Investigation of Root Zone Phytoene Synthase (*PSY*) and Alfin (*ALF*) Gene Expression in Salt Stressed *Zea mays* Inbred Lines Mo17 & B73

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Abstract: Carotenoids are plant pigments that function in photoprotectection, photosynthesis, and abiotic stress response. Soil salinity, an abiotic stressor, negatively impacts crop yields worldwide. Phytoene synthase (*PSY*) is the first dedicated enzyme in the carotenoid biosynthetic pathway, ultimately producing the stress hormone abscisic acid. In maize, *PSY* is encoded by three sub-functionalized genes: *PSY1*, *PSY2*, and *PSY3*, which are differentially expressed in root zones. How *PSY* expression is affected by salt stress is not well understood in US inbred lines. To determine the effects of salinity on *PSY* expression in developing roots, maize seedlings were grown in increasing salt concentrations. Root and shoot growth were assessed and the expression of *PSY* quantified in primary root zones. Expression of two Alfin *(ALF*) genes, also involved in stress responses, were measured. Increasing salt concentrations significantly stunted seedling growth and differentially altered the expression of *PSY* and *ALF* genes in primary root zones.

Keywords: maize, phytoene synthase, salinity, roots

I. Introduction

By 2050, the global population is expected to rise to 9 or 10 billion, requiring significant increase in crop production to sustain.[1] To meet this demand, crop production needs to double by then, with a 2.4% global yield increase per year for the top four staple crops: maize, rice, wheat, and soybeans. Current estimates place yearly increases at 1.6%, 1.0%, 0.9%, and 1.3% for each crop, respectively, far below the necessary yield production.[2] Speculated causes for these estimates include poor agricultural practices, loss of organic matter and nutrients in the soil, waterlogging, climate change, and soil salinity.[1] Soil salinization is one of the most globally prevalent and agriculturally impactful abiotic stresses, and has been cited as an important factor in decreased crop yield.[3, 4] While soils have a natural salt content, crops experience decreased development, yield, and germination rates when this increases to inhibitory levels.[5] Identifying means of improving crops against salt stress presents a necessary challenge to overcome in ensuring the sustainability of the global population.

Stress tolerance mechanisms are influenced by both physiological and biochemical processes and are composed of multigenic traits, which makes direct isolation difficult for crop improvement.[6] Detection of abiotic stressors depends on membrane sensors that identify mechanical shifts induced by stress, initiating different response pathways to mitigate damage. Plant stress signaling mechanisms are directed by primary and secondary signals, which vary depending on the type of stress.[7] Although some may overlap, stress response pathways are not universally utilized for all abiotic stress responses. These responses include the induction of synthesis of certain phytohormomes including abscisic acid (ABA), production of protective metabolites such as polyamines, increased levels molecular chaperones such as heat shock proteins, and increased activity of antioxidant enzymes to detoxify reactive oxygen species.[8, 9]

The primary response to salt stress is directed toward reducing the overaccumulation of Na^+ and Cl^-, restoring the K^+ content, and addressing osmotic stress as water uptake is inhibited by the osmotic potential of the environment as cells become hypertonic.[8] Secondary signals result from the prolonged effects of unattended primary stress, including damaged cellular components and inhibited metabolic functions. Salt stress inhibits photosynthesis, protein and enzyme synthesis, and disrupts crop intracellular osmotic gradients, all of which lead to stunted plant development and growth.[10] An important response to salt stress in plants is the production of ABA, which is synthesized from carotenoid precursors. Carotenoids also provide protection against light damage and play roles in photomorphogenesis, photoprotection, and photosynthesis.[11, 12]

Carotenoid synthesis occurs in plastids beginning with the condensation of two molecules of geranylgeranyl pyrophosphate; this reaction is catalyzed by the rate-limiting enzyme phytoene synthase (*PSY*), producing phytoene. Phytoene is subsequently desaturated, cyclized, and oxidized by a series of enzymes to produce violoxanthin and neoxanthin. These carotenoids are converted to cis-xanthoxin, exported to the cytosol, and metabolized to ABA.[13] ABA is detected by proteins in the PYRABACTIN RESISTANCE 1 (PYR1)/PYR1-like protein (PYL) family. Binding of ABA to a PYL receptor activates a number of kinases and phosphatases, resulting in metabolism changes.[14] ABA-dependent signaling pathways are mediated by a number of transcription factors, including those in the MYB/MYC, WRKY and zinc finger homeodomain families, which activate stress response genes in response to osmotic stress.[10]

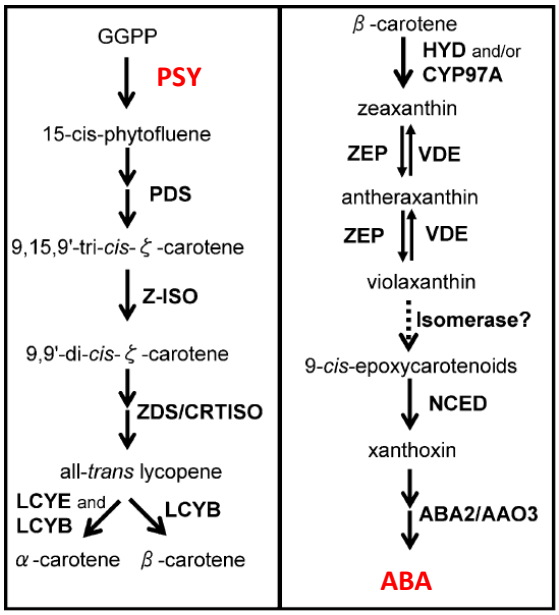

Figure 1. Carotenoid biosynthetic pathway with *PSY* and ABA indicated [3]

ABA regulates carotenoid production in a positive feedback loop to meet stress demands.[12] In a study of *Arabidopsis thaliana*, salt exposure increased ABA production and carotenoid accumulation in roots, connecting carotenoid synthesis with salt tolerance.[15] ABA is able to alter carotenoid expression via *PSY*.[11] Depending on the plant species there can be several nonredundant paralogs of *PSY* expressing functional enzymes that respond to different stimuli and vary in concentration and location within the plant body.[3] In *Zea mays* there are three nonredundant *PSY* paralogs which allow for the differential accumulation of carotenoids in root, leaves, embryo, and endosperm.[3, 16] The third paralog, *PSY*3, has been observed to increase prior to ABA accumulation, particularly to salt stress in the roots.[12] The relation between these events has been explored in several studies, linking *PSY*3 expression with increased salt tolerance.[3, 11, 16]

Alfin (*ALF*) genes are transcription factors found exclusively in plants and were first identified in root development processes.[17] They are also involved in response to abiotic stress. Increased expression of *ALF* paralogs has been associated with improved tolerance to salinity and drought.[18] Previous studies in transgenic *Atriplex hortensis* found certain *ALF* paralogs to increase drought resistance and enhance ABA-mediated responses; similar findings were made for salt tolerance in transgenic *Medicago sativa*.[19] In *Zea mays*, 18 *ALF* paralogs have been identified, many of which are upregulated when exposed to abiotic stressors.[20]

Previous studies have demonstrated a link between *PSY* expression and ABA content of plant tissues, but there is limited research on *PSY* paralog expression among root zones.[12] Root functionality is critical to crop establishment and performance. Three root zones, the meristematic zone (MZ), elongation zone (EZ), and root hair zone (RHZ), represent different developing sections of the root (Figure 2). Active division of new cells occurs within the MZ, which displaces cells into the EZ wherein the cells expand in size. The RHZ is the site of cell differentiation, where the cells specialize and become part of the mature root. Research suggests that roots are the site of ABA synthesis and transport to other parts of the plant body when afflicted by abiotic stress.[13] Examining these regions may delineate key aspects of the stress response in roots to aid in future attempts to improve root performance.

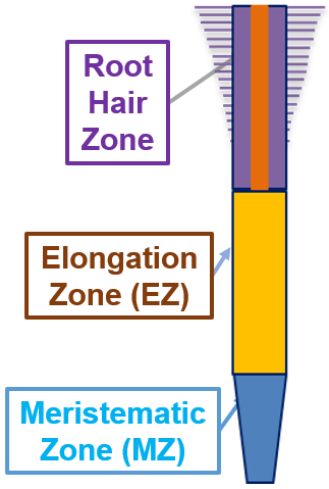

Figure 2. Root zone designations

The purpose of this study was to analyze the effects of salinity on seedling germination and growth, as well as on the expression of *PSY* paralogs and *ALF* genes in the root zones of maize seedling primary roots. ABA should increase as *PSY* and *ALF* expression increases, aiding in salt tolerance mechanisms. *Zea mays* Mo17 and B73 inbred lines were selected to study due to their significant use as genetic models, the extensive research done on them, and their importance to

current US maize agriculture. While studies have been conducted outside the US quantifying salt tolerance in foreign maize inbred lines, none have characterized *PSY* and *ALF* gene expression in root zones in response to saline treatment for these lines.[3, 20, 21, 22, 23]

II. Materials & Methods

NaCl Effect on Germination, Root and Shoot Growth

To determine which NaCl concentrations provide viable seedlings, a germination test was conducted. Mo17 and B73 kernels were surface sterilized in 4% bleach solution for 1 min 30 seconds to eliminate fungus and bacterial growth, rinsed in sterile water, and grown in a 1 mM $CaCl_2$ solution containing 0 mM, 25 mM, 100 mM or 150 mM NaCl.[24] Kernels were grown for five days in the dark at 30°C on germination paper. Germination rates for each group were recorded. On day 5, the wet weight of the shoot and root system were measured, and the number and weight of seminal roots determined, as well as the length of EZ and RHZ in the seedlings' primary roots.

Measurement of PSY & ALF Gene Expression in Maize Seedling Root Zones

B73 and Mo17 kernels (n=45) were divided into three groups, surface sterilized and placed on germination paper in a sterilized tray.[24] The control group was grown in a 1 mM $CaCl_2$ solution containing 25 mM NaCl, while the high salt group were grown in a 1 mM $CaCl_2$ solution containing 100 mM or 150 mM NaCl. Kernels were incubated for 5 days in the dark at 30°C on germination paper. Primary roots were dissected into MZ, EZ and RHZ zones on day 5, frozen in liquid nitrogen and stored at -80ºC. Tissues from five plants were pooled to make a single biological replicate; 5 biological replicates each of low (25 mM NaCl) and high (100 mM and 150 mM NaCl) salt were used in these comparisons. RNA was isolated using the RNeasy method, treated with DNase I to remove genomic DNA, and cDNA was prepared using Invitrogen's First Strand Synthesis kit following the manufacturer's protocols. Quantitative reverse transcription PCR (qRT-PCR) using Power SYBR Select™ (Applied Biosystems) and manufacturer's protocol on an Applied Biosystems StepOnePlus™ instrument. For each experiment, five biological replicates were examined, each performed in triplicate. qRT-PCR was performed using paralog specific primers for *PSY1, PSY2, PSY3, ALF2A* and *ALF2B*. The samples were normalized to the endogenous expression of maize *GAPC* and *actin7* genes and standardized to MZ samples.

III. Results & Discussion

Effects of NaCl on Germination & Seedling Growth

Growth of five-day maize seedlings were significantly stunted by increasing concentrations of salt. Overall, seedlings grew best in the 1 mM $CaCl_2$ solution containing 25 mM NaCl (Figures 3A and 3C). Significant differences in germination rate and seedling growth were observed between the high salt treatments (100 mM and 150 mM NaCl) and control (0 mM NaCl and 25 mM NaCl) seedlings in nearly all measured parameters (Figure 3). Seedlings grown in the 100 mM NaCl or 150 mM NaCl showed no statistically significant difference in shoot, primary root, or seminal root length. However, total root system wet weight was significantly lower in the seedlings grown in 150 mM NaCl. The RHZ length was significantly shorter in the 100 mM and 150 mM NaCl treated B73 and Mo17 seedlings, while only the EZ of B73 was significantly shorter in seedlings grown in high salt. This may suggest a greater capacity for Mo17 to grow under salt stress in the EZ of primary roots.

Not only did the higher salt concentration inhibit seedling growth, it also negatively impacted germination rate. Seeds grown in 0 mM NaCl and 25 mM NaCl each demonstrated an 80% germination rate, while seeds grown in 100 mM NaCl and 150 mM NaCl demonstrated a 40% and 60% germination rate, respectively. Sodium from salt has been found to interfere with potassium uptake, interfering with water transport as the two compete for the ion transport mechanism.[25] However, this data suggests some quantity of salt is beneficial to early root and shoot development, which may align with their micronutrient requirements.

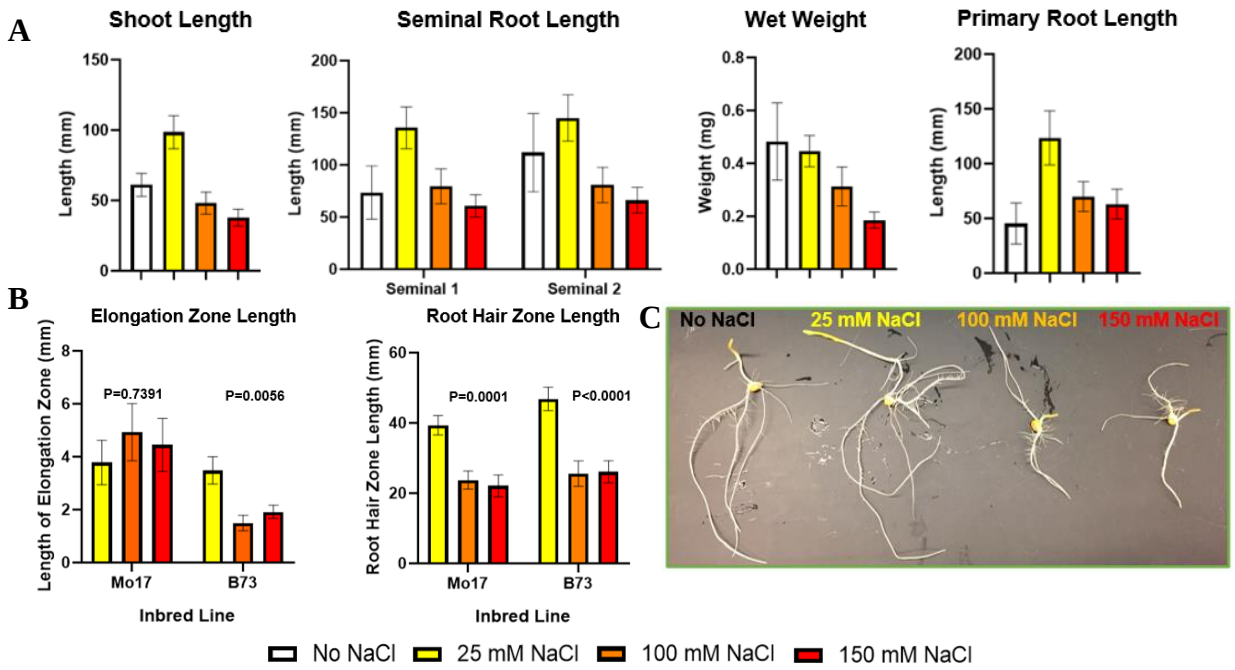

Figure 3. Effect of NaCl Concentration on Maize Seedling Growth (A) Impact of NaCl concentration on Mo17 seedling growth. Four NaCl concentrations were examined: 0 NaCl (control; n=8), 25 mM NaCl (n=16), 100 mM NaCl (n=8), and 150 mM NaCl (n=12). Root system wet weight was taken before root dissection; primary roots, seminal roots, and shoot tissue was harvested by dissecting seedlings at the base of the kernel. Data was evaluated using a one-way ANOVA test. Mean ± SEM are depicted and significance values are noted on graphs. **(B)** Impact of NaCl concentration on growth of primary roots zones of B73 and Mo17 seedlings. Three NaCl concentrations were examined: 25 mM NaCl (n=83), 100 mM NaCl (n=30), and 150 mM NaCl (n=45). **(C)** Representative Mo17 seedlings grown in each NaCl treatment group.

PSY & ALF Expression in Root Zones of Salt Stressed Maize Seedlings

To examine the influence of high salt on *PSY* and *ALF* paralog expression, B73 and Mo17 seedlings were grown in low (25 mM NaCl) or high (100 mM or 150 mM NaCl) salt solutions. Seedlings grown in the high salt solutions showed no significant difference in shoot or root growth and were combined into a single high salt group. The expression of *PSY3* in the EZ and MZ, *ALF2A* in EZ, and *ALF2B* in RHZ was significantly reduced in Mo17 roots treated with high salt (Figure 4). Conversely, high salt treatment induced the expression of *PSY1* and *ALF2B* in the RHZ of these plants. We also observed significant changes in the expression of *PSY* and *ALF* paralogs in B73 seedlings grown in high salt. Statistically significant differences included a reduction in expression of both *PSY1* and *PSY2* in the MZ and RHZ, reduced expression of *PSY2*

in the MZ, and an increase in the expression of both *ALF2A* and *ALF2B* in the RHZ (Figure 4). With the exception of a consistent decrease in the expression of *PSY3* and an increased expression of *ALF2A*, it is interesting to note that these two inbred lines responded differentially to the high salt treatment.

Observed induction of *ALF* paralogs in response to salt stress is consistent with prior studies, although these did not examine responses in different root zones.[19] Our results demonstrate that *PSY3* expression is significantly reduced in response to high salt, which is markedly different from previous research that suggested increased expression in salt stressed roots.[15] This could be due to different experimental designs, where germinating kernels and growing seedlings in a salt solution could cause differential expression of *PSY3* as compared to applying a salt solution to established seedlings. This may also ultimately influence the production of ABA to initiate stress mechanisms. We observed a significant reduction in the expression of *PSY2* only in B73 RHZ in response to high salt. Overall, our data suggests that RHZ in both inbred lines experiences the greatest difference in expression for *PSY* and *ALF* genes under salt stress.

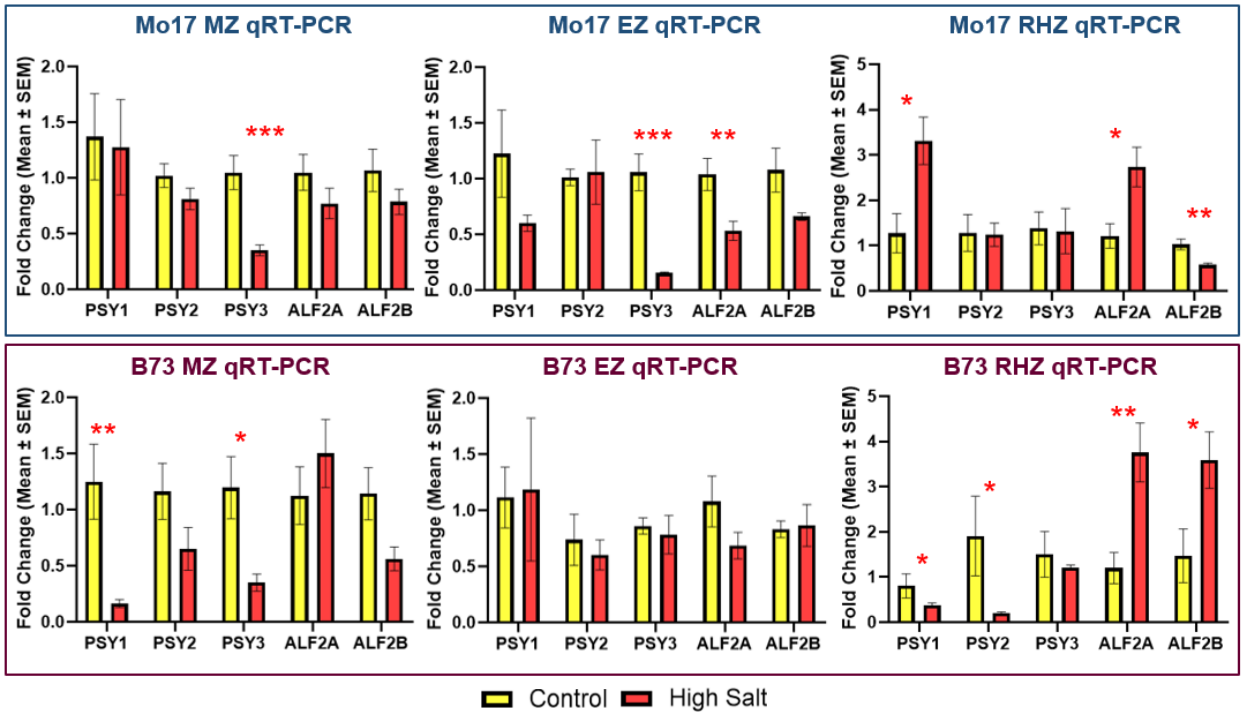

Figure 4. Expression of *PSY* and *ALF* Paralogs in Primary Root Zones of Seedlings Grown in Low and High Salt. Expression of *PSY* and *ALF* paralogs was determined using qRT-PCR and normalized to the endogenous expression of the maize *GAPC* and *actin7* genes. The $\Delta\Delta C_T$ method was used to calculate relative expression and standardized to control (25 mM NaCl) samples. 100 mM NaCl and 150 mM NaCl were combined in the high salt category. Mean ± SEM of 5 biological replicates are shown on graphs. Low and high salt $\Delta\Delta C_T$ were compared via an unpaired T Test. P values are designated as follows: *p<0.05, ** p<0.01, ***p<0.001.

Patterns of Expression of PSY and ALF Paralogs in Root Zones of Salt Stressed Maize Seedlings
To determine whether seedlings grown in high salt impacted the level and pattern of expression of *PSY* and *ALF* paralogs in root zones of B73 and Mo17 seedlings, the qRT-PCR data was

recalculated and standardized to the expression of each paralog in the MZ (Figures 5 and 6). No significant difference in the expression of *PSY* or *ALF* paralogs was observed across the root zones in Mo17 (Figure 5) or B73 (Figure 6) seedlings grown in a low salt solution. However, Mo17 seedlings grown in high salt demonstrated a significantly higher expression of *PSY1*, *PSY3* and *ALF2A* in RHZ. A similar induction in expression of *PSY3* and *ALF2A* was observed in B73 seedlings grown in high salt, as well as an induction of *ALF2B* expression. Growth in high salt decreased the expression of *PSY2* in RHZ of B73 seedlings.

The significant differences seen in the expression of *PSY* and *ALF* paralogs in the high salt treatment data suggests a correlation between high salt exposure and their expression. This is particularly relevant for *PSY3* and both *ALF* genes, which have been previously connected to abiotic responses in salt tolerance in roots. The lack of significant difference between *ALF2B* treatments in Mo17 compared to B73 could suggest a potential difference in gene function between the two inbred lines.

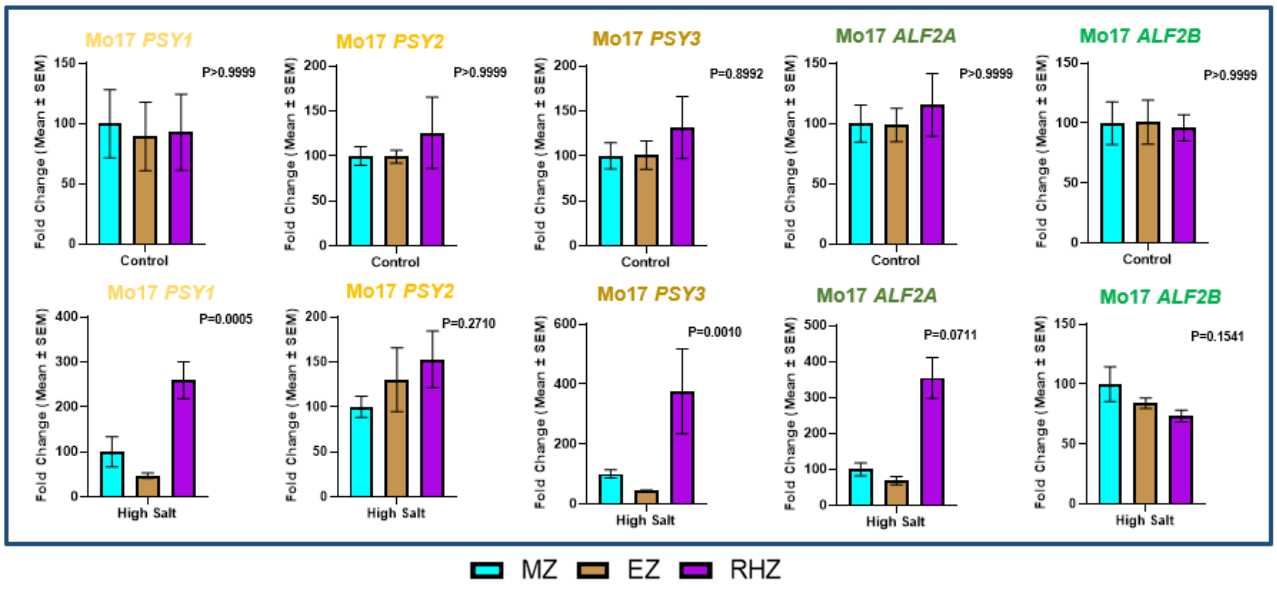

Figure 5. Pattern of Expression of *PSY* and *ALF* Paralogs in Root Zones of Mo17 Seedlings Grown in Low (Top) or High (Bottom) Salt. Expression of *PSY* and *ALF* paralogs in Mo17 primary roots was determined using qRT-PCR and normalized to the endogenous expression of *GAPC* and *actin7* genes. The $\Delta\Delta C_T$ method was used to calculate relative expression of each gene and standardized to MZ values (set at 100). Mean ± SEM for 5 biological replicates are depicted on each graph. A one-way ANOVA test was applied to $\Delta\Delta C_T$ values and significance values for interaction are as noted on graphs.

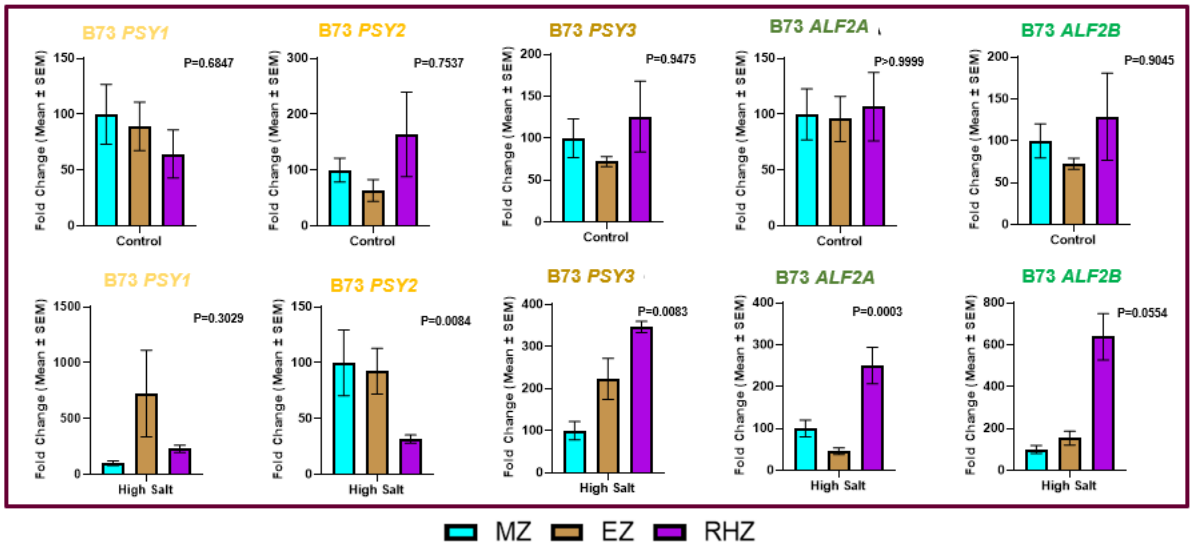

Figure 6. Pattern of Expression of *PSY* and *ALF* Paralogs in Root Zones of B73 Seedlings Grown in Low (Top) or High (Bottom) Salt. Expression of *PSY* and *ALF* paralogs in Mo17 primary roots was determined using RT-PCR and normalized to the endogenous expression of the maize *GAPC* and *actin7* genes. The $\Delta\Delta C_T$ method was used to calculate relative expression of each gene and standardized to MZ values (set at 100). Mean ± SEM for 5 biological replicates are depicted on each graph. A one-way ANOVA test was applied to $\Delta\Delta C_T$ values and significance values for interaction are as noted on graphs.

IV. Conclusion

Thorough comprehension of the *Zea mays* salt stress response mechanism in developing roots is necessary for future research in improving root performance and crop vigor. Maize tolerance to increased levels of saline stress may be mitigated by greater expression of *PSY* and *ALF* paralogs in the roots due to the connection between the carotenoid biosynthetic pathway and the phytohormone ABA. The expression of the three *PSY* paralogs and select *ALF* genes among the primary root zones suggests there is a significantly different reaction to concentrations of salt between these zones when exposed during germination and early growth. Increased salt levels severely stunted both germination rates and overall development of the five-day seedlings. Differences between the inbred lines Mo17 and B73 could suggest slightly different mechanisms in stress tolerance, and may be of interest for study to determine if either demonstrates greater resistances to abiotic stressors. This study attempted to examine the production and effects of ABA in maize roots by determining *PSY* expression, however, a direct method of measurement utilizing an enzyme-linked immunosorbent assay (ELISA) and mass spectrometry analysis would better elucidate the role of ABA in both root salt tolerance and development. Other possible avenues for future consideration include imaging of the root and leaf architecture during different concentrations of salt exposure and expression studies for other *ALF* genes.

Acknowledgements

We acknowledge the Ronald E McNair Program and NSF Plant Genome Award DBI-0820610 for funding for this project. We also thank Dr. Brent Buckner for his assistance in data acquisition, analysis, experimental design suggestions, and feedback, and Evan Reeves for his assistance in seedling dissections.

References

1. Wichelns, D., & Qadir, M. (2015). Achieving sustainable irrigation requires effective management of salts, soil salinity, and shallow groundwater. *Agricultural Water Management, 157*, 31-38. doi:10.1016/j.agwat.2014.08.016
2. Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *PLoS ONE, 8*(6). doi:10.1371/journal.pone.0066428
3. Li, F., Vallabhaneni, R., & Wurtzel, E. T. (2008). PSY3, a new member of the phytoene synthase gene family conserved in the poaceae and regulator of abiotic stress-induced root carotenogenesis. *Plant Physiology, 146*(3), 1333-1345. doi:10.1104/pp.107.111120x
4. Welle, P. D., & Mauter, M. S. (2017). High-resolution model for estimating the economic and policy implications of agricultural soil salinization in California. *Environmental Research Letters, 12*(9), 094010. doi:10.1088/1748-9326/aa848e
5. Ali, S., Khan, M. J., Shah, Z., Naveedullah, N., & Jalal, A. (2019). Genotypic screening of maize (*Zea mays* L.) for salt tolerance at early growth stage under different salinity levels. *Sarhad Journal of Agriculture, 35*(1). doi:10.17582/journal.sja/2019/35.1.208.215
6. Sandhu, D., Pudussery, M. V., Kumar, R., Pallete, A., Markley, P., Bridges, W. C., & Sekhon, R. S. (2019). Characterization of natural genetic variation identifies multiple genes involved in salt tolerance in maize. *Functional & Integrative Genomics, 20*(2), 261-275. doi:10.1007/s10142-019-00707-x
7. Zhu J. K. (2016). Abiotic stress signaling and responses in plants. *Cell*, *167*(2), 313–324. https://doi.org/10.1016/j.cell.2016.08.029
8. Alcázar, R., Bueno, M., & Tiburcio, A.F. (2020) Polyamines: small amines with large effects on plant abiotic stress tolerance. *Cells.* 9(11): 2373. doi: 10.3390/cells9112373
9. Abhinandan, K., Skori,L., Stanic, M., Hickerson, N.M.N., Jamshed, M., & Samuel, M.A. (2018) Abiotic stress signaling in wheat – an inclusive overview of hormonal interactions during abiotic stress responses in wheat. *Frontiers in Plant Sciences*, 9: 734. doi: 10.3389/fpls.2018.00734
10. Hassan, N., Hasan, M. K., Shaddam, M. O., Islam, M. S., Barutçular, C., & Sabagh, A. E. (2018). Responses of maize varieties to salt stress in relation to germination and seedling growth. *International Letters of Natural Sciences, 69*, 1-11. doi:10.18052/www.scipress.com/ilns.69.1
11. Simpson, K., Fuentes, P., Quiroz-Iturra, L. F., Flores-Ortiz, C., Contreras, R., Handford, M., & Stange, C. (2018). Unraveling the induction of *phytoene synthase 2* expression by salt stress and abscisic acid in *Daucus carota*. *Journal of Experimental Botany, 69*(16), 4113-4126. doi:10.1093/jxb/ery207
12. Nisar, N., Li, L., Lu, S., Khin, N., & Pogson, B. (2015). Carotenoid metabolism in plants. *Molecular Plant*, 8(1), 68-82. doi:10.1016/j.molp.2014.12.007
13. Gietler, M., Fidler, J., Labudda, M. & Nykiel, M. (2020). Abscisic acid - enemy or savior in the response of cereals to abiotic and biotic stresses? *International Journal of Molecular Science, 21,* 4607.
14. He, Z., Zhong, J., Sun, X., Wang, B., Terzaghi, W., & Dai, M. (2018). The maize ABA receptors ZmPYL8, 9, and 12 facilitate plant drought resistance. *Frontiers in Plant Sciences*, 9:422. https://doi.org/10.3389/fpls.2018.00422
15. Ruiz-Sola, Arbona, Gómez-Cadenas, Rodríguez-Concepción, & Rodríguez-Villalón, (2014). A root specific induction of carotenoid biosynthesis contributes to ABA

production upon salt stress in Arabidopsis. *PLoS ONE*, 9(3). doi:10.1371/journal.pone.0090765
16. Li, F., Tzfadia, O., & Wurtzel, E. T. (2009). The phytoene synthase gene family in the Grasses. *Plant Signaling & Behavior, 4*(3), 208-211. doi:10.4161/psb.4.3.7798
17. Krochko, J. E., Pramanik, S. K., & Bewley, J. D. (1992). Contrasting storage protein synthesis and messenger RNA accumulation during development of zygotic and somatic embryos of alfalfa (*Medicago sativa L*.). *Plant Physiology*, 99(1), 46-53
18. Winicov I. (1993). cDNA encoding putative zinc finger motifs from salt-tolerant alfalfa (*Medicago sativa L*.) cells. *Plant Physiology*, *102*(2), 681–682. https://doi.org/10.1104/pp.102.2.681
19. Tao, J.J., Wei, W., Pan, W.J., Lu, L., Li, Q., Ma, J., Zhang, W., Ma, B., Chen, S. & Zhang, J. (2018). An *Alfin-like* gene from *Atriplex hortensis* enhances salt and drought tolerance and abscisic acid response in transgenic Arabidopsis. *Scientific Reports*, 8, 2707. https://doi.org/10.1038/s41598-018-21148-9
20. Zhou, W., Wu, J., Zheng, Q., Jiang, Y., Zhang, M., & Zhu, S. (2016). Genome-wide identification and comparative analysis of Alfin-like transcription factors in maize. *Genes & Genomics, 39*(3), 261-275. doi:10.1007/s13258-016-0491-6
21. Soufan, W. & Okla, M.K. (2014). Effect of salt stress on germination, growth and yield of maize (*Zea mays L*. CV. Ghota-1). *Journal of Pure and Applied Microbiology*. 8. 185-194.
22. Singh, P.K., Shahi, S.K., & Singh, A.P. (2015). Effects of salt stress on physico-chemical changes in maize (*Zea mays* L.) *Plants in Response to Salicylic Acid*.
23. Neto, A. D., Prisco, J. T., Enéas-Filho, J., Lacerda, C. F., Silva, J. V., Costa, P. H., & Gomes-Filho, E. (2004). Effects of salt stress on plant growth, stomatal response and solute accumulation of different maize genotypes. *Brazilian Journal of Plant Physiology*, 16(1), 31-38. doi:10.1590/s1677-04202004000100005
24. Rahman, M. M., Ahammad, K. U., & Alam, M. M. (2011). Effect of soaking condition and temperature on imbibition rate of maize and chickpea seeds. *Research Journal of Seed Science*, 4(2), 117-124. doi:10.3923/rjss.2011.117.124
25. Farooq, M., Hussain, M., Wakeel, A. & Siddique, K.H.M. (2105). Salt stress in maize: effects, resistance mechanisms, and management. A review. *Agronomy for Sustainable Development*. 35, 461–481. https://doi.org/10.1007/s13593-015-0287-0