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The Role of the *Arabidopsis* SIAMESE-RELATED Cell Cycle Regulators on Root Growth During Phosphate Starvation and Salt Stress

by

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Undergraduate honors thesis under the direction of

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the Upper Division Honors Program.

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Introduction

Although once thought to be mutually exclusive, the cell-cycle progression and cell differentiation are closely linked. The cell cycle inhibits the onset of differentiation in the most basic example. Another example is endoreplication, also commonly referred to as endoreduplication, continuing during differentiation. Endoreplication is the replication of nuclear DNA during the synthesis phase of the cell cycle without the subsequent completion of mitosis and cytokinesis. Therefore, polyploidy occurs, resulting in large cells with a nuclear DNA content greater than 2C. It is thought one reason for endoreplication is to meet the needs of a larger cell. There is often a positive correlation between cell size and DNA content (Melaragno et al., 1993; Hülskamp et al., 1994). Endoreplication occurs in many cell types in both animals and plants. It is important in agriculture and human development. Endoreplication not only occurs, but it is also essential to symbiotic nitrogen fixation in root nodules of legumes. It also occurs in platelet-producing megakaryocytes and placental trophoblastic cells in mammals. *Arabidopsis thaliana* is a favorite research subject for plant molecular geneticists. The science world has expanded its understanding of genetic, cellular, and molecular biology largely due to research on *Arabidopsis thaliana.* Its shoot epidermal hairs, also called trichomes, are a well-established model for investigating the control switches of endoreplication. Recessive mutations in the *SIAMESE* (*SIM*) gene family of *Arabidopsis thaliana* have a distinct cell cycle–related phenotype: multicellular trichomes, with individual nuclei having reduced levels of endoreplication (Walker et al., 2000). *SIM* overexpression in transgenic plants results in small plants with

leaves containing large cells and increased levels of nuclear DNA. The *SIM* loss-of-function and gain-of-function phenotypes clearly indicate that *SIM* plays a vital role in regulation of endoreplication (Churchman et al., 2006). Cell cycle progression is largely controlled by the expression of cyclin-dependent kinases (CDKs), cyclin (CYC) proteins, and inhibitors of CYC/CDK complexes. *SIM* encodes a protein that has a potential cyclin binding motif and shares a motif with Kip-related proteins (KRPs), or Interactors of Cdc2 kinases (ICKs) (De Veylder et al., 2001; Churchman et al., 2006). A more complete understanding of the onset of endoreplication and the interactions between cell cycle regulators could have big implications for increasing agricultural yield and developing future medicines for treating diseases related to the cell cycle progression, such as cancer. The existence of additional *Arabidopsis SIM* homologs, the SIAMESE-related (*SMR*) genes, suggests that *SIM* may only be one player involved in this regulation and may, in fact, play additional roles beyond those discovered in trichomes (Churchman et al., 2006). While much has recently been learned about this cell cycle regulator in trichomes, little is known about its effects on root growth.

Phosphorous is one of the seventeen essential nutrients for healthy plant growth. It typically exists as an inorganic chemical called phosphate in soil. Phosphate is often a limiting nutrient in environments and is commonly added to soil for farming. Phosphate is a major structural component of the deoxyribonucleic acid (DNA) molecule. In fact, phosphate gives DNA its negative charge and hydrophilic nature. Additionally, adenosine triphosphate (ATP) is the energy

currency of the cell, and cleaving just phosphate groups from this molecule provides the cell with energy to carry out necessary chemical reactions.

Typically, plant growth and development will slow or stop under environmental stresses, such as phosphate starvation and salt stress. Since endoreplication causes a decrease in cell proliferation and overall growth, it is suspected that the genes responsible for inducing endoreplication may be key players for stunting growth during environmental stress. It is hypothesized that plants with mutations in the *SIM* gene family do not have the genetic "intelligence" to slow or stop growth when undergoing environmental stress. That is, those plants are not expected to have the necessary proteins to induce endoreplication, thereby slowing growth and conserving energy.

Methods

Seeds: The wild type (WT) strain used throughout these experiments is a characterized ecotype called Columbia (Col). The mutant line used throughout these experiments is a triple mutant (3M) with mutations in the following genes: *SIM-1, SMR1, SMR2*.

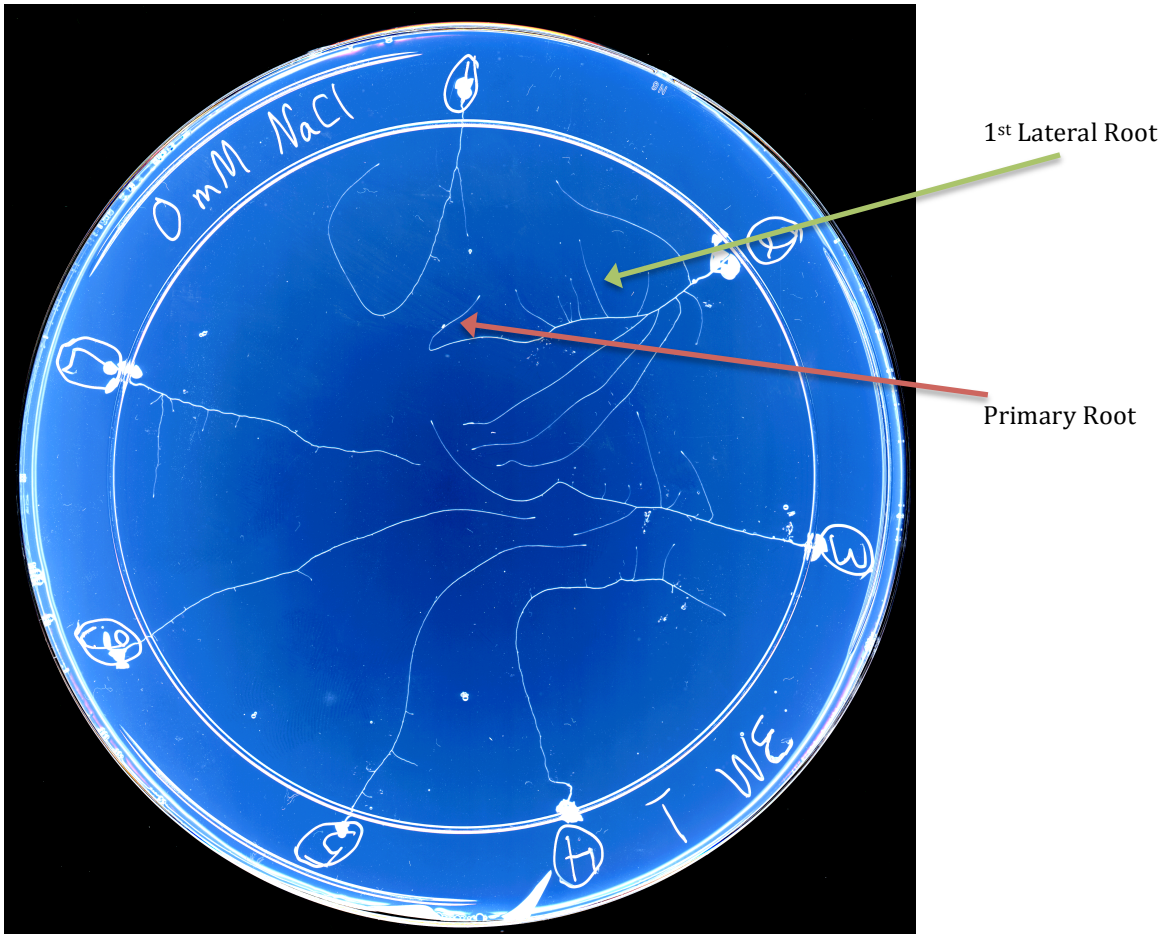
Sterilization: Seeds were first sterilized for ten minutes in a solution of bleach, water, and Tween 20. Seeds were then rinsed several times with sterile water in the sterile hood before being stored with sterile water in a 1.5-mL tube at four degrees Celsius for at least two days. This step promotes synchronized germination.

Planting: After a minimum of two days, seeds were planted on ½ strength Murashige and Skoog (MS) media in square Petri plates using sterile technique.

Square plates were used rather than round to allow all plant roots to grow the same distance before hitting the bottom of the plate. All Petri plates were divided in half and properly labeled: one side for wild type and the other side for triple mutant. Fifteen seeds were planted on each side of each plate. Parafilm was used to seal the Petri plate and prevent contamination. The Petri plates were then placed vertically in a growth chamber (22 degrees Celsius; 16 hour photoperiod).

Transplanting: After seven days of growth, sterile technique was used to transplant seedlings onto media prepared to induce the desired environmental stress (salt stress or phosphate starvation). In the phosphate starvation experiments, all seedlings were germinated on MS media, which contains phosphate. Then, half of all seedlings were transferred to MS media, and the other half of seedlings were transferred to media that lack phosphate. This medium is identical in composition other than the absence of phosphate. Seedlings being transplanted onto media without phosphate were gently but thoroughly rinsed in sterilized, deionized water to prevent phosphate contamination. Essentially, it prevents the introduction of phosphate onto the phosphate-free media. In the salt stress experiment, all seedlings were germinated on MS media with 0 milliMolar (mM) NaCl. Then, seedlings were transplanted onto media that contains 0 mM NaCl, 50 mM NaCl, and 100 mM NaCl. After transplanting Parafilm was again used to seal the Petri plate and prevent contamination. All Petri plates were then returned to the growth chamber and allowed to grow until the first seedling nearly reached the bottom of the vertical plate (5-7 days). No seedling was allowed to touch the bottom of the Petri plate to disallow a conditional difference between seedlings.

Measuring: Seedlings were removed at the end of the growth period and preserved in ethyl alcohol in properly labeled 1.5-mL tubes (one seedling per tube) at four degrees Celsius. Ethyl alcohol bleaches the seedlings, and the bleached seedlings are better visualized during the scanning process. Next, the roots of the seedlings were carefully spread onto agar and water in properly labeled Petri plates in such a way that no roots overlapped. The non-overlapping roots enable easier measuring and more accurate data. The Petri plates were then scanned (**Pic 1**) and saved as an image to a computer. ImageJ software (http://rsb.info.nih.gov) was downloaded and used to measure the lengths of the primary roots and first lateral roots.



Pic 1: A scanned image showing the primary root (red arrow) and a first lateral root (green arrow).

Results

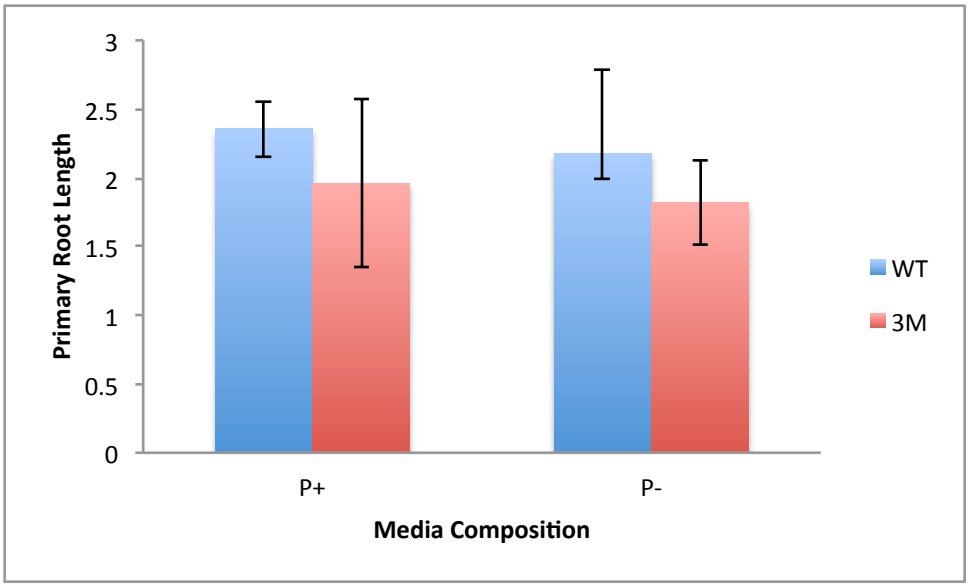


Figure 1: Primary root length of wild type and triple mutant during phosphate starvation (P+: P = 0.072; n (WT) = 15; n (3M) = 13) (P-: P = 0.127; n (WT) = 15; n (3M) = 12).

There is no significant difference in the primary root lengths between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

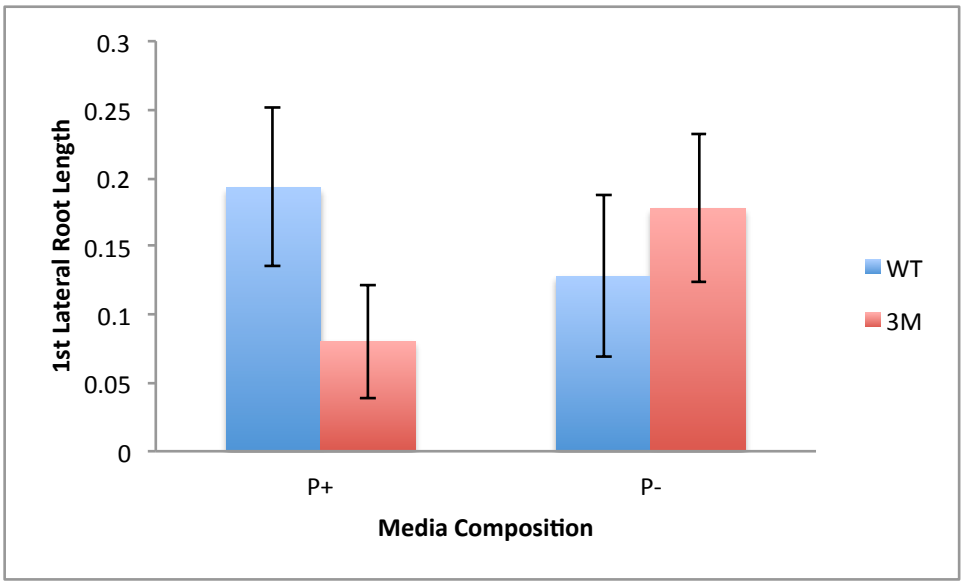


Figure 2: 1st lateral root length of wild type and triple mutant during phosphate starvation ((P+: P = 0.033; n (WT) = 190; n (3M) = 90) (P-: P = 0.271; n (WT) = 103; n (3M) = 106)).

There is a significant difference in the first lateral root lengths between wild type and triple mutant under normal conditions. However, there is no significant difference between wild type and triple mutant under conditions of phosphate starvation.

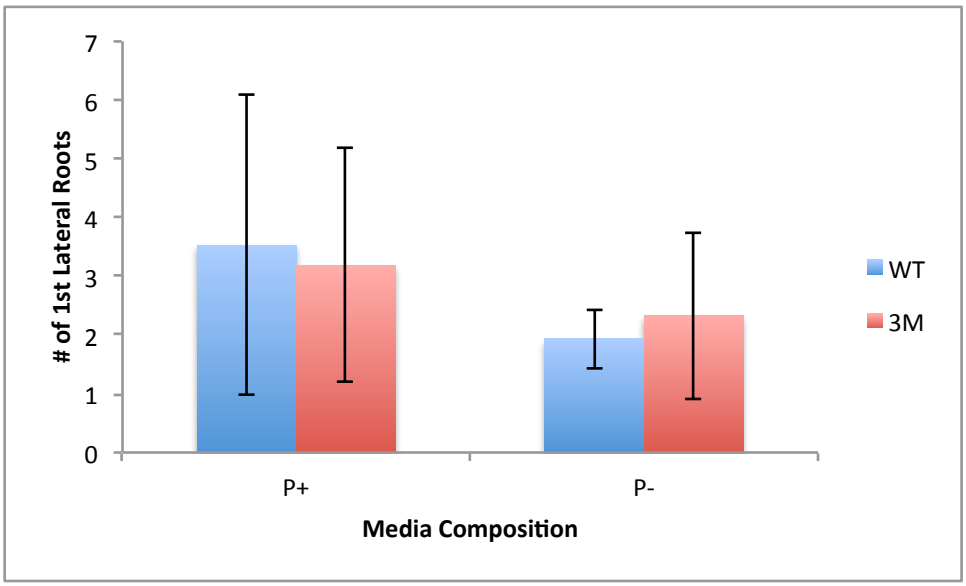


Figure 3: Number of 1st lateral roots of wild type and triple mutant during phosphate starvation ((P+: P = 0.623; n (WT) = 15; n (3M) = 13) (P-: P = 0.588; n (WT) = 15; n (3M) = 12)).

There is no significant difference in the number of first lateral roots between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

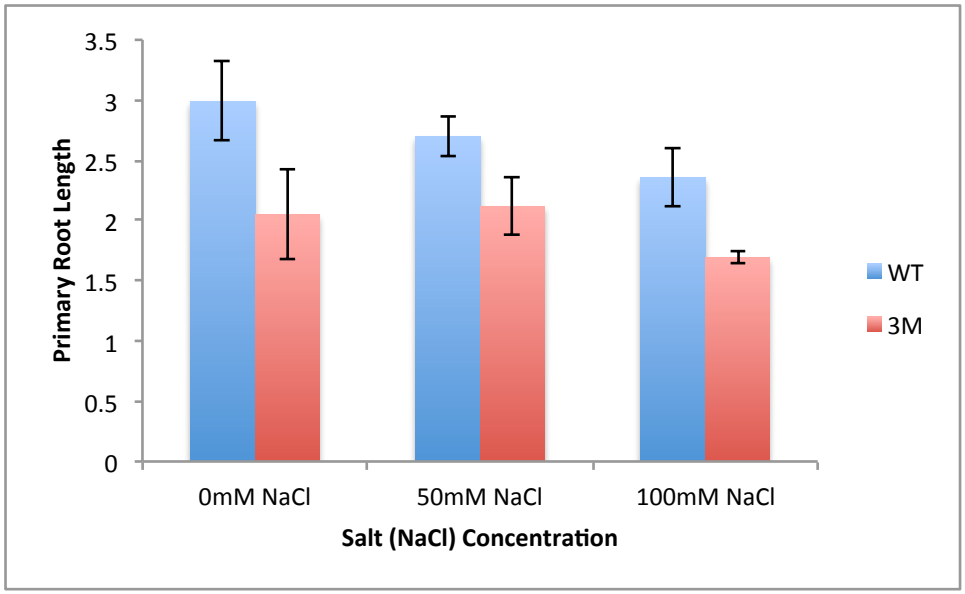


Figure 4: Primary root length of wild type and triple mutant during salt stresses ((0mM NaCl: $P < 0.001$; n (WT) = 21; n (3M) = 19) (50mM NaCl: $P = 0.002$; n (WT) = 20; n (3M) = 21) (100 mM NaCl: $P < 0.001$; n (WT) = 21; n (3M) = 21)).

There is a significant difference in primary root lengths between wild type and triple mutant under the various salt stresses (50 mM NaCl and 100 mM NaCl) and the controlled, salt-free condition (0 mM NaCl).

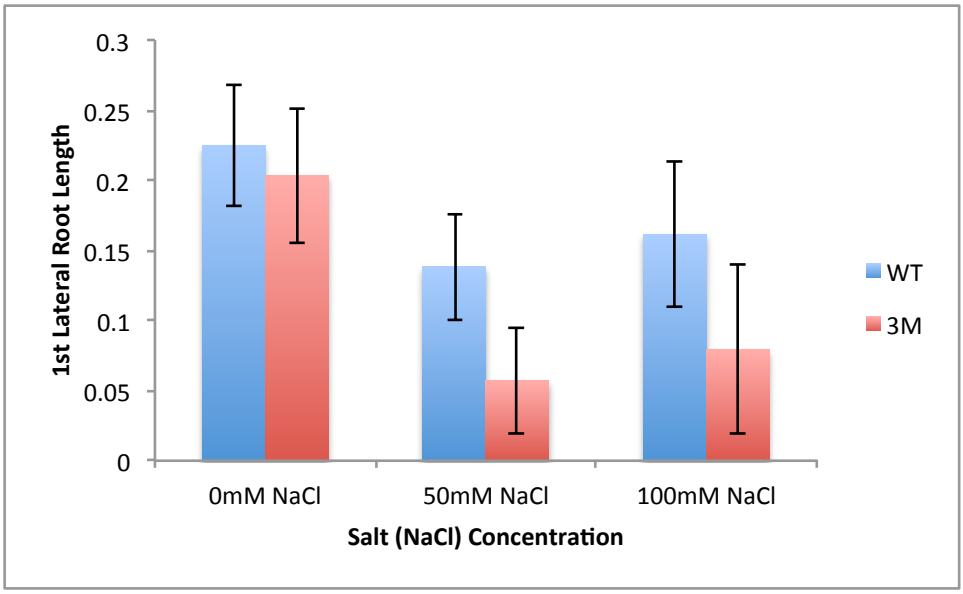


Figure 5: 1st lateral root length of wild type and triple mutant during salt stresses ((0mM NaCl: P = 0.254; n (WT) = 182; n (3M) = 105) (50mM NaCl: P = 0.006; n (WT) = 57; n (3M) = 25) (100 mM NaCl: P = 0.122; n (WT) = 47; n (3M) = 43)).

There is a significant difference in first lateral root lengths between wild type and triple mutant under the 50 mM NaCl condition. However, there is no significant difference in first lateral root lengths between wild type and triple mutant under the 0 mM NaCl and 100 mM NaCl conditions.

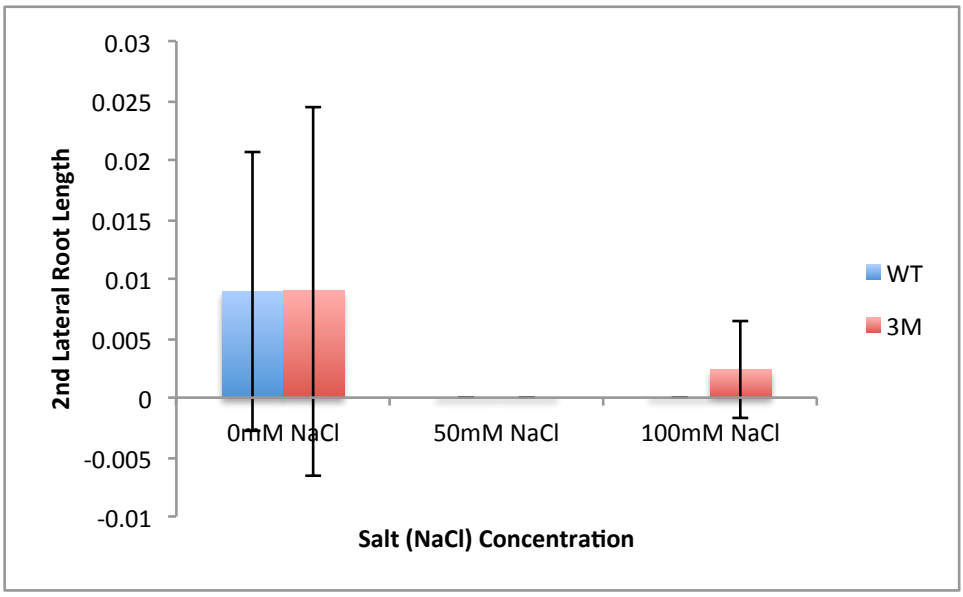


Figure 6: 2nd lateral root length of wild type and triple mutant during salt stresses.

There are no significant differences in 2nd lateral root lengths between wild type and triple mutant under any condition.

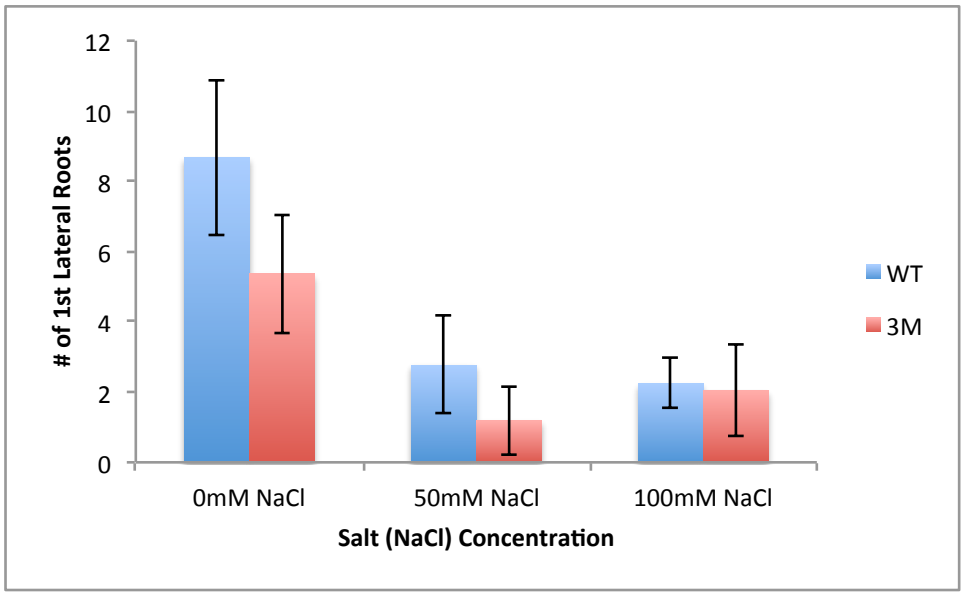


Figure 7: Number of 1st lateral roots of wild type and triple mutant during salt stresses ((0mM NaCl: P = 0.120; n (WT) = 21; n (3M) = 18) (50mM NaCl: P = 0.058; n (WT) = 20; n (3M) = 20) (100 mM NaCl: P = 0.262; n (WT) = 21; n (3M) = 21)).

There are no significant differences in the number of first lateral roots between wild type and triple mutant under any condition.

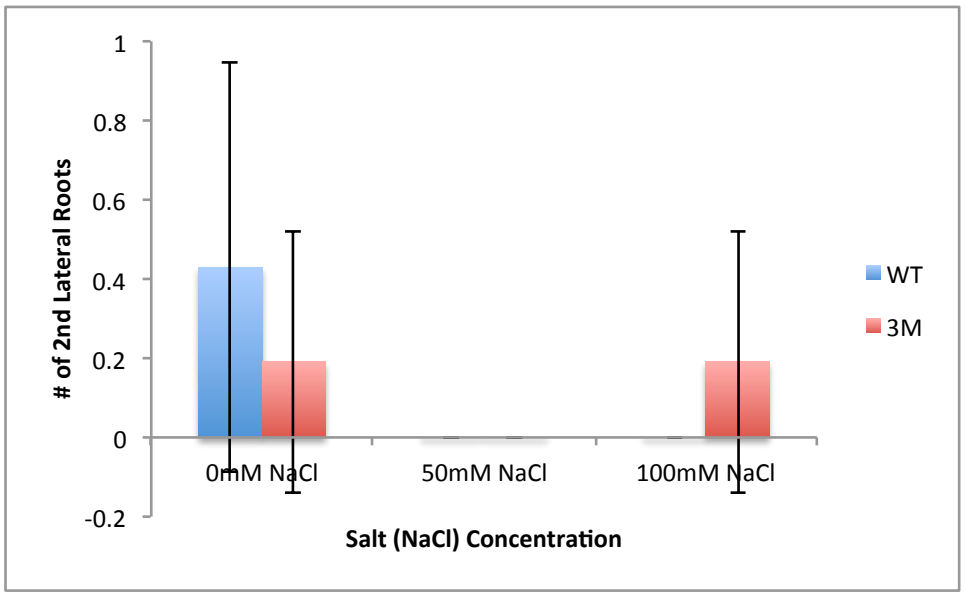


Figure 8: Number of 2nd lateral roots of wild type and triple mutant during salt stresses.

There are no significant differences in 2nd lateral root lengths between wild type and triple mutant under any condition.

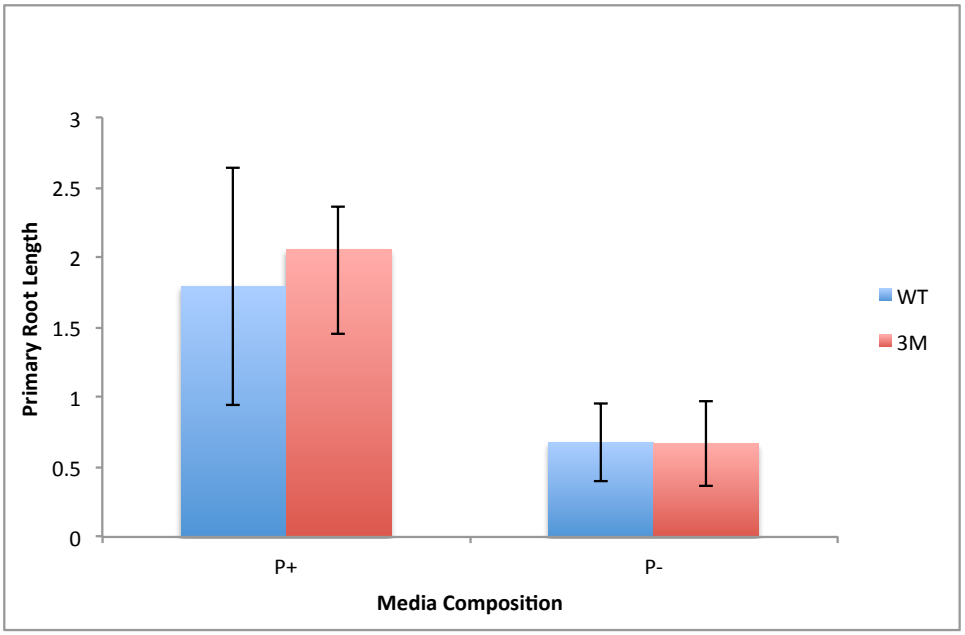


Figure 9: Primary root length of wild type and triple mutant during phosphate starvation ((P+: P = 0.326; n (WT) = 26; n (3M) = 18) (P-: P = 0.810; n (WT) = 23; n (3M) = 21)).

There are no significant differences in the primary root lengths between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

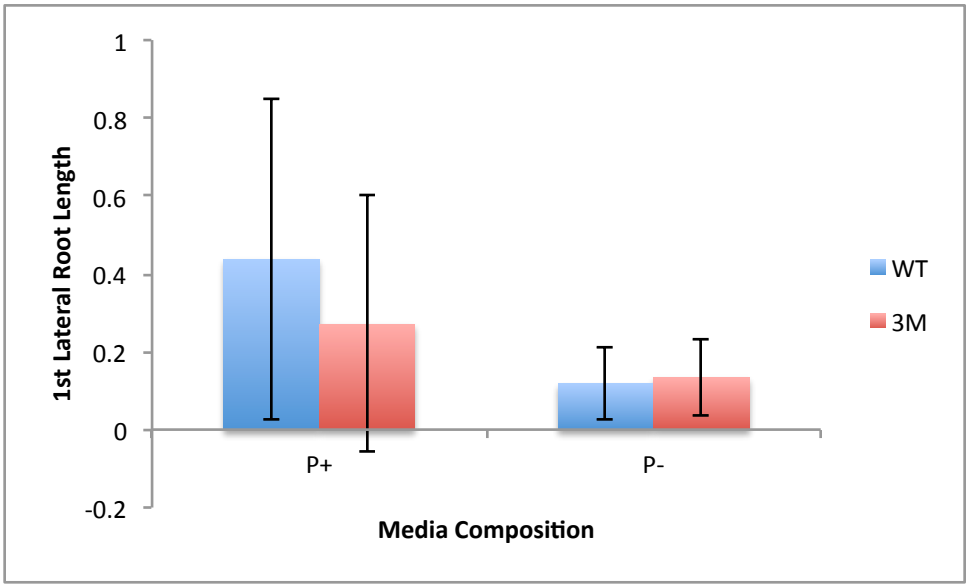


Figure 10: 1st lateral root length of wild type and triple mutant during phosphate starvation ((+P: P = 0.330; n (WT) = 190; n (3M) = 90) (-P: P = 0.271; n (WT) = 103; n (3M) = 106)).

There are no significant differences in the first lateral root lengths between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

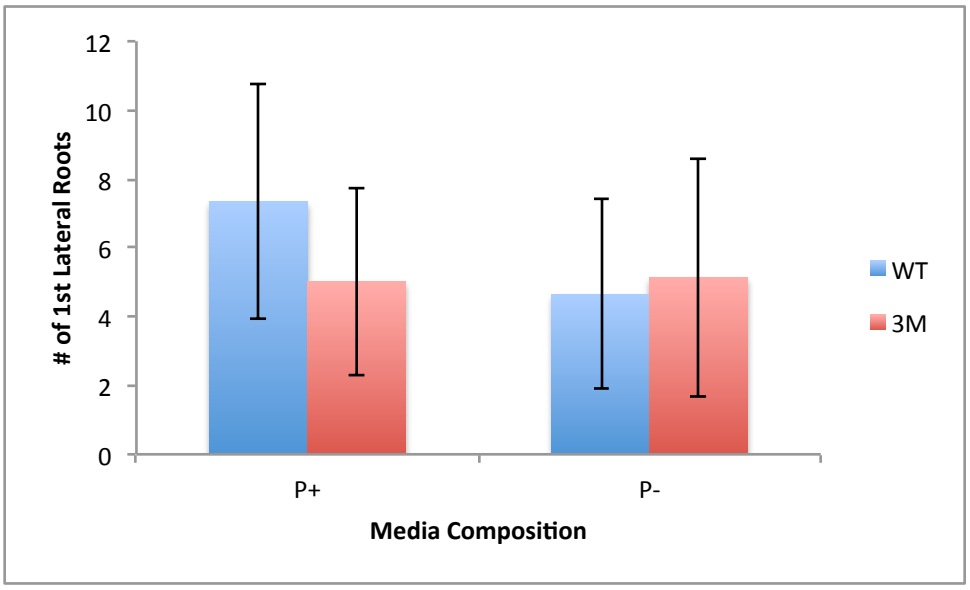


Figure 11: Number of 1st lateral roots of wild type and triple mutant during phosphate starvation ((+P: P = 0.443; n (WT) = 26; n (3M) = 18) (-P: P = 0.953; n (WT) = 23; n (3M) = 21)).

There are no significant differences in the number of first lateral roots between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

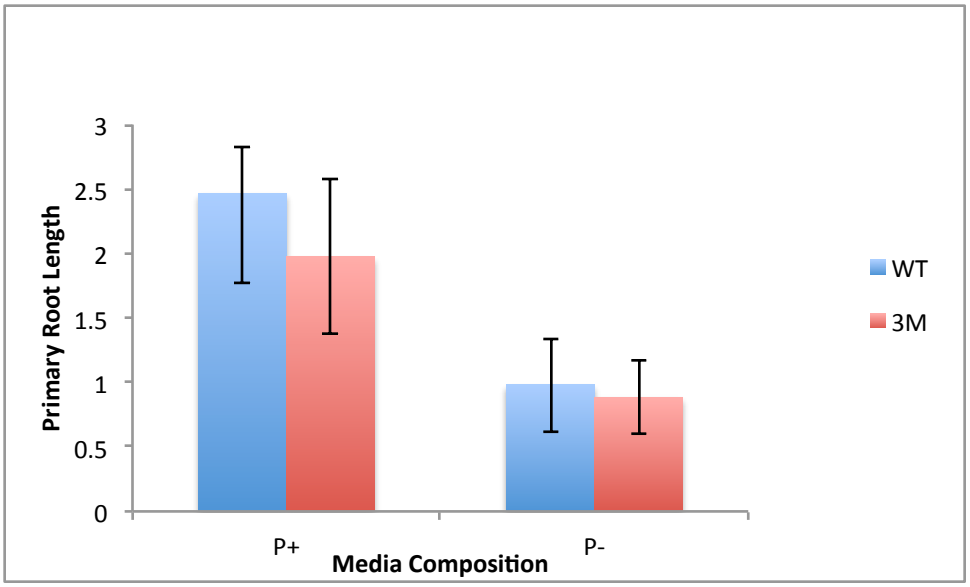


Figure 12: Primary root length of wild type and triple mutant during phosphate starvation ((+P: P = 0.071; n (WT) = 30; n (3M) = 22) (-P: P = 0.765; n (WT) = 25; n (3M) = 26)).

There are no significant differences in the primary root lengths between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

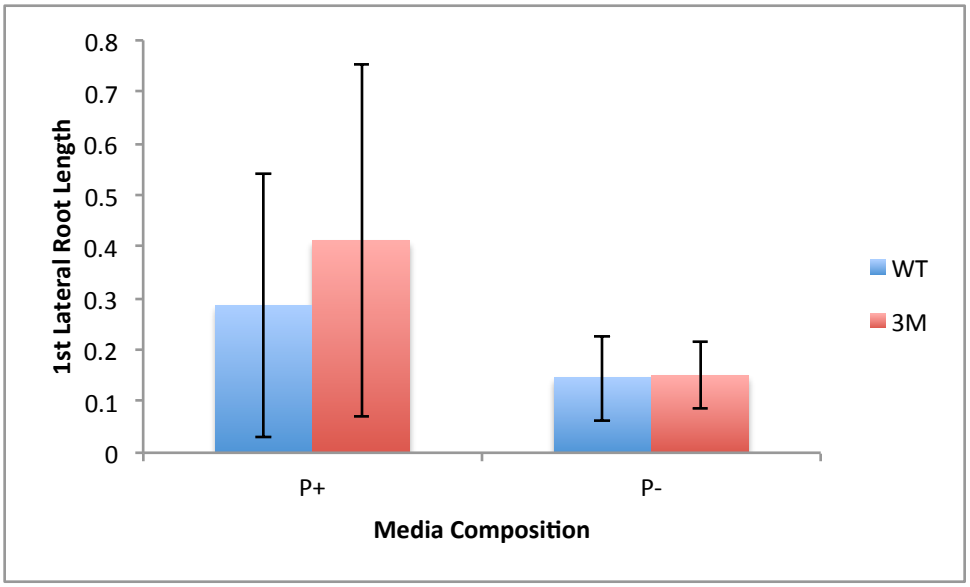


Figure 13: 1st lateral root length of wild type and triple mutant during phosphate starvation ((+P: P = 0.267; n (WT) = 72; n (3M) = 66) (-P: P = 0.940; n (WT) = 130; n (3M) = 101)).

There are no significant differences in the first lateral root lengths between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

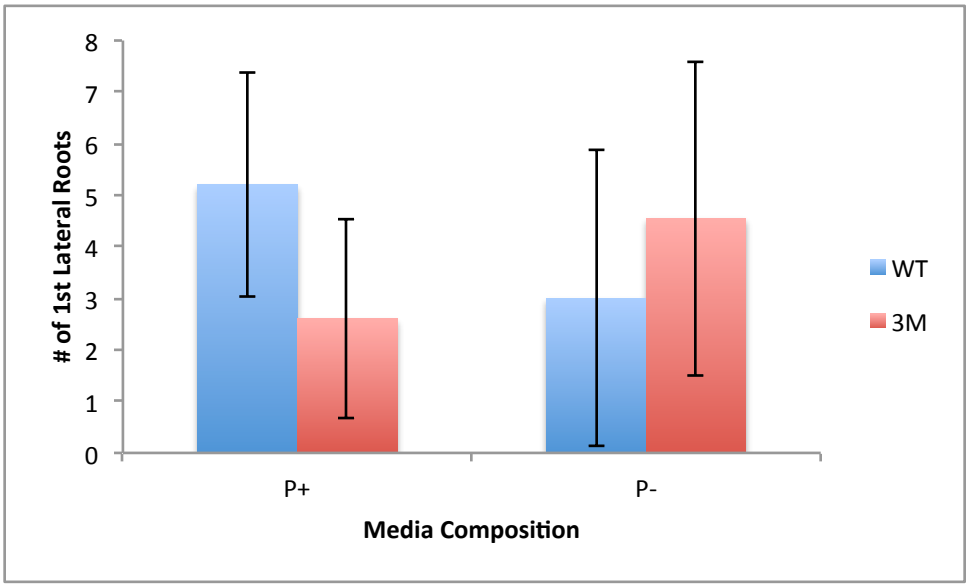


Figure 14: Number of 1st lateral roots of wild type and triple mutant during phosphate starvation ((+P: P = 0.600; n (WT) = 25; n (3M) = 26) (-P: P = 0.624; n (WT) = 28; n (3M) = 18)).

There are no significant differences in the number of first lateral roots between wild type and triple mutant under normal conditions and conditions of phosphate starvation.

Discussion

The only significant difference found in the phosphate starvation experiments was under the controlled condition (media with phosphate). This shows no significance with respect to phosphate deprivation. Furthermore, the lack of phosphate appears to have little or no effect on root growth during the entire first trial of experiments (**Fig 1, 2, and 3**). It was observed that the seedlings in this experiment did not show the typical phosphate starvation growth responses, such as inhibition of primary root elongation. This seems unlikely based on the essential nature of phosphate. After researching literature it was discovered that the phosphate-free salt mix used actually contained some phosphate (Jain et al. 2009). Therefore, the media used in the experiment was not truly phosphate deficient due to contamination from the salt mix. Essentially, this was not a phosphate starvation experiment after all. This is consistent with the results (or lack thereof) found in the experiments (**Figure 1, 2, and 3**). A different phosphate-free salt mix was used for the second and third trials of this experiment.

There was a significant difference in the primary root lengths in both of the different salt concentrations (**Figure 4**). The triple mutant showed significantly less growth than wild type under all conditions (0 mM NaCl, 50 mM NaCl, 100 mM NaCl). These results do not support the hypothesis that the triple mutant would grow more than wild type when the environmental stress was induced. The graph (**Figure 4**) shows that the triple mutant had little variation in average primary root length across all conditions. Visually, the salt far more adversely affected wild type. The triple mutant seems to grow more poorly under normal (non-salt) conditions but is

less affected by the introduction of salt. These results support the hypothesis that perhaps the *SIM* gene family is involved in the plant's ability to detect stress within its environment. Suppression of this gene may disrupt the sensor that detects the concentration of phosphate.

A significant difference was found in first lateral root length between wild type and triple mutant on 50 mM NaCl media (**Figure 5**). It seems that if this effect were due to the salt, the difference would be exaggerated with a higher salt concentration. However, there was no significant difference between wild type and triple mutant on 100 mM NaCl media. Furthermore, both wild type and triple mutant actually grew more with the increased salt concentration. The plant may be spending more energy to grow its lateral roots rather than its primary root in an effort to branch out and escape the salt.

There were no second lateral roots in either group when grown on 50 mM NaCl media (**Figure 6 and 8**). However, this has very little significance because there were very few second lateral roots in total. This explains why there is such great variance in these two bar graphs (**Figure 6 and 8**). It is very difficult to get results from such a small sample size. Moreover, there were just too few second lateral roots in the other two trials of the phosphate starvation experiment to gain any kind of insight. The sample size was too small and the variance was too large. Therefore, the graphs for second lateral roots were not included.

There were no significant differences in the number of first lateral roots between wild type and triple mutant in the various salt concentrations (**Figure 7**). However, this graph provides a good visual representation of the stunted growth

due to salt stress. Although not statistically significant, the triple mutant again grew less during salt stress than wild type.

The last two trials of the phosphate starvation experiment showed no significant differences between wild type and triple mutant for any of the parameters under any conditions (**Figure 9-14**). However, it was very evident that the media without phosphate had an obvious effect on the root growth of both wild type and triple mutant. As expected, both wild type and triple mutant showed stunted growth in an environment without phosphate.

Unfortunately, the third trial of the phosphate starvation experiment contained microbial contamination (**Figure 12-14**). The contamination was only observed on the media lacking phosphate. It is suspected that the contamination was introduced from the water used to rinse the seedlings during transplanting because the seedlings grown on normal media (with phosphate) were not rinsed with water and did not have any contamination. Therefore, we must consider that this presence of microbial growth may affect root growth.

Conclusion

Overall, the results do not support the hypothesis that seedlings with mutations in the *SIM* gene family would grow more than wild type during phosphate starvation and salt stress. However, it was intriguing to see how salt stunted the primary root growth of wild type much more than the triple mutant (**Figure 4**). There may be a connection between *SIM* and the plant's ability to recognize it is in a stressful environment. The salt experiments should be repeated in the future to see whether this pattern occurs again.

Future efforts might include repeating both the salt stress and phosphate starvation experiments. It will be interesting to see if future research shows that wild type is much more adversely affected by salt than a *SIM* mutant. Also, more varying or higher salt concentrations could provide better results when this experiment is repeated in the future. The first and third trial of the phosphate starvation experiment each had major problems due to phosphate and microbial contamination, respectively. Therefore, repeat experiments are needed. In addition to root length and number of roots, root density would also be an interesting parameter to investigate. Larger Petri plates could be used to allow longer growth periods. This could allow a few extra days of growth since the seedlings were removed just prior to their roots hitting the bottom of the plate. It would also be interesting to compare growth rates by somehow measuring each plant's daily growth.

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