). The nuclear lamina in plants, such as *Arabidopsis thaliana*, consists of coiled-coil domain proteins, called CRWN (CROWDED NUCLEI). The *CRWN* proteins are considered to be examples of Nuclear Matrix Constituent Proteins (NMCPs), named after prototypes first discovered in carrots (Masuda et al., 1997). NMCP and NMCP-related proteins are thought to be the major components of the reticulated complex that composes the plant nuclear lamina. Though it is not known for certain, the CRWN proteins likely dimerize and aggregate to form filaments, by analogy with other proteins containing extensive coiled-coil domains. CRWN proteins interact with the SUN-domain proteins that are inserted into the inner nuclear membrane and, via intermediary proteins, link the cytoskeleton to the nuclear lamina (Graumann, Runions, & Evans, 2010; Zhou, Groves, & Meier, 2015). Figure 1 illustrates the proteins of the plant nuclear lamina and their various interactions. While *CRWN* and *NMCP* are plant-specific, their coiled-coil domain structure and nuclear localization suggest their proteins' functions are analogous to those of the animal lamins (Ciska, Masuda, & Moreno, 2013). However, CRWN proteins share no amino acid similarity with these animal lamin proteins, suggesting that they may be an example of convergent evolution (Wang, Dittmer, & Richards, 2013). This evidence for convergent evolution is an indication that two different mechanisms or machineries exist to

encompass eukaryotic nuclei. Discoveries made by investigating protein interactions with CRWN proteins can inform us about the underlying principles of how coiled-coil domains function in both plant and animal nuclear laminas.

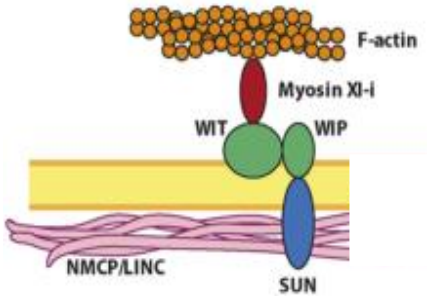

Figure 1: Illustration of proteins composing plant nuclear lamina. Alongside the CRWN and SUN proteins, WIP/WIT proteins are inserted into the outer nuclear membrane and anchor Myosin XI-i (Tamura et al., 2013). Actin filaments of the cytoskeleton then bind to Myosin XI-i. Image adapted from Ciska & Moreno (2014).

A small family of genes encode four different CRWN protein paralogs. CRWN 1, 2, and 3 are closely related and belong to one clade in phylogeny based on amino acid sequence similarity in the coiled-coil domain (see Figures 2 and 3), while CRWN4 and its orthologs in other species constitute a separate clade (Wang et al., 2013). This research project focuses on CRWN4, which plays a role in specifying the size and shape of the nucleus, as well as the number of chromocenters (large aggregates of silent chromatin) present during interphase (Sakamoto & Takagi, 2013; Wang et al., 2013). The *CRWN4* gene encodes a protein that is 1,042 amino acids long (ThaleMine, n.d.). Wild-type plants have spindle shaped nuclei and contain about 10 chromocenters per nucleus (corresponding to the diploid chromosome number). Mutant *crwn4* nuclei lose their structure and become smaller and more spherical in shape. These mutants can display a wide variety of chromocenter numbers, ranging from 2 to 27, as the heterochromatin either aggregates or breaks into smaller puncta (Wang et al., 2013). Beyond the nuclear alterations, there is no phenotypic effect on the overall plant's architecture, growth, or size. The *crwn4-2* allele, on which this research is centered, is the result of a non-conservative

missense mutation that changes a single amino acid from Lysine (K) to Leucine (L) at position 923 (see Figure 3). This mutation disrupts a possible nuclear localization signal (NLS) domain, but it is unknown whether CRWN4 relies on this NLS or gains access to the nucleus by interaction with CRWN 1, 2, or 3, which contain a well-defined and verified NLS motifs (Endia Blunt, personal communication). The importance of the putative NLS in CRWN4 will be addressed in this thesis.

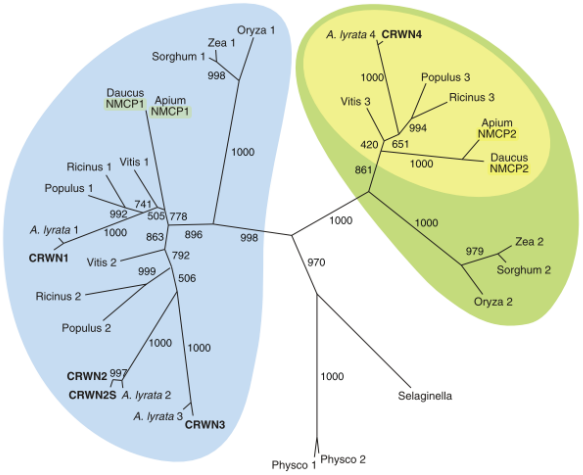

Figure 2: An illustration of the CRWN phylogenetic tree. Image from Wang et al. (2013).

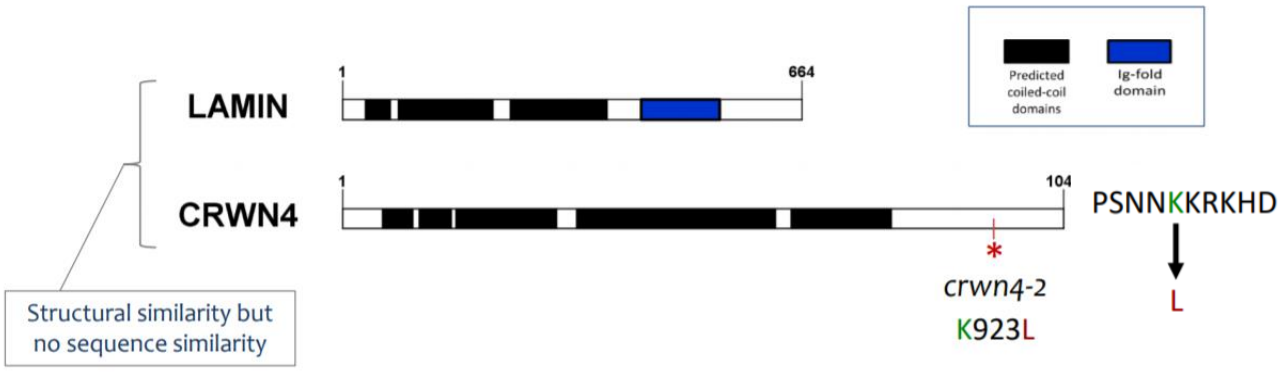

Figure 3: Depiction of an animal lamin protein and the CRWN4 protein. The animal and plant lamin demonstrate no similarity in amino acid sequence but still have similar function and share domain structure, indicating possible convergent evolution. The site of the *crwn4-2* mutation and its effect on the amino acid sequence is marked by an asterisk.

One approach to understanding the mechanism of CRWN4's action is to identify other proteins that interact, either functionally or physically, with this nuclear lamina protein. This

project is focused on identifying CRWN4 interactors through a genetic suppressor screen. Suppressor screens can identify novel genes in a pathway or connect known genes in a previously unidentified manner. In a study that analyzed hundreds of suppression experiment papers, the majority of interactions identified were novel (Friesen et al., 2016). Either new genes were discovered altogether, or proteins not thought to previously be related were found to have close interactions. Thus, a suppression experiment of *crwn4*-2 is a logical method for discovering novel genes or protein-protein interactions present at the plant nuclear lamina.

There are several types of suppressors that can be identified with this type of screen (see Figure 4), and each one could provide a better understanding of the function of CRWN4. For example, a suppressor could work by bypassing the original mutation through the use of a different pathway or through epistasis by altering the function of a pathway step downstream of the original mutation. These types of suppression would be the result of a suppressor mutation that is gene-specific and allele-nonspecific, meaning that this mutation would suppress any *crwn4* loss-of-function or hypomorphic mutation (*i.e.*, one with less gene function) (Guarente, 1993). Alternatively, the mutation might act as an interactional suppressor, in a situation where the two proteins physically interact, causing a compensatory conformation change in an interacting protein. In this case, the mutation would be gene- and allele-specific and only suppress the *crwn4-2* allele and no other *crwn4* mutations (Guarente, 1993). Conversely, the mutation could be an informational suppressor that alters the processing of the original mutation, often through RNAs. This type of suppressor typically behaves in a gene-nonspecific and allele-specific manner. (Guarente, 1993). Figure 4 illustrates these various forms of suppression.

This thesis describes the isolation and characterization of a *crwn4* extragenic suppressor mutation. My results support the hypothesis that the *crwn4-2* mutation affects the abundance of

mutant CRWN4 protein in the nucleus. I will discuss the different interpretations of my findings as well as possible next steps in characterization of the suppressor mutation and to understand its effect on the CRWN4 protein.

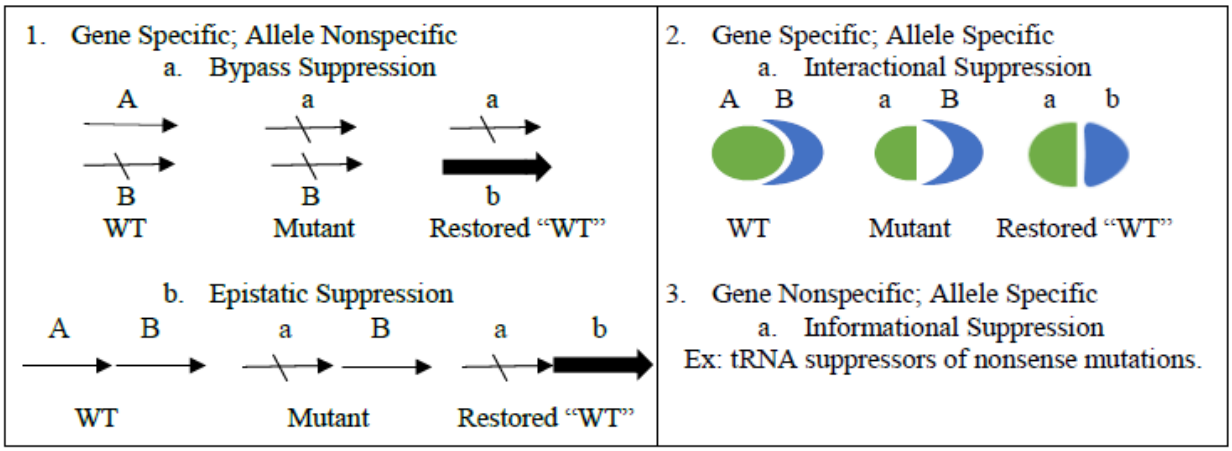

Figure 4: Depiction of each form of suppression as grouped by gene- and allele- specificity.

Materials and Methods

The starting population for this experiment was a Columbia strain of *Arabidopsis thaliana* homozygous for the *crwn4-2* mutation. This *crwn4-2* strain carries a NLS-GFP-GUS transgene within its genome. This transgene encodes a large protein called β-glucuronidase (GUS), which is tagged with a nuclear localization signal (NLS) and green fluorescent protein (GFP), allowing the protein to be visualized in the nucleus under a fluorescent microscope (Chytilova, Macas, & Galbraith, 1999). The size of the fusion protein ensures that it does not leak out of the nucleus (Chytilova et al., 1999). The transgene is expressed using a strong constitutive Cauliflower Mosaic Virus 35S promotor (Chytilova et al., 1999). To induce mutations that might suppress the *crwn4-2* phenotype, these seeds were treated with the chemical mutagen ethylmethansulfonate (EMS). The EMS-treated seeds were then grown to produce M1 plants, which were partitioned into pools of approximately 100 plants and self-pollinated to

generate M2 seeds segregating for induced mutations. Seedlings from the M2 generation were then screened, as described below, for either revertant or suppressor mutations that led to the formation of spindle-shaped or elongated nuclei. Figure 5 illustrates the process of EMS mutagenesis.

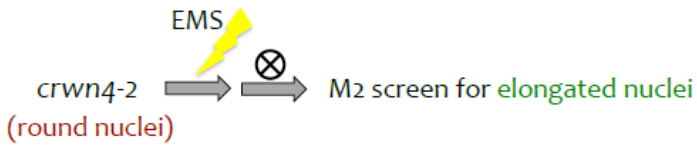

Figure 5: Illustration of the EMS treatment including the starting genotype and phenotype and the subsequent selfing process and M2 screening.

The M2 seeds were then plated onto 1X Murashige and Skoog (MS), 1% sucrose, 0.7% phytoagar plates and grown vertically in an environmental growth chambers under long-day conditions (16-hour light and 8-hour dark cycles) at approximately 23˚C at 60% relative humidity. About 20 seeds were sown on each plate and plates were cold treated at 4˚C overnight to ensure that the seeds germinated simultaneously. After one week of growth, the roots of the seedlings were phenotyped under a Leica M205 fluorescence stereomicroscope. True breeding wild-type and unmutagenized *crwn4-2* seeds were grown on separate plates as controls.

Approximately 900 M2 seedlings were screened from 4 pools and putative revertant/suppressor plants were transplanted from the MS phytoagar plates to soil and returned to grow in the environmental growth chamber. When transplanted plants began flowering, anther filaments were harvested, fixed in 3:1 ethanol:acetic acid solution and nuclei were stained with the fluorescent dye DAPI. Nuclei were imaged using a Leica DM5500B epifluorescence microscope to test whether or not the nuclear phenotype seen in the roots of the seedling was present in adult tissues.

Genomic DNA was extracted from plants using the urea lysis miniprep protocol (Cocciolone & Cone, 1993). To test the presence or absence of the original *crwn4-2* allele, two sets of primers were used on a wild-type plant, an unmutagenized *crwn4-2* plant, and a plant carrying the suppressor. A wild-type primer set (a4WTF and a41kbR2) recognized the wild-type *CRWN4* sequence. A mutant primer set (a4MutF and a41kbR2) recognized the same region of the gene, but included the original *crwn4-2* point mutation (a dinucleotide change, corresponding to the 3' end of the a4MutF primer). The entire *CRWN4* gene was also amplified and sequenced using standard Sanger sequencing technology from double-stranded PCR amplicon templates. To check for possible suppressor mutations in a known interactional partner, the *CRWN1* gene was also amplified and sequenced from the suppressor line. Supplementary Table 1 contains the sequences of the primers used to amplify and sequence these genes.

To conduct the various genetic crosses, the candidate M2 suppressor line was self-pollinated to yield M3 and M4 generations. Individuals in the M3 generation were crossed to true-breeding lines of *crwn4-1* and unmutagenized *crwn4-2*. The F1 seeds of each cross were sown on phytoagar and grown in the conditions detailed previously. The roots of the seedlings were phenotyped after one week and the anthers were phenotype when the plants began flowering. Segregating F2 families from backcrossed F1 individuals were generated and characterized. F3 lines were also generated and characterized as well. In the F3 generation, DNA was extracted from individuals in true-breeding families identified phenotypically as "spindle" and pooled together. The same was done for individuals in families that exhibited only "round" nuclei. Genomic DNA was extracted from these plants using a urea lysis miniprep protocol (Cocciolone & Cone, 1993). This DNA will be used for future Whole Genome Sequencing (WGS).

To test the abundance of CRWN4 protein in the nucleus, a western blot was conducted on wild-type, unmutagenized *crwn4-2*, and M3 suppressor seedlings. 50 seeds from each group were grown in 1X MS, 1% sucrose liquid cultures for 10 days in the dark at room temperature. Briefly, crude nuclear preps were prepared by homogenizing seedling tissue in Honda buffer (0.44 M sucrose,1.25% Ficoll, 2.5% Dextran T40, 20 mM HEPES-KOH pH 7.4,10 mM $MgCl_2$, 0.5% Triton X-100) to disrupt chloroplasts. The nuclei are harvested from the suspension by a low speed centrifugation followed by washing the pellet in the same Honda buffer. The proteins were size-fractioned using SDS-PAGE prior to electroblot transfer to nylon membranes. The blot was probed using a CRWN4 antisera as a primary antibody, followed by detection using an anti-rabbit secondary antibody and enzyme-linked chemiluminescent technology. Protein loading was normalized using a parallel probing of membranes with anti-histone H3 antisera, as well as inspection of Coomaisse-stained gels. The blot was imaged using a Storm instrument (Molecular Dynamics) and quantification was done using ImageJ analysis.

Results

Isolation of *crwn4-2* suppressor mutation

A total of 896 EMS-treated M2 seeds were analyzed and 1 phenotypically suppressed seedling was identified that had elongated nuclei in root cells. This seedling was labeled as individual 16-177/1. Figure 6 includes images of the DAPI stained anther filament cell nuclei from the candidate suppressed line as well as the wild-type and unmutagenized *crwn4-2* controls. Note that the wild-type anthers filament cell nuclei contain an average of about ten chromocenters and have a spindle, or elongated, shape. In the unmutagenized *crwn4-2* cells the

nuclei are more spherical in shape and the number of discernable chromocenters within each nucleus has decreased, averaging 5 or less. In the EMS-treated, suppressed *crwn4-2* seedling, the spindle shape had been restored and the chromocenter numbers have increased back to 8-10.

The first concern was that the putative 'suppressed' individual was a contaminant. However, the plant carried a nuclear GFP marker, indicating that the isolate was unlikely to be simple seed contamination from an unrelated wild-type stock. The presence of GFP does not rule out the possibility of pollen contamination, but outcrossing to wild-type pollen can be monitored easily by checking for the presence of a *CRWN4* allele. Another possible concern was that the isolate might be a true revertant in which the original mutation was corrected in the phenotypically 'suppressed' line. To check for a true revertant, the isolate was genotyped using allele-specific PCR. The *crwn4-2* and *CRWN4* alleles were amplified via PCR with the resulting gel electrophoresis pictured in Figure 7. Individual 16-181, a wild-type plant, contained a band for the wild-type primer set and no band for the *crwn4-2* primer set. Individual 16-182, an unmutagenized *crwn4-2* plant, contained a band in the *crwn4-2* primer set and no band in the wild-type primer set. Individual 16-177/1, the potential suppressor, presented a band with the *crwn4-2* primer set and not with the wild-type primer set. Therefore, the 16-177/1 isolate was homozygous for the *crwn4-2* mutation and not a contaminant or a revertant.

Phenotypic suppression breeds true but is variable

The M2 suppressor candidate individual was self-pollinated to generate a M3 generation and subsequently an M4 generation after another round of self-pollination (see Figure 8). In the M3 generation, 5 plants were phenotyped and identified to show incomplete suppression. These plants had roots whose nuclei were nonhomogeneous. While mostly spindle nuclei were observed, the full wild-type phenotype was not restored. In the M4 generation, 8 root and anther

nuclei were examined to be incompletely suppressed as well. The M4 nuclei were determined to be less round than unmutatgenized *crwn4-2*, but less elongated than wild-type; however, chromocenter number was increased to over 5 in this population. The apparent weakening in the suppression may be the real behavior of the suppressor allele, but it might be due to other confounding variables such as increased phenotyping precision in the M3 and M4 generation, or continued segregation of modifying mutations.

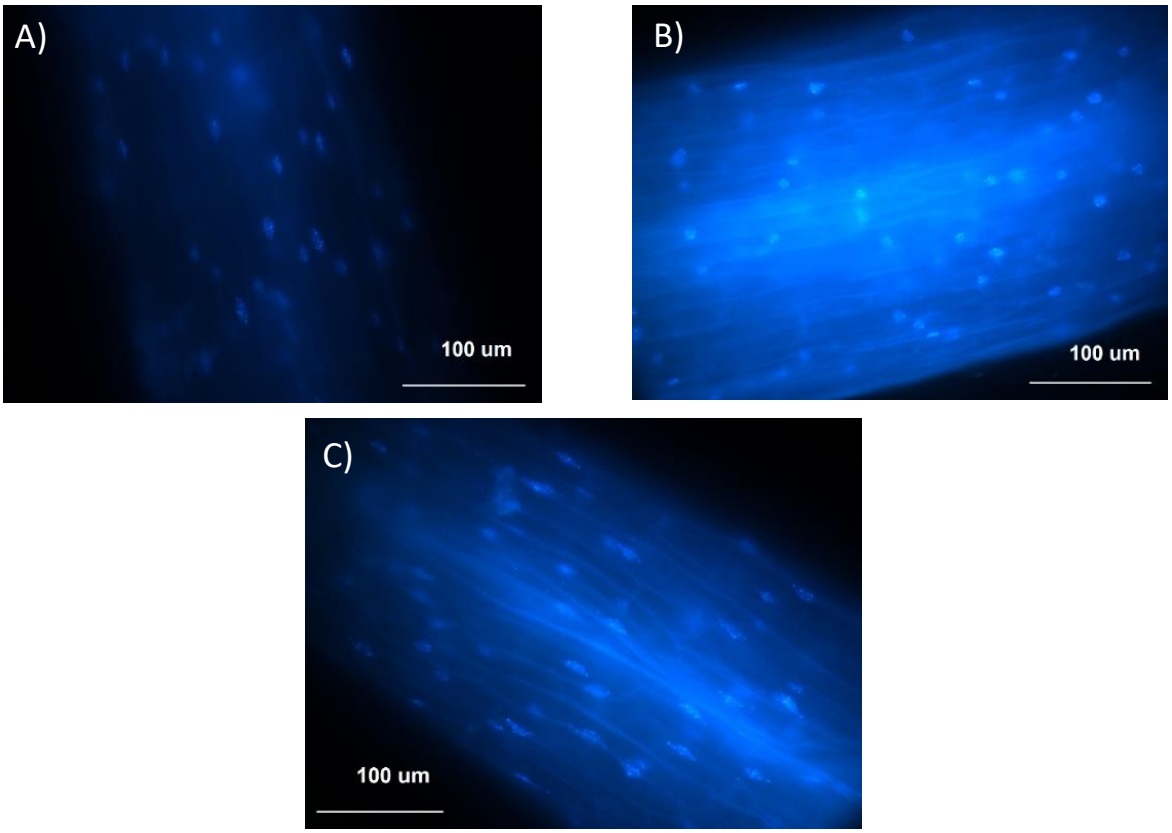

Figure 6: A) Panel A is an image of anther filament cell nuclei in a wild-type individual from family 16-181. B) Panel B is an image of anther filament cell nuclei in an unmutagenized *crwn4-2* individual from family 16-182. C) Panel C is an image of individual 16-177/1, which shows phenotypic suppression resulting in nuclei that resemble wild-type.

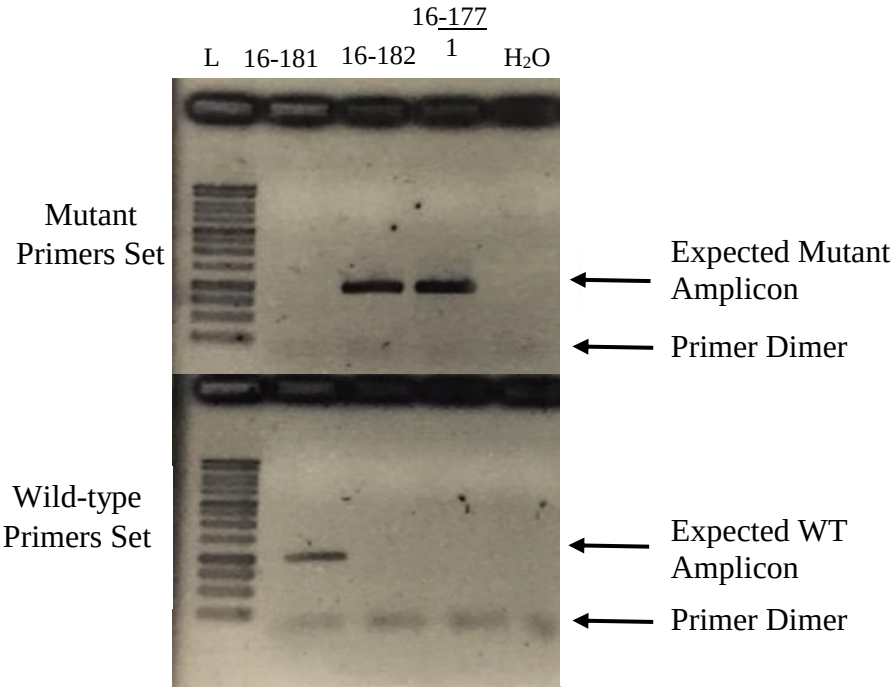

Figure 7: Image of gel electrophoresis from PCR amplification of *crwn4-2* via the mutant primer set and *CRWN4* via wild-type primer set. L designates a 1kb Ladder, 16-181 is a wild-type individual, 16-182 is an unmutagenized *crwn4-2* individual, 16-177/1 is the potential suppressor, and H_2O indicates a 'minus template' negative control.

M0	15-509/14	-Unmutagenized *crwn4-2* seeds, round nuclei 2-5 chromocenters
	EMS ↓ ⊗	
M1	15-713/A	-Mutagenized *crwn4-2* seeds, round nuclei 2-5 chromocenters
	↓ ⊗	
M2	16-177/1	-Putative suppressor, spindle nuclei, 8-10 chromocenters
	↓ ⊗	
M3	16-278/1~5	-Incomplete suppression, 3-7 chromocenters
	↓ ⊗	
M4	16-506 & 16-507	-Incomplete suppression, 3-7 chromocenters

Figure 8: An outline of the M0 to M4 generations of the suppressor screen, including observed phenotypes.

Phenotypic suppression is caused by an extragenic suppressor

The next possibility addressed was that the *CRWN4* gene contained an intragenic suppressor. Therefore, the entire *CRWN4* gene was sequenced. No mutations outside of the *crwn4-2* dinucleotide substitution were found in the coding region, the introns, or the immediate flanking non-transcribed regions. This result indicates that the suppressed line most likely contains an extragenic suppressor at a second site, although a promotor mutation some distance from the gene cannot be ruled out at this time. The subsequent sequencing of *CRWN1* identified no mutations within the gene, ruling out the possibility that suppression in the new isolate is caused by a mutation in this gene encoding a known CRWN4-interacting protein (Goto, et. al. 2014; Endia Blunt, personal communication).

Genetic behavior of the extragenic suppressor mutation

A backcross of the suppressor line to a true-breeding *crwn4-2* line was conducted to begin to understand the mode of inheritance. Specifically, individual 16-278/3 from the M3 generation was backcrossed to the true-breeding *crwn4-2* parental line. The F1 generation resulted in a 'mixed' phenotype in the roots and anther filament cells of the 6 individuals examined. The mixed phenotype presents with mostly spindle-shaped nuclei, but some round nuclei can be observed sporadically. The chromocenters appear to be more defined than in unmutagenized *crwn4-2* controls and typically numbered from 5 to 8 chromocenters, with the caveat that this assessment was qualitative and based on low-resolution epifluorescence. The F1 generation of the backcross is outlined in Figure 9, including an image of a typical F1 anther exhibiting a mixed nuclear morphology phenotype. The presence of the mixed phenotype in

heterozygous individuals of the backcross indicates that the suppressor mutation is semi-dominant.

F0 16-278/3 x 16-277/1

$\frac{crwn4-2}{crwn4-2};\frac{sup^-}{sup^-}$ x $\frac{crwn4-2}{crwn4-2};\frac{sup^+}{sup^+}$

↓

F1 16-501

$\frac{crwn4-2}{crwn4-2};\frac{sup^-}{sup^+}$

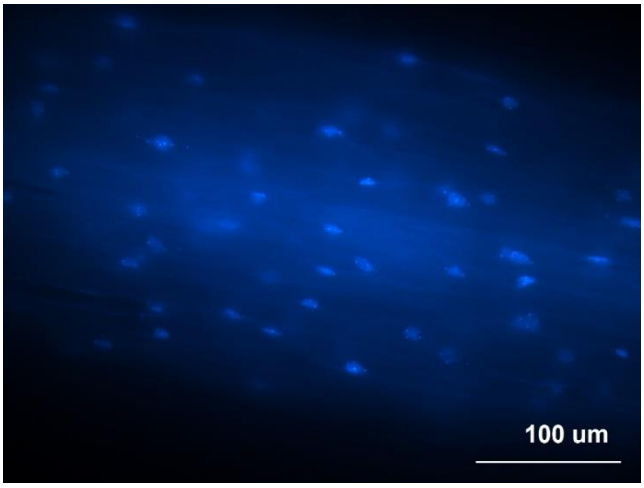

Figure 9: Left) An outline of the F1 generation of the backcross including suspected genotypes. *sup*$^+$ indicates the wild-type allele of the suppressor, while *sup*$^-$ indicates the suppressor allele. Right) Image of anther filament cell nuclei in individual 16-501/1, a typical mixed F1 seedling.

The specificity of the suppressor allele can be determined using an outcross to a line carrying a null *crwn4* mutation. Accordingly, individual 16-278/3 from the M3 generation was outcrossed to a true breeding *crwn4-1* line, which carries a T-DNA insertion within the *CRWN4* gene. Seven F1 individuals from this cross were phenotyped by examining roots and anthers. The nuclei in these seedlings were all round, and chromocenter numbers were in the range of 3 to 7. Nuclei in the *crwn4-2* and *crwn4-1* controls were round and contained 2-5 chromocenters. Figure 10 below details the outcross and includes a picture of a representative F1 anther. The results of the outcross to *crwn4-1* lines points to the allele specificity of the suppressor mutations because the level of phenotypic suppression is greater in *crwn4-2* homozygotes than in *crwn4-1/crwn4-2* heterozygotes.

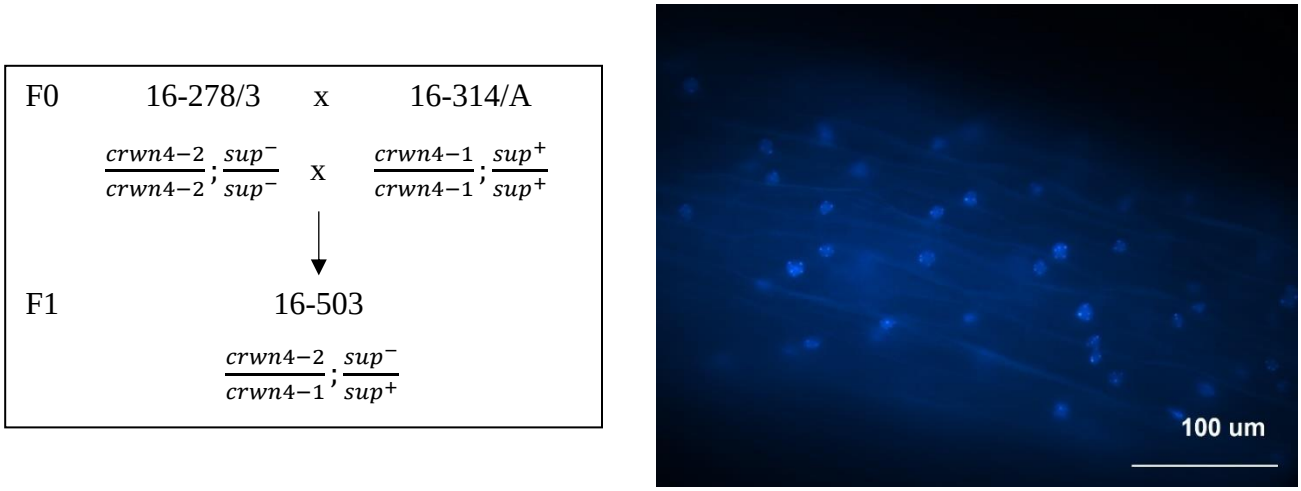

Figure 10: Left) An outline of the outcross including suspected genotypes. Sup$^+$ indicates the wild-type allele of the suppressor, while sup$^-$ indicates the suppressor allele. Right) Image of individual 16-503/3, a typical round F1 seedling.

Next, backcrossed F1 individuals were self-pollinated to generate a family fixed for the *crwn4-2* mutation, but segregating for the extragenic suppressor mutation. In one F2 family, 249 seedlings were phenotyped for nuclear morphology. 166 were classified as 'spindle' (66.6%), and 83 were classified as 'round' (33.3%). The expected values based on the predicted semi-dominant pattern of segregation are 25% spindle, 50% mixed, and 25% round. The results of the first F2 family deviate from the expected values for a semi-dominant allele. The precision of the phenotyping may contribute to this discrepancy, or the results could indicate a dominant mode of inheritance compared to the previously determined semi-dominant inheritance pattern.

Having confirmed the segregation of the suppressor mutation in a family fixed for the *crwn4-2* allele, I sought to generate material that would allow for molecular identification of the suppressor mutation via Whole Genome Sequencing (WGS). Bulk segregant pooling of true-breeding suppressed sublines (with elongated nuclei) and non-suppressed sublines (with round nuclei) would be ideal for comparison of genomes with and without the suppressor allele, respectively. To generate the bulk segregant pools, a second set of F2 families was grown,

phenotyped, and then self-pollinated to create sublines in the F3 generation (see Figure 11). The second set of F2 families generated 41‘spindle’ (46%), 21 ‘mixed’ (24%), and 27 ‘round’ plants (30%) from a total of 89 individuals. Compared to the previously stated expected values, these observed values are more consistent and identify the mixed phenotype unlike the first set of F2 families. Eleven individuals were picked from the 3 phenotypic classes to generate an F3 progeny test (3 spindles, 2 mixed, and 6 round seedlings). The spindle F2 plants produced spindle and mixed nuclei in the F3 generation. These seedlings were pooled as the true-breeding suppressor plants (presumptive sup^{-}/sup^{-}) for WGS. The round F2 plants produced only round nuclei in the F3 generation. These seedlings were pooled as the true-breeding wild-type plants (presumptive sup^{+}/sup^{+}) for WGS. The progeny of the mixed F2 plants included all three phenotypes, consistent with the assignment of the parents as heterozygous for a semi-dominant suppressor mutation. The entire backcross, segregation, and progeny testing program is outlined in Figure 11.

The *crwn4-2* mutation decreases the abundance of CRWN4 in the nucleus, while the suppressor mutation restores wild-type levels of CRWN4

To investigate the possibility that the *crwn4-2* mutation destroys a functional NLS motif, the abundance of CRWN4 in both a *crwn4-2* and a suppressed *crwn4-2;*sup^{-} line was quantified using a western blot (see Figure 12). First, the abundance of CRWN4 in the nuclear extractions of the *crwn4-2* sample is significantly reduced relative to the wild-type control. Second, the level of CRWN4 in the nuclear extracts of the M3 suppressor line was comparable to the wild-type control. The reduced abundance of CRWN4 in the nucleus of the *crwn4-2* plants suggests that the *crwn4-2* mutation might be affecting a functional NLS domain, but other mechanisms are possible – as will be considered further in the Discussion. Regardless of the specific mechanism of the original *crwn4-2* mutation, the extragenic suppressor mutation acts to restore the level of

CRWN4 protein in the nucleus – thereby providing a molecular explanation for the ultimate effect of the suppressor mutation.

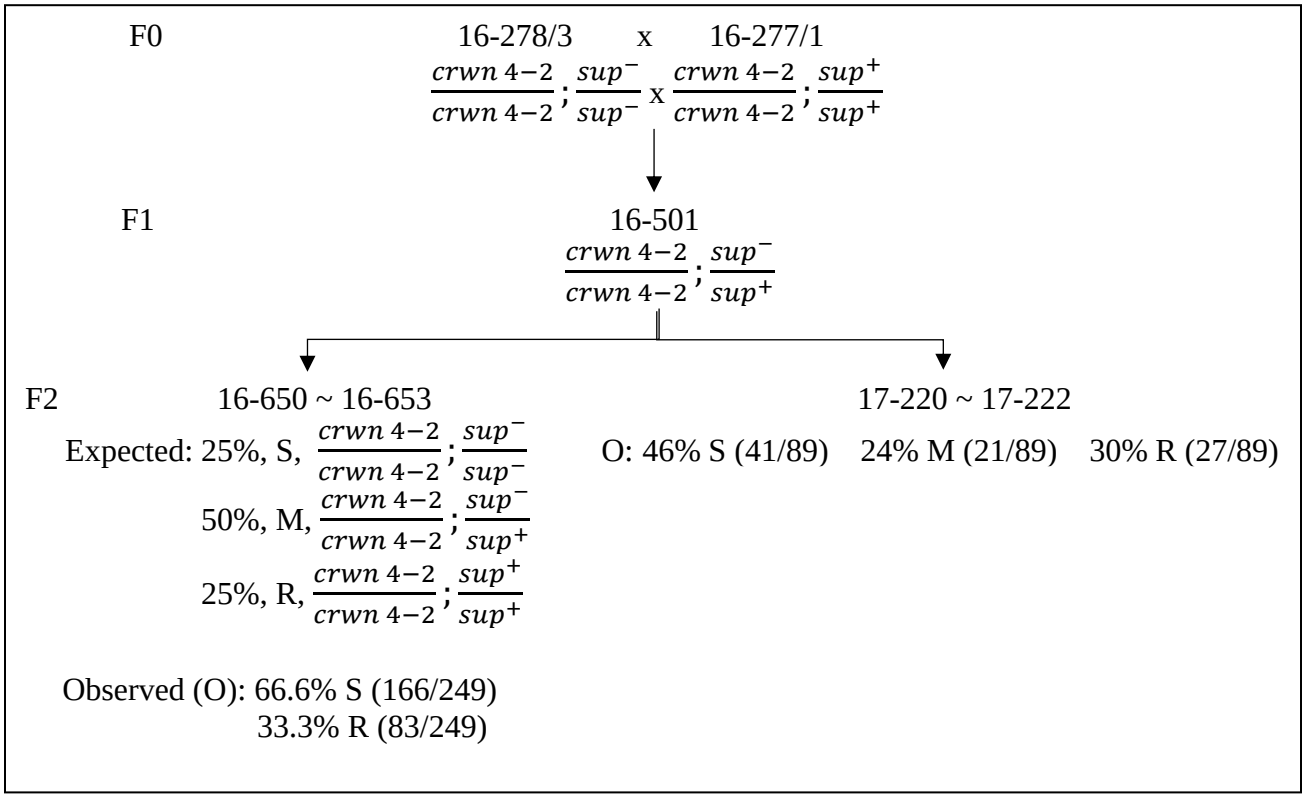

Figure 11: Top) An outline of the backcross including suspected genotypes. Sup$^+$ indicates the wild-type allele of the suppressor, while sup$^-$ indicates the suppressor allele. S indicates Spindle, M indicates Mixed, and R indicates Round. Expected and observed values are indicated where necessary.

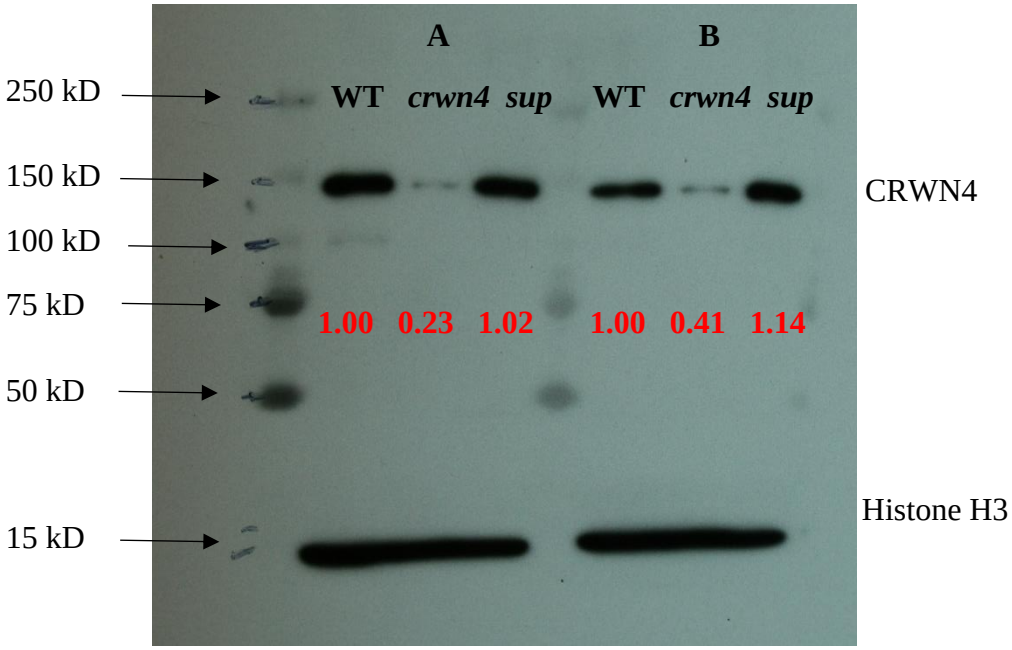

Figure 12: Image of a western blot to quantify the amount of CRWN4 in the nuclei from *crwn4-2* mutants and suppressed lines. Wild-type (WT), unmutagenized *crwn4-2*, and a *crwn4-2;sup*$^{-}$ (*sup*) samples were run. Sample set 'A' indicates run where equal amounts of protein extract were loaded on the gel. Sample set 'B' indicates lanes in which the loading was adjusted for the amount of input tissue weight. Note that two separate blots are shown. The signal at the bottom of the image corresponds to signal from the loading control, histone H3 (at 15 kD; this portion of the blot was probed separately). The top portion of the blot was probed with anti-CRWN4 antisera and the expected band at 150 kD was detected. The relative abundances of the signals are indicated in red.

Discussion

I conducted a suppressor screen to identify new proteins or pathways involved in plant nuclear lamina structure and function. Here, I describe the isolation of an extragenic suppressor mutation that compensates for a missense mutation in the *CRWN4* gene of *Arabidopsis thaliana*. *CRWN1* was the first suspected candidate gene for the location of the suppressor allele as it is a known interactional partner of *CRWN4*. However, the sequencing of the *CRWN1* gene uncovered no mutations, effectively ruling out this gene as the suppressor. This result is promising as this

study was launched to find novel genes, and identifying *CRWN1* as the suppressor would provide insight into the protein's function, but would not lead to the discovery of previously unknown loci.

The newly isolated suppressor mutation behaves in a semi-dominant manner and appears to be allele-specific. The *crwn4-2* backcross produced an F1 generation whose individuals were homozygous for the *crwn4-2* mutation, and heterozygous for the suppressor allele. In these individuals, the nuclei were of mixed phenotype and chromocenter numbers fell in between those of wild-type and *crwn4-2* controls, although a quantitative measurement of chromocenter number remains to be conducted. These observations suggest that the suppressor is inherited in a semi-dominant pattern, producing a third, distinct phenotype in heterozygotes. Compared to the *crwn4-2* backcross, the *crwn4-1* outcross resulted in little phenotypic suppression in the F1 generation. The nuclei of this cross were round and contained slightly elevated numbers of chromocenters. My interpretation is that the weak suppression observed in the progeny reflects the allele-specificity of the suppressor. In this F1 outcross, the *crwn4-2/crwn4-1* plants are heterozygous for the suppressor allele. In this case, the suppressor is not only semi-dominant, but is acting on only one of the two mutant *crwn4* alleles. With one less *crwn4-2* allele to act on, the level is suppression is lower than in *crwn4-2* homozygotes. These observations are consistent with an allele-specific suppressor which, based on the classes of suppressor put forth by Guarente (1993), likely operates as an interactional or informational suppressor.

In order to confirm the allele specificity, and compensate for the possible dosage effect, it will be necessary to continue the outcross to the F2 generation. In this generation, individuals should be genotyped to identify homozygous *crwn4-2* and *crwn4-1* plants as well as the suppressor mutation. If the suppressor allele is allele-specific, phenotyping nuclear morphology

in these plants should show a significantly higher proportion of spindle and mixed nuclei, as well as lower proportions of round nuclei, in the *crwn4-2* individuals than in the *crwn4-1* individuals. Chromocenter number should also be higher and chromocenters more distinct in the *crwn4-2;sup*$^{-}$ plants compared to *crwn4-1;sup*$^{-}$.

The selfing of the suppressor line to the M4 generation demonstrated that the suppressor line is true breeding, but variability in the strength of suppression was observed. Despite incomplete suppression, all generations descendant from the original suppressor exhibited some level of suppresion. It can thus be inferred that the original M2 plant carrying the suppressor was homozygous for the suppressor allele. The phenotypes of the M3 and M4 generations do indicate that there is phenotypic variation within the suppressor. The suppression in these generations was not as strong as in the original M2 suppressor; however, in terms of both nuclear shape and chromocenter number, they still exhibit clear signs of suppression. The nuclei are less circular than in *crwn4-2* controls and have greater numbers of chromocenters, but fall short of appearing phenotypically wild-type. The weakening suppression observed throughout the selfing process may be due to more precise phenotyping or possibly the segregation of other factors caused by the EMS treatment. The EMS treatment generates many mutations that are segregating in addition to the suppressor allele. These extraneous mutations may be altering nuclear size and shape as well, influencing the phenotype observed. The lack of full suppression in these subsequent generations indicates that the suppressor allele is inherently variable in its expressivity. This inherent variation complicates the backcross and outcross phenotyping as the distinction between homozygous and heterozygous suppressors is less clear and more subjective.

Based on a semi-dominant mode of inheritance, the first set of F2 plants from the *crwn4-2* backcross (families 16-650 to 16-653), were expected to contain 25% spindle nuclei, 50%

mixed nuclei, and 25% round nuclei. Instead, for families 16-650 to 16-653, a 66.6% spindle and 33.3% round split was observed. These results fall in line with the expected percentages of a fully dominant mode of inheritance (75% spindle and 25% round). The confounding variable of phenotypic variation may account for this discrepancy. As stated above, the subjective phenotyping of the suppressor makes it difficult to distinguish the spindle, homozygous individuals from the mixed, heterozygous individuals, because plants homozygous for the suppressor allele do not appear purely spindle, but typically contain some mixed nuclei. It is likely that such a subjective error was made during the first phenotyping of the F2 generation. It is believed that the absence of mixed nuclei is due to categorizing the mixed nuclei as spindle. Some nuclei may also have been misclassified as round, but the majority were likely phenotyped as spindle because of their similarity. If this postulation is correct, then the expected percentages could be considered 75% spindle plus misclassified mixed and 25% round. Given these expected percentages that account for the subjective nature of the phenotyping, the observed percentages of 66.6% spindle and 33.3% round are more conceivable.

Looking at the backcross's second set of F2 plants (families 17-220 to 17-222), the observed percentages (46% spindle, 24% mixed, and 30% round), are more consistent with the semi-dominant mode of inheritance. Note that at this stage of the study, the mixed phenotype was being identified separately from the spindle phenotype. This more precise phenotyping was confirmed during progeny testing where these individuals were selfed to the F3 generation. For better comparison to the first F2 family (66.6% spindle and 33.3% round), the spindle and mixed results of the second F2 family can be combined to observe 70% spindle/mixed and 30% round. Recognizing that the spindle and mixed phenotypes are difficult to distinguish, we are observing consistent results across independent plantings: essentially 70% spindle/mixed versus 30%

round. The F2 spindle and round individuals bred true in the F3 progeny test. These individuals are believed to be homozygous for the mutant and wild-type suppressor alleles, respectively. F2 mixed individuals believed to be heterozygous for the suppressor allele generated all three phenotypic classes in the F3 generation. This result was observed, considering the phenotypic variation in the suppressor that creates some mixed nuclei even in true breeding plants. The F3 generation of the backcross further confirms the semi-dominant nature of the suppressor allele. The progeny testing of the second F2 family does provide greater confidence in the phenotypic appearance and segregation of the suppressor compared to the first F2 generation where I failed to identify the mixed phenotype. Despite the insufficient sample size in the second family to make clear conclusions about the inheritance pattern of the mutation, the F1 backcross provided enough information to demonstrate that the suppressor behaves like a semi-dominant factor.

In preparation for Whole Genome Sequencing, DNA was extracted from F3 plants originating from the true breeding spindle and round F2 individuals. Since these individuals come from the backcross, the only significant difference in their genomes will be the suppressor allele that is present in the spindle plants and not the round plants. However, other mutations are being carried in the backcross that do not influence the identified phenotype. These mutations were originally caused by the EMS treatment and are segregating with the suppressor allele. However, these extraneous mutations will be present in both spindle individuals and those phenotyped as round. When the WGS results are returned, the data will be analyzed for mutations present in the spindle population and not the round. While many such mutations may be identified, only one of these will be the suppressor allele, as the rest are co-segregating byproducts of the EMS treatment. By analyzing the genes in which the mutations occurred, a candidate suppressor gene may be identified. For example, a candidate gene may be a known

nuclear lamina protein, or perhaps a gene involved in nuclear import. These genes are more likely to interact with CRWN4 than other, random mutations in the genome. Once a list of candidate genes is established, the segregation of the suppressed phenotype will be compared to the segregation of the candidate gene's mutation. A segregating population of suppressor plants will be phenotyped and genotyped. If the suppressed phenotype overlaps with the genotyping of the candidate gene's mutation, then the candidate gene is likely the cause of the suppression. If the segregation patterns do not coincide, then the candidate gene can be discarded from consideration. The identification of the gene responsible for the observed suppression will provide insights into the mechanism of suppression.

The results of the western blot (see Figure 12) indicate that the abundance of CRWN4 in the nucleus is decreased in unmutagenized *crwn4-2* seedlings. This decrease in abundance supports the suggestion that the NLS domain where the *crwn4-2* mutation occurs is functional and allows for proper localization, and therefore function, of the CRWN4 protein. The abundance of protein is restored to wild-type levels in the suppressor line. This restoration indicates that the suppressor mutation may be compensating for the loss of CRWN4's NLS. One way to envision how the suppressor might be working is by facilitating co-import of CRWN4 into the nucleus with other proteins containing an NLS. Perhaps the suppressor allele enhances the ability of CRWN4 to bind to CRWN1, 2, or 3, whose NLS domains have been verified. Sequencing of *CRWN1* proved that the suppressor allele was not present in this gene, so therefore only CRWN2 and 3 remain as possible options. The co-localizing protein does not have to be a member of the CRWN family, but possibly another nuclear lamina protein, such as SUN or KAKU4. In this scenario, the suppressor mutation would act by increasing the efficiency of nuclear transport through a 'piggy-back' mechanism. Alternatively, the *crwn4-2* mutation

could be decreasing the expression of the gene. An undetected promotor mutation may be present, or the missense mutation of *crwn4-2* could lead to less efficient translation. In this model, the suppressor would be compensating by increasing CRWN4 gene expression. These possibilities could be tested by looking at *CRWN4* transcript levels across the various genotypes. In another model, the *crwn4-2* mutation might lead to protein misfolding and increased protein turnover where the CRWN4 protein is less stable and/or experiences increased protease-mediated degradation. In this case, nuclear transport of the protein may be normal, but the amount of protein imported, or its stability once imported, is limiting its abundance in the nucleus. The suppressor mutation could then be affecting protein folding through a chaperone, or protein degradation through a protease. These possibilities could be tested with a separate western blot that uses total tissue extracts and not nuclear extracts to quantify the total CRWN4 protein levels. Many other possibilities exist outside these three, and it is impossible to rule out any without further experimentation. The identification of the suppressor mutation's identity through WGS will help distinguish among these possibilities, or shed light on new ones.

Conclusion

The current findings of this thesis project identified a suppressor allele of the *crwn4-2* mutation that is inherited in a semi-dominate pattern while hinting at allele specificity. With the proposed allele-specific nature of the suppressor allele, the interactional and informational forms of suppression are most likely. The sequence and function of a candidate gene to be identified through future Whole Genome Sequencing, in accordance with the known properties of the *crwn4-2* mutation, will assist in narrowing down the possibilities to one specific mechanism. Knowledge of interactions of genes and their proteins in the plant nuclear lamina is crucial for

comparison to similar mechanisms and pathways found in animals. To understand better the convergent evolution observed in how plants and animals construct and regulate their respective nuclei, a comprehensive knowledge of both plant and animal nuclear lamina proteins is necessary. The results of this study set the foundation for identifying novel genes, interactions, and pathways involving the nuclear lamina in *Arabidopsis thaliana*.

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Supplementary Info

Table 1: Listed below is the name, sequence, and use of each primer from this study

Name	Sequence	Use
a4WTF	TGAGACTAGCGAACCAAGCAATAACAA	PCR amplification of *CRWN4*
a41kbR2	TCCAGCCGAACACTCTTGCTGTTG	PCR amplification of *CRWN4* and *crwn4-2*
a4MutF	TGAGACTAGCGAACCAAGCAATAACCT	PCR amplification of *crwn4-2*
CRWN4 KpuI F2	TCATTTTTTCTACCGATGTGCATATAT	PCR amplification of *CRWN4*
CRWN4 KpuI R2	CTAGAGGATCCCCGGCAGGTGCACA	PCR amplification and DNA sequencing of *CRWN4*
LINC4 Seq Primer 1	ATTCGCAACACATGCTTTGA	DNA sequencing of *CRWN4*
LINC4 Seq Primer 2	ATTTCCGATAACGCCGAGTA	DNA sequencing of *CRWN4*
LINC4 Seq Primer 3	CAACACAACATGGGTCTTCTC	DNA sequencing of *CRWN4*
LINC4 Seq Primer 4	TGAAACGAAAGAGAACGAAATG	DNA sequencing of *CRWN4*
LINC4 Seq Primer 5	CTAATTGAGAACGTCTTAGCGAATC	DNA sequencing of *CRWN4*
LINC4 Seq Primer 6	GGAAGAGTTAAGAAAAGAAGCAGA	DNA sequencing of *CRWN4*
LINC4 Seq Primer 7	GTTGCATTAGATGATATGTCCATG	DNA sequencing of *CRWN4*
LINC4 Seq Primer 8	CCCAAAAAATATTCTGATGAAGCTGA	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 9	CCATACAGTCGTGACTCTTGA	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 10	AGGTTGAGATTGTTGTATGGG	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 12	AGATTGCGTTAGCCTTGTGT	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 15	GAGAAAGAGAAGAATTTGGTTGC	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 16	ACTCTGGAGATGAAACTTAAGGAGG	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 18	TGCTGAGAGGTTGGAGATC	DNA sequencing of *CRWN4*

CRWN4 Seq Primer 22	CCTCAGGATAACTTCTTGATTCGCT	DNA sequencing of *CRWN4*
CRWN4 Seq Primer 23	GCAACCAAATTCTTCTCTTTCTCA	DNA sequencing of *CRWN4*
CRWN1 Seq01	AGCCCTCCTAAGTAGCTACTA	PCR amplification and DNA sequencing of *CRWN1*
CRWN1 Seq02	CTGCACCACATTCGTGCTTA	PCR amplification and DNA sequencing of *CRWN1*
CRWN1 Seq03	TCAGAACGACCAGTTTCACC	DNA sequencing of *CRWN1*
CRWN1 Seq04	CACCAGTAACTGGCAATCCTA	DNA sequencing of *CRWN1*
CRWN1 Seq05	GTCTGCATATCAGACTCGAGT	DNA sequencing of *CRWN1*
CRWN1 Seq06	ATCTCCTTATCCTCGAGCAG	DNA sequencing of *CRWN1*
CRWN1 SeqChk R1	AGCGGGAACAATTCATTAGTGAG	PCR amplification and DNA sequencing of *CRWN1*
CRWN1 SeqChk R2	ATGCTGCTAACCTCGCTGT	PCR amplification and DNA sequencing of *CRWN1*

References Cited

Chang, W., Worman, H. J., & Gundersen, G. G. (2015). Accessorizing and anchoring the LINC complex for multifunctionality. *Journal of Cell Biology*, *208*(1), 11–22. https://doi.org/10.1083/jcb.201409047

Chytilova, E., Macas, J., & Galbraith, D. (1999). Green Fluorescent Protein Targeted to the Nucleus , a Transgenic Phenotype Useful for Studies in Plant Biology. *Annals of Botany*, *83*, 645–654.

Ciska, M., Masuda, K., & Moreno, S. (2013). Lamin-like analogues in plants : the characterization of NMCP1 in Allium cepa. *Journal of Experimental Botany*, *64*(6), 1553–1564. https://doi.org/10.1093/jxb/ert020

Cocciolone, S. M., & Cone, K. C. (1993). Pl-Bh, an Anthocyanin Regulatory Gene of Maize That Leads to Variegated Pigmentation. *Genetics*, *135*(2), 575–588.

Friesen, H., Koschwanez, J., Mattiazzi, M., Pechlaner, M., Takar, M., Matej, U., … Boone, C. (2016). Exploring genetic suppression interactions on a global scale. *Science*, *354*(6312), 599. https://doi.org/10.1126/science.aag0839

Goto, C., Tamura, K., Fukao, Y., Shimada, T., & Hara-Nishimura, I. (2014). The Novel Nuclear Envelope Protein KAKU4 Modulates Nuclear Morphology in Arabidopsis. *The Plant Cell*, *26*(5), 2143–2155. https://doi.org/10.1105/tpc.113.122168

Graumann, K., Runions, J., & Evans, D. E. (2010). Characterization of SUN-domain proteins at the higher plant nuclear envelope. *The Plant Journal*, *61*, 134–144. https://doi.org/10.1111/j.1365-313X.2009.04038.x

Gruenbaum, Y., Margalit, A., Goldman, R. D., Shumaker, D. K., & Wilson, K. L. (2005). The Nuclear Lamina Comes Of Age. *Nature Reviews Molecular Cell Biology*, *6*(January), 21–31. https://doi.org/10.1038/nrm1550

Guarente, L. (1993). Synthetic enhancement in gene interaction : a genetic tool come of age. *Trends in Genetics*, *9*(10), 362–366.

Masuda, K., Xu, Z., Takahashi, S., Ito, A., Ono, M., Nomura, K., & Inoue, M. (1997). Peripheral Framework of Carrot Cell Nucleus Contains a Novel Protein Predicted to Exhibit a Long a -Helical Domain. *Experimental Cell Research*, *181*(232), 173–181.

Mattout, A., Cabianca, D. S., & Gasser, S. M. (2015). Chromatin states and nuclear organization in development — a view from the nuclear lamina. *Genome Biology*, 1–15. https://doi.org/10.1186/s13059-015-0747-5

Meinke, P., & Schirmer, E. C. (2015). LINC'ing form and function at the nuclear envelope. *FEBS Letters*, *589*(19), 2514–2521. https://doi.org/10.1016/j.febslet.2015.06.011

Sakamoto, Y., & Takagi, S. (2013). LITTLE NUCLEI 1 and 4 regulate nuclear morphology in arabidopsis thaliana. *Plant and Cell Physiology*, *54*(4), 622–633. https://doi.org/10.1093/pcp/pct031

Tamura, K., Iwabuchi, K., Fukao, Y., Kondo, M., Okamoto, K., & Ueda, H. (2013). Report Myosin XI-i Links the Nuclear Membrane to the Cytoskeleton to Control Nuclear Movement and Shape in Arabidopsis. *Current Biology*, *23*(18), 1776–1781. https://doi.org/10.1016/j.cub.2013.07.035

"ThaleMine: mRNA LINC4 *A. thaliana*." *Thalemine*, Araport, apps.araport.org/thalemine/report.do?id.

Wang, H., Dittmer, T. a, & Richards, E. J. (2013). Arabidopsis CROWDED NUCLEI (CRWN) proteins are required for nuclear size control and heterochromatin organization. *BMC Plant Biology*, *13*(200). https://doi.org/10.1186/1471-2229-13-200

Zhou, X., Groves, N. R., & Meier, I. (2015). Plant nuclear shape is independently determined by the SUN-WIP-WIT2-myosin XI-i complex and CRWN1. *Nucleus (Austin, Tex.)*, *6*(2), 144–53. https://doi.org/10.1080/19491034.2014.1003512