Linking Performance of Almond Rootstocks to Underlying Physiological and Genetic Determinants of Salinity Tolerance

Project No.: **HORT26.Sandhu**

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A. Summary *(In laymen's terms – emphasize key findings and recommendations)*

Salinity is one of the most important abiotic stresses that adversely affect plant growth and productivity globally. To tackle this complex problem, it is important to link the biochemical and physiological responses with the underlying genetic determinants. In an effort to understand underlying genetic and biochemical mechanisms for the salt tolerance process, we have evaluated 16 commercial almond rootstocks for salt tolerance. To determine importance of specific ions toxicities we applied 5 different treatments of irrigation water that included control (T1), sodium sulfate-based solution (T2), sodium chloride-based solution (T3), sodium dominant water with mixed anions (chloride and sulfate) (T4), and calcium and magnesium dominant water with mixed anions (chloride and sulfate) (T5). We studied the effect of different treatments on trunk diameter, survival rate, physiological and biochemical traits. The survival rate and the change in trunk diameter analyses suggested that mostly Na and, to a lesser extent, Cl concentration in irrigation water are the most critical ion toxicities for almond rootstocks. Photosynthesis showed the highest correlation with change in trunk diameter, followed by correlations with stomatal conductance and chlorophyll content. Different rootstocks were characterized based on their ability to store Na and Cl in their leaf tissues. For the most part, ion analysis was correlated with trunk diameter and survival rate assessments.
Leaf proline content showed significant correlation with ability of plants to exclude Na and Cl and negative correlation with the survival rate. These findings suggest that proline can be used as a useful biochemical marker for screening genotypes tolerant to salinity.

The expression analysis of 10 genes known to play important roles in salt tolerance revealed that treatments where Na and Cl were the main ions in irrigation water (T3 and T4) led to induction of most genes, suggesting importance of both the chloride and sodium toxicities during salt stress in almonds. The *HKT1* and *AKT1* genes displayed the highest upregulation (expression) in salinity treatments in roots and *NHX1*, *SOS3* and *AKT1* were highly upregulated in salinity treatments in leaves. In addition to the 10 genes known to play import roles in Na^+ transport, we studied 8 genes involved in Cl transport. This analysis showed that root-to-shoot Cl^- transport and sequestration of Cl^- into root vacuoles are two important traits in Prunus that regulated Cl^- toxicity.

The RNA sequencing (RNAseq) analysis of one most tolerant and one most sensitive rootstock resulted in identification of differentially expressed genes (DEGs) between salinity versus control treatments, root versus leaves, and also salt-tolerant versus salt-sensitive rootstocks. Several candidate genes involved in salinity stress were identified. These genes include both previously known genes and several novel genes. We have conducted functional validation of Prunus genes, *PpHKT1* and *PpSOS2*, into Arabidopsis mutants. Both genes fully complemented salt tolerance function in Arabidopsis mutants. The candidate genes will be further explored to develop a link between the predicted function and their functional relevance to the physiological or the biochemical mechanisms involved in salt tolerance. Combining different components of the salt tolerance mechanism may lead to the development of superior salt tolerant rootstocks. Improving salt tolerance in almond rootstocks will not only improve crop yield, but will also provide incentives to expand the use of alternative/degraded waters that will allow almonds to be cultivated in new lands and at lower costs for irrigation.

B. Objectives *(300 words max.)*

The overarching goal of proposed study is to identify rootstocks tolerant to salinity and to understand genetic, physiological and biochemical mechanisms involved in salinity tolerance. The functional characterization of candidate genes involved in salt tolerance will help in the development of tools for marker-assisted selection, which may become instrumental in screening almond germplasm to select novel sources for salt tolerance.

1. Year 1, Objective 1. Evaluate diverse rootstocks for tolerance to salinity of solutions of mixed salt composition.
2. Year 1, Objective 2. Characterize physiological and biochemical markers associated with salt tolerance and salt composition of irrigation water in almond rootstocks.
3. Year 1, Objective 3. Identify and characterize the genes involved in salinity tolerance in almond rootstocks.
4. Year 2, Objective 1. Evaluation of almond rootstocks to determine their tolerance response to a range of salt concentrations.
5. Year 2, Objective 2. Characterizing different almond genotypes based on different components of salt tolerance mechanism.
6. Year 2, Objective 3. Study global changes in the gene expression profiles under normal versus salt stress conditions in almond rootstocks.
7. Year 3, Objective 1. Evaluation of selected almond rootstocks for their effects on scion performance under salinity stress and identification of underlying genetic components.
8. Year 3, Objective 2. Using priming as an alternate approach to mitigate salinity stress in almond rootstocks.
9. Year 3, Objective 3. Functional validation of almond genes involved in salt tolerance using model plants

C. Results *(This is the core function of this report)*

Year 1, Objective 1. Evaluate diverse rootstocks for tolerance to salinity of solutions of mixed salt composition.

In trial 1, we have analyzed sixteen (non-grafted) rootstocks in mixed salt compositions. Different irrigation water treatments included control (T1), Na-SO_4 based irrigation water (T2), Na-Cl based irrigation water (T3), Na-Cl/SO_4 based irrigation water (T4) and Ca/Mg-Cl/SO_4 based irrigation water (T5). Before initiating salinity treatments, trunk diameter was measured for all the plants in July, 2017. Trunk diameter was again measured in May 2018 (Figures 1, 2,

3 and 4). Percent change in trunk diameter was calculated and relative change in trunk diameter with respect to T1 was calculated. As expected, T1 showed the highest change in trunk diameter followed by T5 and T2 (Figure 3). The T3 and T4 treatments displayed the lowest increase in trunk diameter. A smaller increase in all three Na based treatments (T2, T3 and T4) indicated importance of Na^+ toxicity in almonds. A smaller increase in trunk diameter in T3 (Na-Cl based treatment) versus T2 ($Na\text{-}SO_4$ based treatment) suggests importance of both Na^+ and Cl^- toxicities during salt stress (Figure 3).

Figure 1. Experimental setup for the salt tolerance experiment on 16 almond rootstocks. Students measured trunk diameter before the application of salt treatment.

Figure 2. Physical appearance of 16 almond rootstocks irrigated with Na-Cl based water for one year showing differences in survival rates.

Out of 16 tested rootstocks, Rootpac 40 displayed highest relative change in trunk diameter (73.6%) under salinity versus control (Figure 4). Other rootstocks that performed well in salinity treatments include Empyrean 1, BB106, Cornerstone, Viking and F x A (Figure 4). The rootstocks that performed poorly in salinity treatments were Rootpac 20, Guardian, Lovell and Krymsk 86.

One year after the initiation of salt treatments, plants were analyzed for survival rates and the toxicity symptoms on leaves. T3 was the harshest treatment for the plants with the lowest average survival rate (17.4 %) followed by T4 (31.9%), T2 (54.9%), T5 (76.4%) and T1 (85.4%) (Figure 5). Rootpac 40 displayed very high relative survival rate (92.5%) for four salinity treatments, followed by Empyrean 1 (90.0%), Cornerstone (67.9%), Bright's 5 (64.3%), BB 106 (64.3%), and FxA (63.9%) (Figure 6). On the other hand, Lovell (18.8%), Guardian (25.0%), and Rootpac 20 (25.0%) had low relative survival rates for the four salinity treatments. While many of the rootstocks were dead in T3, Rootpac 40 had minimal toxicity symptoms (Figure 2).

The comparison of 16 different almond rootstocks for tolerance to salinity of various solutions of different salt compositions showed that Na and to a lesser extent Cl concentration in irrigation water are the most critical ion toxicities for almond rootstocks. This investigation demonstrated that both Na and Cl are critical for salt stress in almonds. Our previous work showed that Na toxicity is known to play an important role during salt stress in salt tolerant crops such as alfalfa (Cornacchione and Suarez 2017; Sandhu et al. 2017); however Cl toxicity plays a major role in salt-sensitive crops like strawberries and avocado (Suarez and Grieve 2013). For

some plants, such as faba beans, concentrations of both Na and Cl ions are critical during salt stress (Tavakkoli et al. 2010).

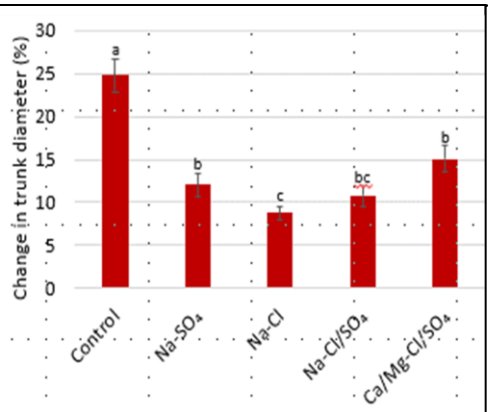

Figure 3. Change in trunk diameter of almond rootstocks in four mixed salt ion combinations of irrigation waters. Error bars represent standard error. Means followed by the same letters are not significantly different according to LSD (0.05)

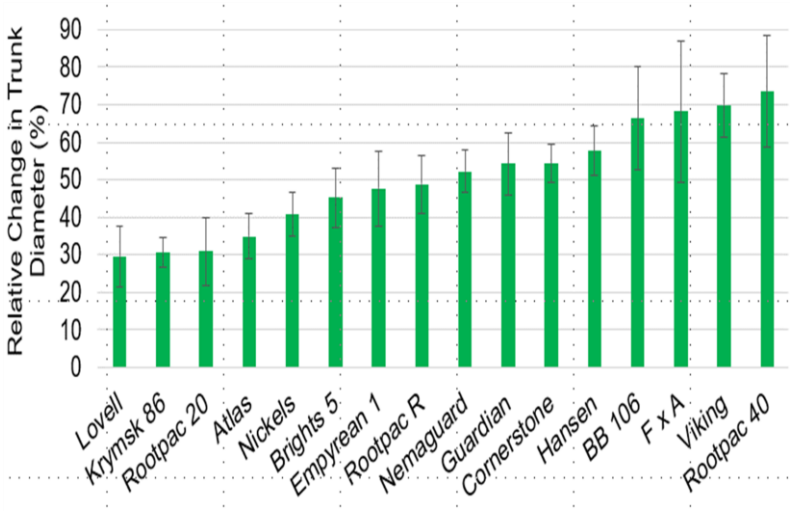

Figure 4. Relative change in trunk diameters of 16 almond rootstocks in salinity treatment with respect to control after one year of salinity treatment. Error bars represent standard error.

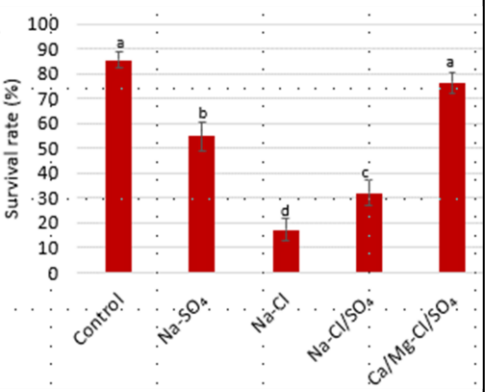

Figure 5. Survival rates of almond rootstocks in four mixed salt ion combinations of irrigation waters. Error bars represent standard error. Means followed by the same letters are not significantly different according to LSD (0.05)

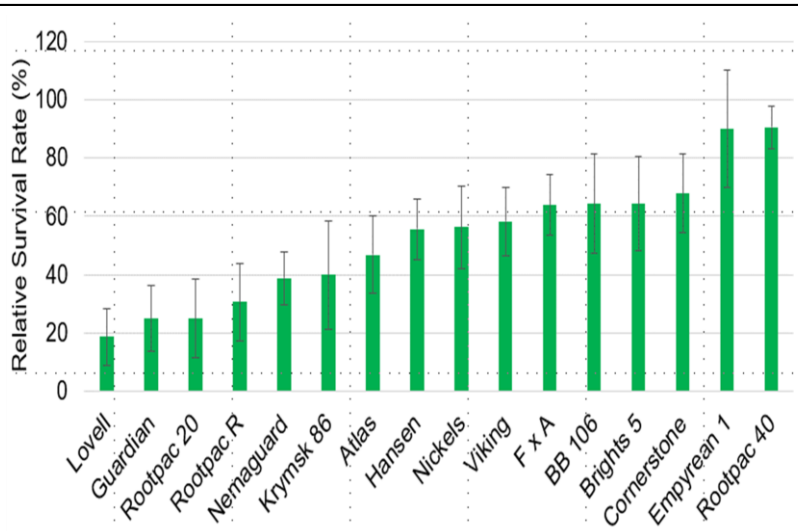

Figure 6. Relative survival rates of 16 almond rootstocks in salinity treatments with respect to control after one year of salinity treatment. Error bars represent standard error.

Year 1, Objective 2. Characterize physiological and biochemical markers associated with salt tolerance and salt composition of irrigation water in almond rootstocks.

The analyses of physiological parameters for 16 almond rootstocks treated with five treatments of irrigation water suggested that there were significant differences among treatments and among rootstocks ($P<0.001$) for the net photosynthetic rate (*Pn*), leaf stomatal conductance (*gs*) and *SPAD* chlorophyll content. T3 showed maximum reduction for *Pn*, *gs* and *SPAD* as

compared to the control (T1) (Figure 7). On the other hand, T5 had least reduction in *Pn* and *SPAD* as compared to the control and T4 exhibited least reduction for *gs*.

Pn had the highest correlation (R^2 = 0.88) with trunk diameter, followed by *gs* (R^2 = 0.63), then *SPAD* (R^2 = 0.53). High correlation between *Pn* and the trunk diameter suggests that *Pn* is an important physiological parameter and can be used an indicator of salinity stress (Figure 7A).

Comparison of different rootstocks in T3 revealed that Nickels, Empyrean 1, Hansen and Brights 5 had high *Pn* values as compared to the other rootstocks. Nemaguard was the worst performer for *Pn* in T3. Hansen performed well in all four salinity treatments (T2 – T5). Nickels, Emyprean 1 and Hansen were also the top three performers for *gs* in T3 suggesting that observations for *Pn* and *gs* were somewhat consistent for different rootstocks. For the *SPAD* chlorophyll content Hansen, Brights 5, Cornerstone, BB106 and Nickels were good performers in T3. Hansen was the best performer for *SPAD* readings among all four salinity treatments.

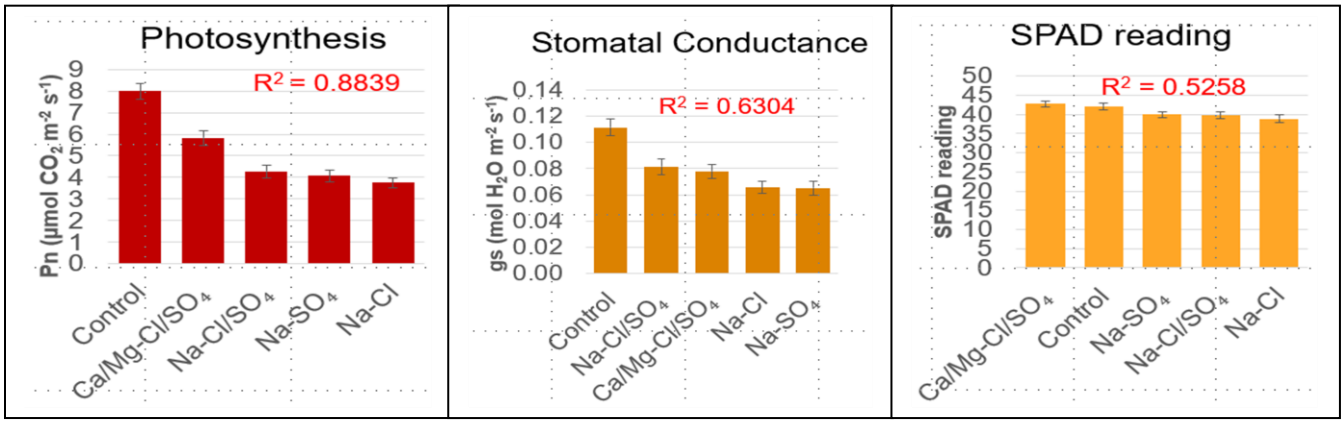

Figure 7. Physiological measurements in almond rootstocks under different salt treatments. Data for all the rootstocks was pooled for each treatment. A, Net photosynthesis (*Pn*); B, stomatal conductance (*gs*); C, *SPAD* reading for Chlorophyll content. Error bars represent standard error.

The biochemical response of 16 rootstocks were evaluated in response to the different salinity treatments. The biochemical markers chosen were proline, antioxidant capacity (ORAC), and total phenolics in leaves of rootstocks exposed to different treatments. Regarding both antioxidant capacity and total phenolics, all rootstocks had high leaf antioxidant capacities measured by ORAC with values ranging from 840 (Hansen) to 2933 µmoles TE/g DW (Krymsky 86) and total phenolics ranging from of 19.5 (Hansen) to 47.7 mg GAE/g DW (Krymsky 86). However, these markers either increased or decreased without a clear trend across the rootstocks or regarding their abilities to exclude Na or Cl, or to their survival rates. However, proline leaf concentrations, expressed as the difference between the high NaCl and the control treatment in the 16 rootstocks, had a significant positive correlation to their ability to exclude Na, Cl, and an inverse correlation to their survival rate. Leaf proline concentrations at salinity control levels ranged from 1.0 (Krymsky 86) to 4.2 mg/g DW (Nemaguard), while at the salinity concentration of NaCl of 3.0 dS/m proline ranged from 1.5 (Nickels) to 7.3 mg/g DW (Lovell). When the differences of leaf proline between high and low NaCl treatments were correlated with the Na and Cl exclusion of the rootstocks, the correlations were statistically significant (r^2 = 0.424 for leaf Na exclusion and r^2 = 0.344 for leaf Cl exclusion) and the rootstocks with the lower leaf proline differences were the 5-6 best Na and best Cl excluders (Figure 8).

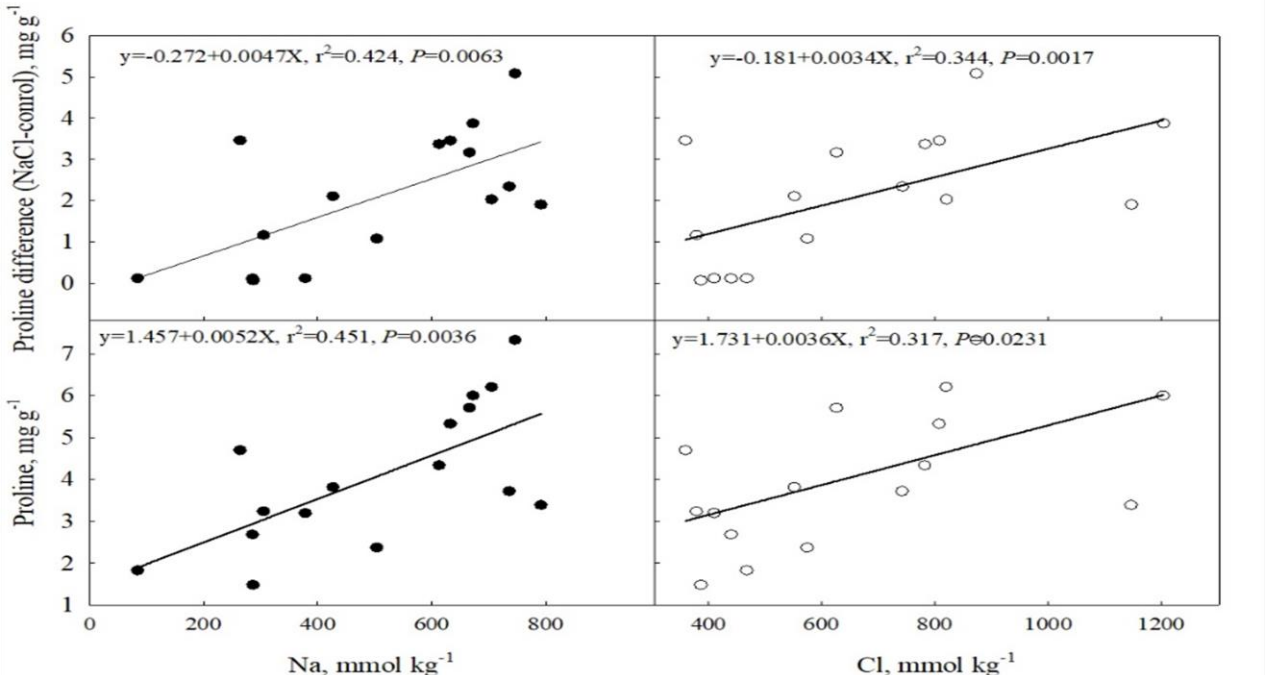

Figure 8. Leaf proline concentration (mg/g DW) and concentration differences related to leaf Na and Cl accumulation in 16 almond rootstocks. Dots represent different rootstocks, mean values.

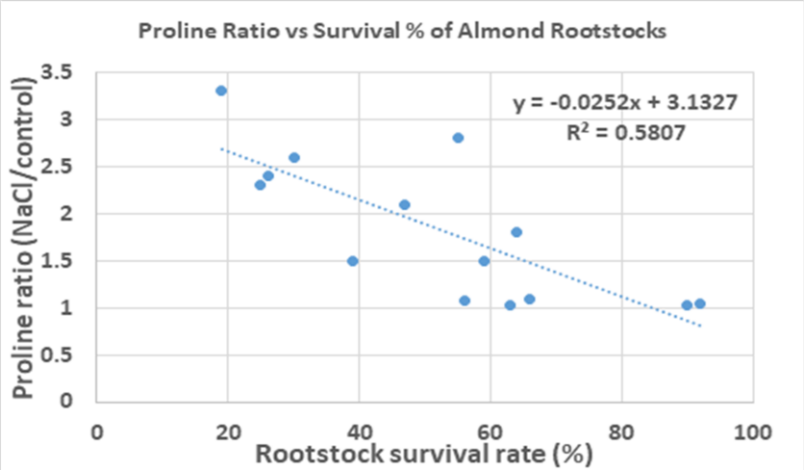

Figure 9. Leaf proline concentrations (mg/g DW) and concentration differences related to leaf Na and Cl accumulation in 16 almond rootstocks. Dots represent different rootstocks, mean values.

Regarding survival rates, an inverse correlation of $r^2 = 0.5807$ between proline differences and survival rate was found (Figure 9) and the three rootstocks with best survival rates (Cornerstone, Empyrean 1, and Rootpac 40) had the lowest proline ratios (ranged from 1.03 to 1.1) between salt and control treatments, while the four rootstocks with the worse survival rates (Lovell, Guardian, Rootpac 20, and Rootpac R) had proline ratios ranging from 2.3 to 3.3 mg/g DW. Thus, the significant leaf correlations between proline differences between high NaCl and control treatments and the rootstock's ability to exclude Na and Cl, and the inverse correlation between rootstock survival rate and proline ratios can be interpreted as indicators of the rootstock's ability to tolerate irrigation with recycled waters that are moderately high in salts and up to an electrical

conductivity of the irrigation water (EC_{iw}) of 3.0 dS/m.

Year 1, Objective 3. Identify and characterize the genes involved in salinity tolerance in almond rootstocks.

Expression analysis was carried out for a set of 10 genes selected for their involvement in salt stress. These include genes known to be associated with Na^+ efflux from root to soil (SOS1, SOS2 and SOS3), genes involved in sequestration of Na^+ in vacuoles (NHX1, NHX2 and AVP1), genes important for retrieving Na^+ from xylem (*HKT1* and *AKT1*), and genes involved in signal transduction during salt stress (SAL1 and SERF1). The expression analyses for 10 genes revealed that Treatment 3 and Treatment 4 both led to induction of majority of salt associated genes during salt stress, suggesting importance of both the chloride and sodium toxicities during salt stress in almonds. *NHX1*, *SOS3,* and *AKT1* were highly upregulated in salinity treatments in leaves (Figure 10). Both *HKT1* and *AKT1* genes displayed the highest upregulation in salinity treatments in roots (Figure 11).

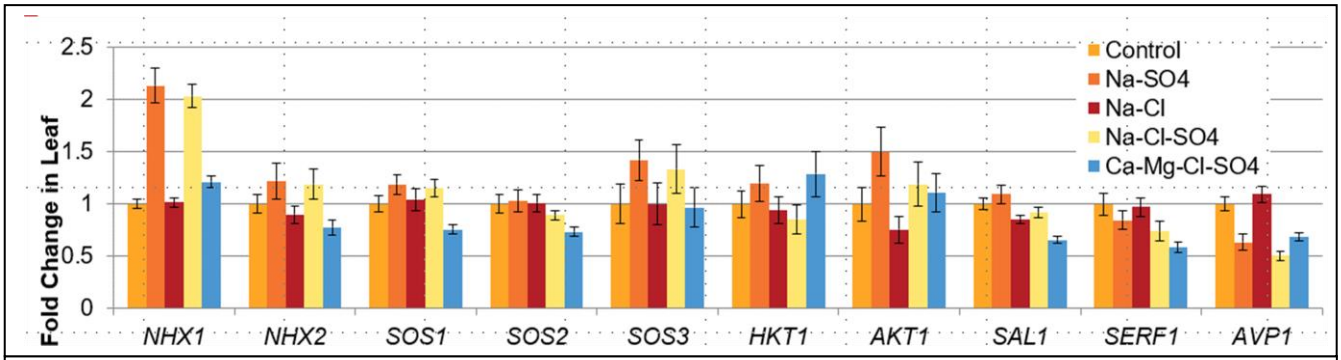

Figure 10. Expression of genes involved in the salt tolerance mechanism in leaves of 16 almond rootstocks under different saline treatments. Error bars represent standard error.

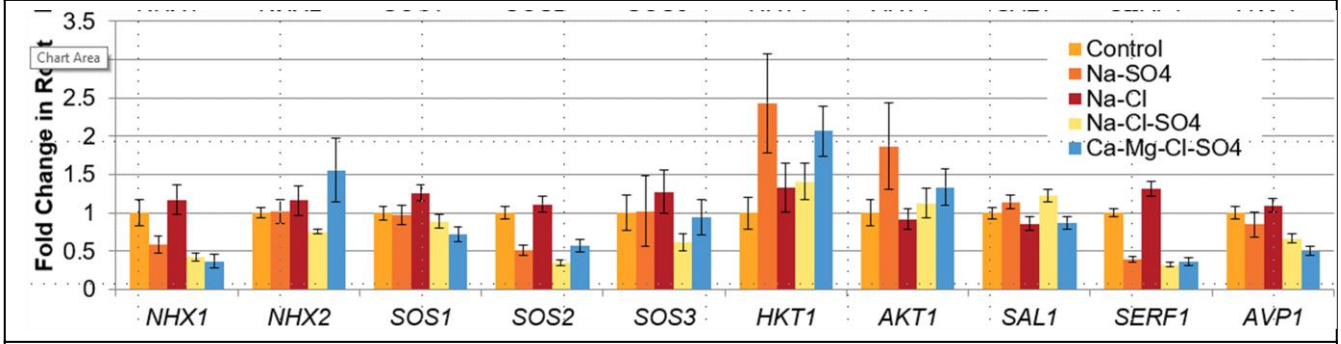

Figure 11. Expression of genes involved in the salt tolerance mechanism in roots of 16 almond rootstocks under different saline treatments. Error bars represent standard error.

Year 2, Objective 1. Evaluation of almond rootstocks to determine their tolerance response to a range of salt concentrations.

Ion analysis was done for digested leaf samples for Na, Cl, K, Ca, Mg, P, S, B, Cu, Fe, Mn, Mo and Zn. Of the 16 rootstocks, Rootpac 40 stored least amount of Na in the leaves in the control and Nemaguard stored the most (Figure 12). In all other 4 salinity treatments, among all

rootstocks, Rootpac 40 stored least amount of Na^+. Other rootstocks that stored low amount of Na^+ include Cornerstone, Empyrean 1, Viking, Atlas, Nickels, BB 106 and Brights 5.

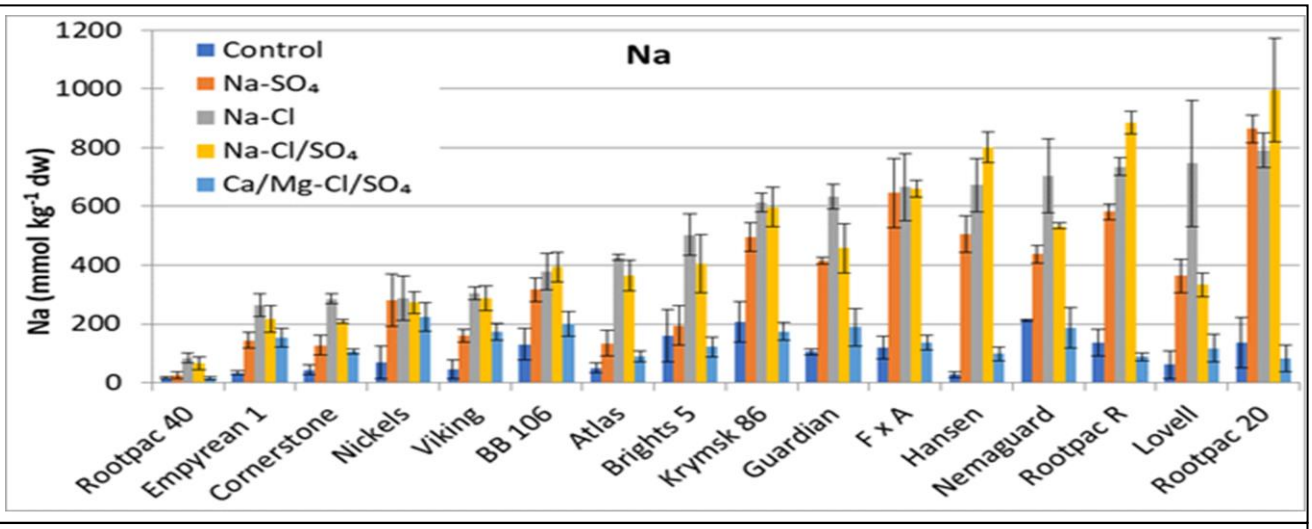

Figure 12 Shoot Na concentrations of 16 almond rootstocks treated with 5 irrigation water treatments. Error bars represent standard errors of three biological replicates.

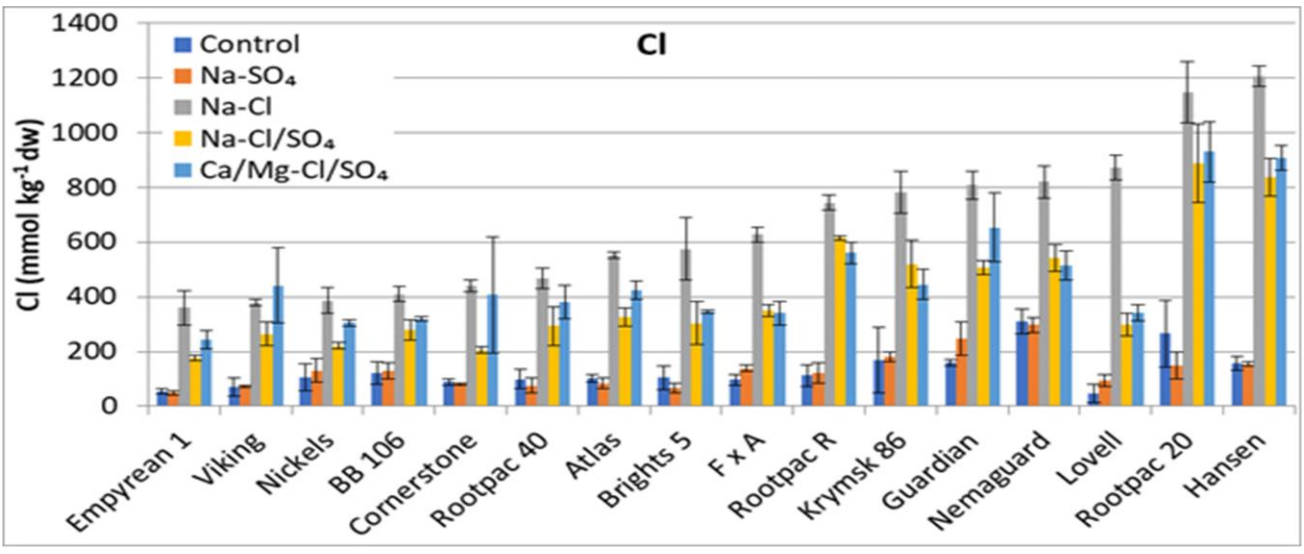

Figure 13. Shoot Cl concentrations of 16 almond rootstocks treated with 5 irrigation water treatments. Error bars represent standard errors of three biological replicates.

In general, Empyrean 1 stored the least amount of Cl in the salinity treatments, followed by Nickels and BB 106 (Figure 13). Interestingly, Rootpac 40 was also among the rootstocks that stored low amount of Cl in their leaves.

In most rootstocks, the total K content in the leaves was reduced in all four salinity treatments as compared to the control (data not shown). However, Rootpac 40 displayed clear increase in K content in T3 (Na-Cl based irrigation water) and T5 (Ca/Mg-Cl/SO_4 based irrigation water) and maintained K levels in T4 (Na-Cl/SO_4 based irrigation water) as compared to the control. Reduction in K content in salinity treatments was consistent with many other plant

species and can be explained as some protein channels play important role in transport of both Na^+ and K^+.

Different rootstocks showed variation in tissue Ca concentrations in different treatments T2 to T4 did not show much change in tissue Ca concentrations as compared to T1 in most genotypes (data not shown). However, T5, which contained high amount of Ca in irrigation water displayed high tissue Ca as compared to T1 in most genotypes. In many plant species the ability to maintain high Ca content in plant tissue under salinity is associated with salinity tolerance. However, in almond rootstocks we did not observe any association between tissue Ca concentration and salinity tolerance.

Year 2, Objective 2. **Characterizing different almond genotypes based on different components of salt tolerance mechanism**.

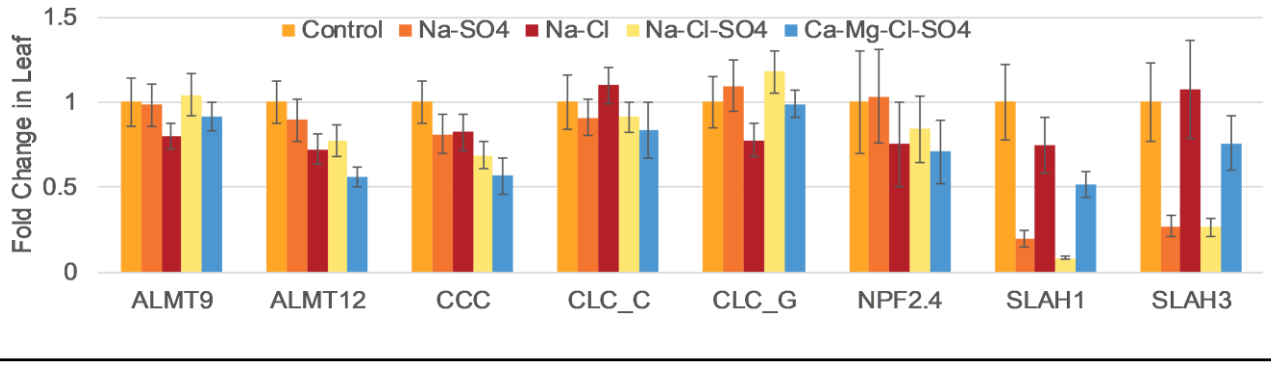

Figure 14. Expression analysis of Cl related genes in almond leaves

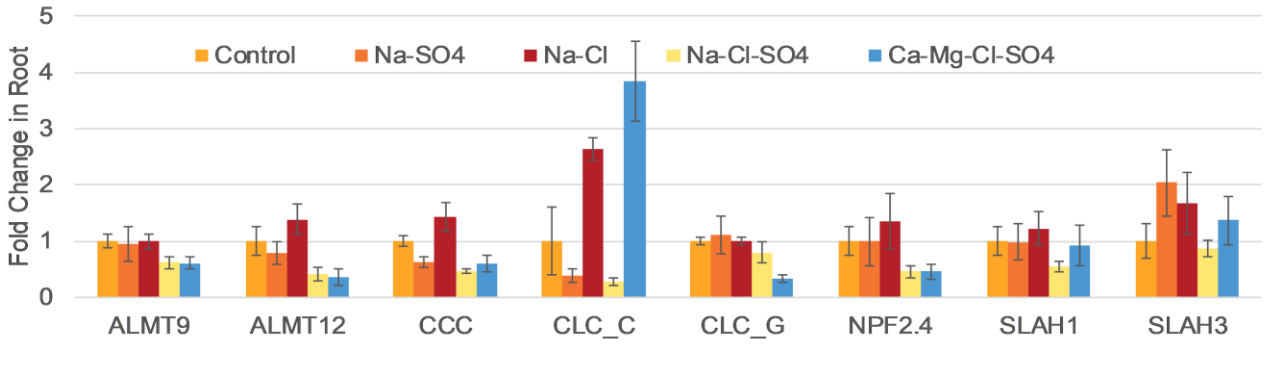

Figure 15. Expression analysis of Cl related genes in almond roots

In this objective, we established a relationship between genes involved in salt stress and phenotypic differences among different genotypes in response to salinity treatment. In addition to 10 genes involved in Na transport evaluated in Year 1, we studied 8 genes involved in Cl transport (Figure 14 and 15). The SLAH1 and SLAH3 gene were significantly downregulated in leaf tissue under salinity treatment. On the other hand, CLC_C and SLAH3 were induced under salinity treatments. AtSLAH1, a homolog of the slow type anion channel AtSLAC1, interacts with AtSLAH3 in controlling root-to-shoot Cl^- transport. It makes perfect sense that SLAH3 was down regulated in leaves whereas it was upregulated in roots. Cl^- channels (CLCs) regulate Cl^-

homeostasis by sequestering Cl^- into root and leaf vacuoles. Hence under salinity, CLC_C was upregulated in roots. Our results indicate that Prunus species primarily regulate Cl concentrations either by controlling root-to-shoot transport or by sequestering Cl^- into root vacuoles. Different rootstocks differ component traits involved in regulation Cl. Previously we have shown that it is also true of Na^+ transport. This information will be extremely valuable to almond breeders and geneticists in making crosses and combining different components of salt tolerance mechanism into a single genotype that may result in development of a highly tolerant rootstock to salt.

Year 2, Objective 3. Study global changes in the gene expression profiles under normal versus salt stress conditions in almond rootstocks.

Based on our findings from Year 1, we were able to select a salt-tolerant rootstock (Rootpac 40) and a salt sensitive rootstock (Nemaguard) for our RNA sequencing (RNAseq) analysis. RNAseq analysis allowed us to identify differentially expressed genes (DEGs) between salinity and control treatments, between root and leaves and also between salt-tolerant and salt-sensitive rootstocks (Table 1).

Table 1. Differentially expressed genes in different sample comparisons. In group name, the first letter represents control (C) or treatment (T), the second letter represents Rootpac 40 (R) or Nemaguard (N) and the third letter represents root (R) or leaf (L).

Group	Up- regulated	Down-regulated
TRR vs TNR	1856	1831
TRL vs TNL	2760	2289
CRR vs CNR	2158	1558
CRL vs CNL	4657	3932
TRR vs CRR	5	2
TRL vs CRL	2	10
TNR vs CNR	85	73
TNL vs CNL	7	61
TRL vs TRR	6943	8009
TNL vs TNR	6739	8065
CRL vs CRR	6550	7789
CNL vs CNR	7230	8301

Some DEGs include already known genes such as transporters, transmembrane receptors, SOS1, SOS2, NHX2, in addition some unique DEGs with no direct association to salt stress such as sucrose synthesis, auxin response factors, terpenoid cyclase and translocan at inner chloroplast membrane were also identified (Table 2). DEGs involved in hormonal-signaling, calcium-signaling, redox-signaling, and transcriptional regulation showed treatment specific (control *vs.* salinity) and genotype specific (salt-tolerant *vs.* salt-sensitive) differences and resulted in identification of various candidate genes involved in salinity stress response.

Our gene ontology (GO) analysis in Treatment, Nemaguard, Root (TNR) *vs.* Treatment, Rootpac 40, Root (TRR) showed enrichment of “ion transport” (GO:0006811), “transmembrane transporter” (GO:0055085), “response to oxidative stress” (GO:0006979) and “response to salt

stress" (GO:0009651). These observations suggest that the enhanced salinity tolerance of Rootpac 40 may be due to its ability to manage different ions in the root tissues. In addition, GO analysis in Control, Nemaguard, Leaf (CNL) *vs.* Treatment, Nemaguard, Leaf (TNL) showed enrichment of "stomatal closure" (GO:0090332), suggesting importance of mechanism to regulate amount of water uptake in response to salinity stress.

Several families of transcription factors (TFs) and transcription regulators (TRs) were differentially expressed in control *vs.* salt comparisons and in salt-tolerant *vs.* salt-sensitive comparisons. These observations suggest that transcriptional regulation may be a critical aspect of plant's response during salinity stress in almonds. Detailed characterization of theses TFs and TRs will reveal their exact roles during salinity stress.

Table 2. Some differentially expressed genes between Treatment-Rootpac 40-Root (TRR) and Treatment-Nemaguard-Root (TNR)

Gene ID	TRR Read count	TNR Read count	Predicted function
ppa000268m.g	378	92	Transmembrane receptors
ppa000453m.g	4354	1730	SOS1
ppa000636m.g	692	73	Sucrose synthesis
ppa001557m.g	1255	667	Auxin response factor
ppa001817m.g	382	108	Terpenoid cyclases
ppa002104m.g	8267	14679	Transporter family
ppa003695m.g	4	280	Translocon at inner chloroplast membrane
ppa003943m.g	2545	1280	NHX2
ppa004837m.g	1054	454	Ion efflux
ppa005344m.g	2583	4690	SOS3-interacting
ppa005575m.g	4057	1977	CBL-interacting protein kinase
ppa005619m.g	178	64	Mg regulation
ppa006520m.g	401	105	Cation Efflux
ppa006584m.g	616	1339	Cation transporter
ppa013318m.g	64	10	Salt induced protein

Year 3, Objective 1. Evaluation of selected almond rootstocks for their effects on scion performance under salinity stress and identification of underlying genetic components.

In Year 1, we evaluated 16 ungrafted rootstocks and learned that Na^+ and Cl^- are critical for tissue toxicity. Based on biochemical and genetic analyses, we selected the 5 best salt-tolerant rootstocks. Currently, we are studying grafted plants to evaluate if genetic control is similar in grafted and ungrafted plants. Plants of 5 selected rootstocks, grafted with Nonpareil and Monterey were transplanted into pots in 3 replications, with 3 plants/ replication in June 2019. Plants are being field evaluated under three irrigation waters: Control (EC 1.4), Saline (EC 3.5); Saline + 20mg Zn/kg of soil, with 270 total plants (5 rootstocks, 2 scions, 3 water types, 3 replications, 3 plants/ replication). Survival rates and stem girth reading will be taken in May 2020. Understanding the roles played by Na^+ or Cl^- in salt toxicity and the genetic interactions between rootstock and scion will help in refining tools and techniques used to study salt tolerance, which in turn maybe utilized to enhance the subsequent breeding efforts for almonds rootstocks.

Year 3, Objective 2. Using priming as an alternate approach to mitigate salinity stress in almond rootstocks.

"Priming" (exposing plants to chemicals before applying stress) is one of the alternate approaches that succeeded in mitigating salinity in many crops. These chemicals include growth regulating hormones, signal molecules and other compounds. We are evaluating 10 rootstock/scion (5 rootstocks and 2 scions) combinations for the effect of priming on salinity tolerance. Different priming agents used are H_2S, melatonin, and salicylic acid (SA). Two different levels of salinity: control salinity (EC 1.4 dS m^{-1}) and EC 3.5 dS m^{-1}, were used. Priming treatments were initiated in August 2019. One-week later salinity treatment was initiated. Plants are currently growing, and we will take survival rate and change in trunk diameter in May 2020. Novel tools such as "priming" may be critical in providing a fast way to mitigate salt toxicity in almonds.

Year 3, Objective 3. Functional validation of almond genes involved in salt tolerance using model plants

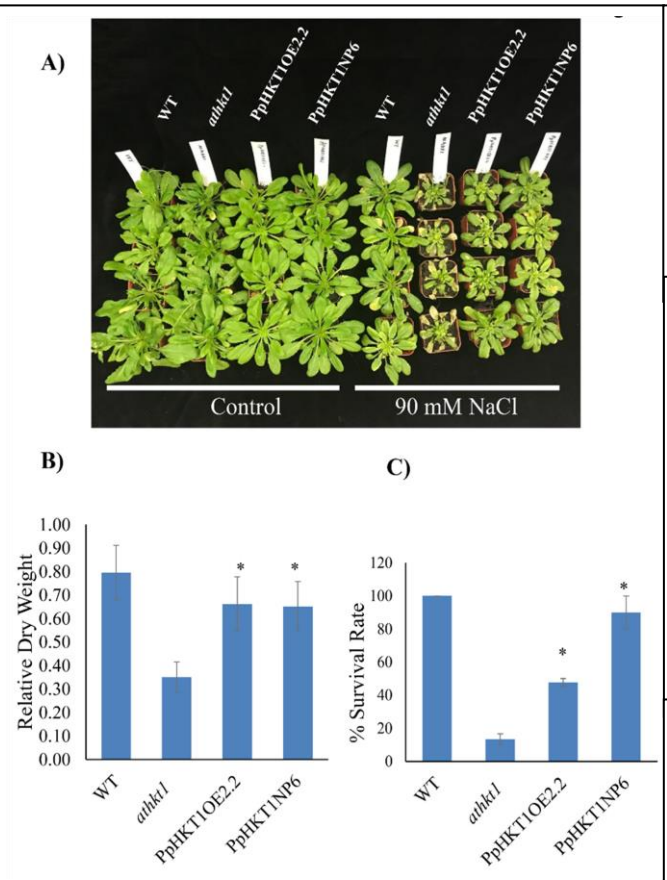

Figure 17. Functional complementation of Prunus *HKT1* (*PpHKT1*) in Arabidopsis *hkt1* mutant. A. Salt assay; B. Relative dry weight; C. % survival rate. WT, wildtype; *athkt1*, Arabidopsis mutant; PpHKT1OE2.2, transgenic line with overexpression of *PpHKT1*; PpHKT1NP6, transgenic line with native expression of *PpHKT1*.

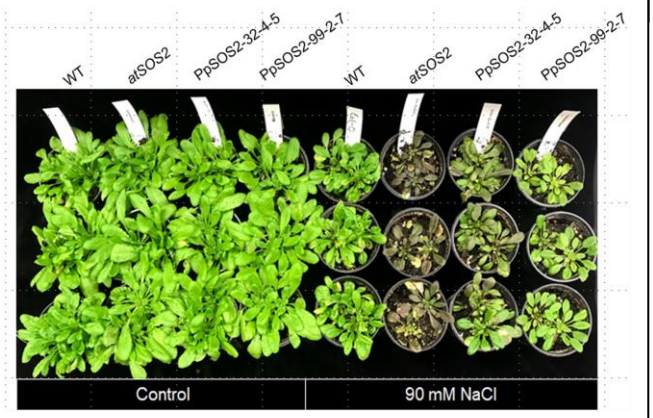

Figure 18. Functional complementation of Prunus *SOS2* (*PpSOS2*) in Arabidopsis *sos2* mutant. WT, wildtype; *athkt1*, Arabidopsis mutant; PpSOS2-32-4-5, transgenic line with overexpression of *PpSOS2*; PpSOS2-99-2-7, transgenic line with native expression of *PpSOS2*.

Results from the first two years of genetic analyses identified almond genes important for salt tolerance. We selected the *HKT1* and *SOS2* genes for functional validation. The respective almond genes were expressed under the dual CaMV *35S* promoter (*2x-35S)* and the native promoter of the gene in Arabidopsis mutants (*athkt1*). The developed transgenic lines were used to study salt stress tolerance along with the mutants and wildtype Arabidopsis lines. Both transgenic lines for Prunus *HKT1* (*PpHKT1*) survived salt concentrations up to 120 mM NaCl, however mutants died after 18 days of salinity treatment (Figure 17). Under moderate salinity

treatment, the dry weight of Arabidopsis mutant (*athkt1*) decreased significantly compared to the transgenic lines (Figure 17). Transgenic lines also performed better for lateral root formation, electrolyte leakage and relative water content, suggesting that transgenic plants coped well with increase salt concentrations by maintain the integrity of the membranes. These experiments confirmed that over-expression and native expression of *PpHKT1* can complement salt tolerance function in the Arabidopsis mutant.

In the second experiment we complemented *PpSOS2* in Arabidopsis *sos2* mutant (Figure 18). Both transgenic lines containing *PpSOS2* performed better than the *atsos2* mutant under salinity stress. These experiments showed that *HKT1* and *SOS2* functions are conserved in Prunus and these genes can be utilized to develop enhanced almond rootstocks tolerant to salinity.

D. Discussion and Conclusions *(This is the core function of this report)*

In a comprehensive three-year experiment by evaluating a large number of commercial almond rootstocks under salinity stress, we have shown that:

- There was maximum reduction in trunk diameter when irrigation water was high in Na and Cl suggesting that mostly Na and to a lesser extent Cl concentration in irrigation water are the most critical ion toxicities for almond rootstocks
- Photosynthesis showed the highest correlation with change in trunk diameter followed by correlations with stomatal conductance and chlorophyll content.
- Of the biochemical markers (proline, antioxidant capacity and total phenolics) studied on almond rootstocks for salinity stress, proline showed significant correlation with ability of plants to exclude Na and Cl and negative correlation with the survival rate. These findings suggest that proline can be used as a useful biochemical marker for screening genotypes tolerant to salinity.
- Rootpac 40, Cornerstone, Empyrean 1, Viking, Nickels and BB106 stored least amount of Na in leaves
- Empyrean 1, Viking, Nickels, BB106, Cornerstone and Rootpac 40 stored least amount of Cl in leaves
- NHX1, SOS3 and AKT1 were highly upregulated in salinity treatments in leaves and HKT1 and AKT1 showed the highest upregulation (expression) in salinity treatments in roots suggesting their importance in Na regulation under salinity stress.
- CLC_C and SLAH3 were highly upregulated in salinity treatments in roots and SLAH1 and SLAH3 showed significant downregulation under salinity treatments in leaves signifying their role in Cl homeostasis.
- RNA-seq analysis resulted in identification of several genes involved in different pathways that are induced on salt treatment and also differentially expressed between the sensitive and resistant rootstocks.
- Understanding of genetic networks regulating salt tolerance will help in developing new rootstocks for saline conditions

- With the development of new genomics resources in Prunus, the genes validated to have roles in salt tolerance can be used to improve successful rootstocks using genetic transformation or other new technologies such as CRISPR-Cas9.

E. Materials and Methods *(500 word max.)*

Experimental set up and salt treatments: Sixteen rootstocks (Atlas, BB106, Bright's 5, Cornerstone, Empyrean 1, Flordaguard x Alnem (F x A), Guardian, Hansen, Krymsk 86, Lovell, Nemaguard, Nickels, Rootpac 20, Rootpac 40, Rootpac R, and Viking) were obtained from various nurseries. Experiment was set up in a randomized complete block design with 16 genotypes, 3 replications, 3 plants per replication and 5 treatments of saline water (total 720 trees). The five different treatments were as follows:

1. *Treatment 1:* Non-saline control
2. *Treatment 2:* mixed cations (Ca^{2+} = 1.25Mg^{2+} = .25 Na^{+}) with predominantly sulfate (Cl^{-} = 0.2 SO^{2-}_4)
3. *Treatment 3:* mixed cations (Ca^{2+} = 1.25Mg^{2+} = .25 Na^{+}) with predominantly chloride (SO^{2-}_4 = 0.2 Cl^{-})
4. *Treatment 4:* mixed anions SO_4-Cl (SO^{2-}_4=Cl^{-}), predominantly Sodium (Ca^{2+} = 1.25Mg^{2+} = .25 Na^{+})
5. *Treatment 5:* mixed anions SO^{2-}_4-Cl^{-} (SO^{2-}_4=Cl^{-}), predominantly Ca^{2+} and Mg^{2+}. (Ca^{2+} = 1.25 Mg^{2+} = 5 Na^{+})

Treatment 1 was the control treatment with irrigation water salinity with Electrical Conductivity (EC) of 1.36 dS m^{-1} and salinity of Treatments 2 through 5 was maintained at 3.0 dS m^{-1}.

Trunk diameter and ion analysis: The trunk diameter was recorded 10 cm above the soil level in the beginning before and after one year of salt treatment to calculate the change. The survival rates of different rootstocks were also be recorded. Leaf samples were collected 8-weeks after the initiation of salt treatments to determine tissue ion composition.

Physiological and biochemical analysis: Photosynthetic parameters and stomatal conductance were measured with a Li- Cor (Li-Cor Biosciences) 8 weeks from the initiation of the salt treatments. Leaf samples were collected, frozen in liquid nitrogen, and lyophilized for analyses of total phenolics, hydrophilic antioxidant capacity, and proline.

Primer design for expression analyses: Almond genes involved in different mechanisms leading to salt tolerance were selected based on functional conservation with the Arabidopsis genes. These gene sequences were used in Basic Local Alignment Search Tool (BLAST) analyses to identify corresponding sequences from the peach genome (Verde et al. 2013).

Expression analyses: Tissue samples for RNA isolation were taken 24 hours after the initiation of salt treatment. To remove contaminating DNA, RNA was treated with DNase I. The qRT-PCR amplification was carried out in a BioRad CFX96 System. The peach EF2 and Ubiqutin were used as reference genes for the qRT-PCR analyses (Tong et al. 2009). The cycle threshold values of each gene to the reference gene were used to calculate the relative expression and differentially expressed genes were identified.

RNAseq analysis: The most-tolerant (Rootpac 40) and the most-sensitive (Nemaguard) rootstocks were selected for RNAseq. Our approach involved total RNA preparation from the roots and the leaves of the normal and salt stressed plants. cDNA libraries were prepared for each sample with a unique index to facilitate pooling for several samples in a single lane for sequencing. Sequencing was done at Novogene Inc. Sequences were aligned with the peach genome (Verde, et al. 2013) and the bioinformatic analysis was performed and differentially expressed genes were identified.

F. Publications that emerged from this work

1. Peer reviewed publications

a) Kaundal A, Sandhu D, Duenas M, Ferreira JFS. 2019. Expression of the *high-affinity K^+ transporter 1 (PpHKT1)* gene from almond rootstock 'Nemaguard' improved salt tolerance of transgenic Arabidopsis. PLOS ONE 14 (3): e0214473. DOI: 10.1371/journal.pone.0214473

b) Sandhu D, Kaundal A, Forest T, Ferreira JFS and Suarez DL. 2020. Linking diverse salinity responses of 16 almond rootstocks with physiological and genetic determinants. (in preparation for Scientific Reports).

c) Sandhu D, Duenas M, Acharya B, Skaggs T, Ferreira JFS and Suarez DL. 2020. Transcript profiling of a salt-tolerant and a salt sensitive almond rootstock to salinity stress. (in preparation for Functional and Integrative genomics)

2. Other publications (e.g. outreach materials)

a) Sandhu D and Acharya BA. 2019. Mechanistic insight into the salt tolerance of almonds. Progressive Crop Consultant 4 (5): 44-49.

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G. References Cited

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Suarez DL, Grieve CM (2013) Growth, yield, and ion relations of strawberry in response to irrigation with chloride-dominated waters J Plant Nutr 36:1963-1981. DOI 10.1080/01904167.2013.766210

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