Subcellular and molecular characterization of salinity tolerance in almonds with novel tools

Project No.: **18 HORT23B.Drakakaki**

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A. Summary

We established a microscopy-based system to distinguish the mechanisms of various rootstocks coping with the salt stress and identify the cellular, structural, and molecular traits of the premier rootstocks with high salt tolerance. Using our established methodologies we screened diverse rootstocks and identified differences in salt ion transport and sequestration which was correlated with salt tolerance. Besides observing the macrophenotype of rootstock salt resistance in the soil, we established a hydroponics system coupled with live recording to monitor rootstock water uptake and root growth under salinity stress. These are important traits to facilitate rootstock adaptability and support of the scion with high economic value during our current climate changes. We will use our microscopy-based methodologies to validate the selected premier rootstock traits at cellular level, including high sodium exclusion upon salt stress, and use our hydroponic system to facilitate the high throughput selection of high salt tolerance rootstocks.

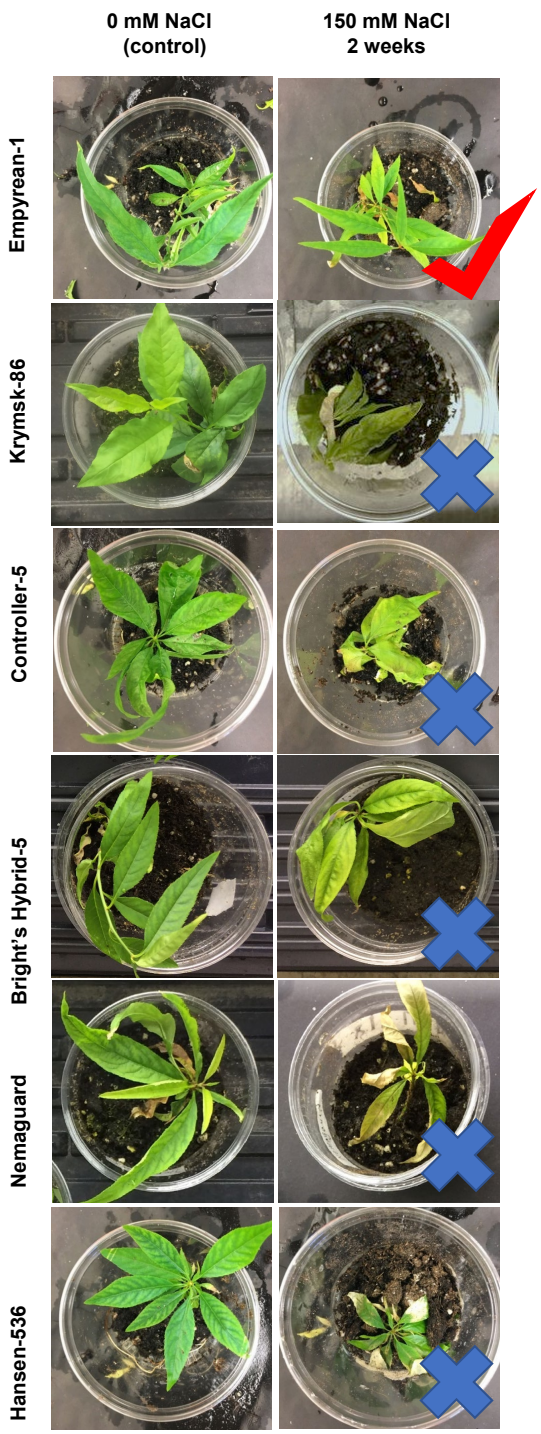
B. Objectives

Aim I: Development/refinement of confocal based assays for the detection of salt ions in almond cells. Characterization of sodium accumulation across a developmental gradient of various rootstocks, under control and salt stress conditions. Examine selected commercially available rootstocks with our current methodologies.
Aim II: Using in live imaging of root growth, analyze root growth dynamics of different rootstocks.
Aim III: Characterize the root structure of almond rootstocks. Correlate phenotypic and cellular data of rootstocks with root structure and development at cellular level.

C. Results

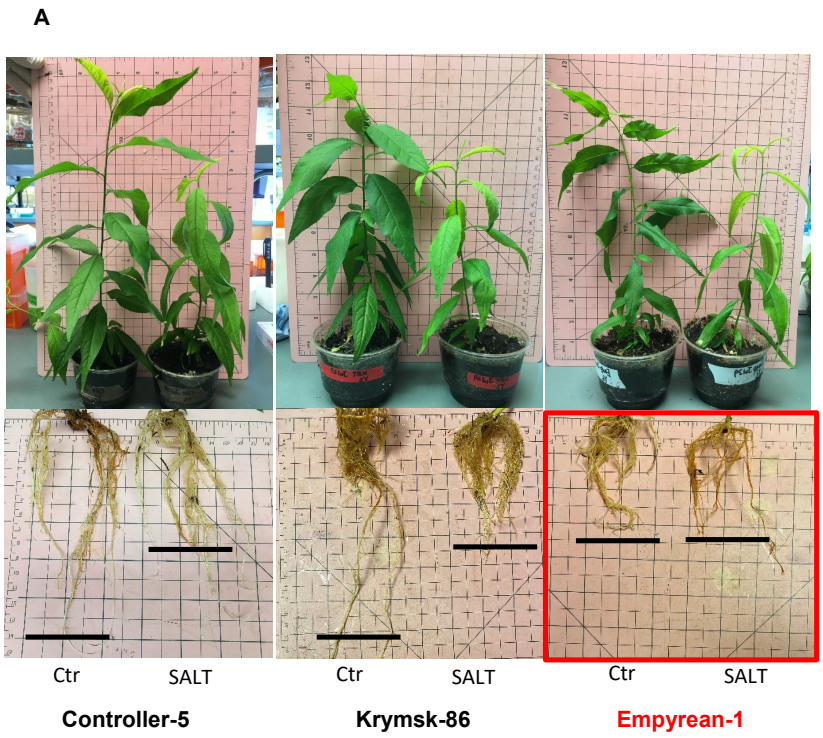
We are currently summarizing our findings of this work into a manuscript that will be submitted within the next 2 months for publication.

Result 1 are the evidences of realization of **Aim I**, **Result 2** realizes **Aim II**, and **Result 3-5** realize **Aims I and III**.



Result 1: Total of eight different rootstocks were phenotypically observed under salt treatment.
Six of eight rootstocks were included in this report for their macrophenotypic response towards salt treatment (**Figure 1**). Two rootstocks, Titan and Viking, were examined, however require further analysis (Data not shown). It is found in most rootstocks, symptoms of leaf water loss and necrosis developed after two weeks of 150 mM NaCl treatment. One rootstock, Empyrean-1, stood out in the initial screening, as little leaf necrosis and no water loss in leaf was observed. To quantify the root biomass change, three rootstocks were selected for further analysis. Empyrean-1 did not show inhibition of root length and mass after two weeks of salt stress (**Figure 2A&B**), and showed significantly higher root to leaf mass ratio, compared to the other two rootstocks.

Figure 1. Phenotypic observation of various rootstocks upon salinity stress. After transferred from rooting medium to soil for three weeks, rootstocks were watered by 150 mM NaCl solution and photos were taken after two weeks.



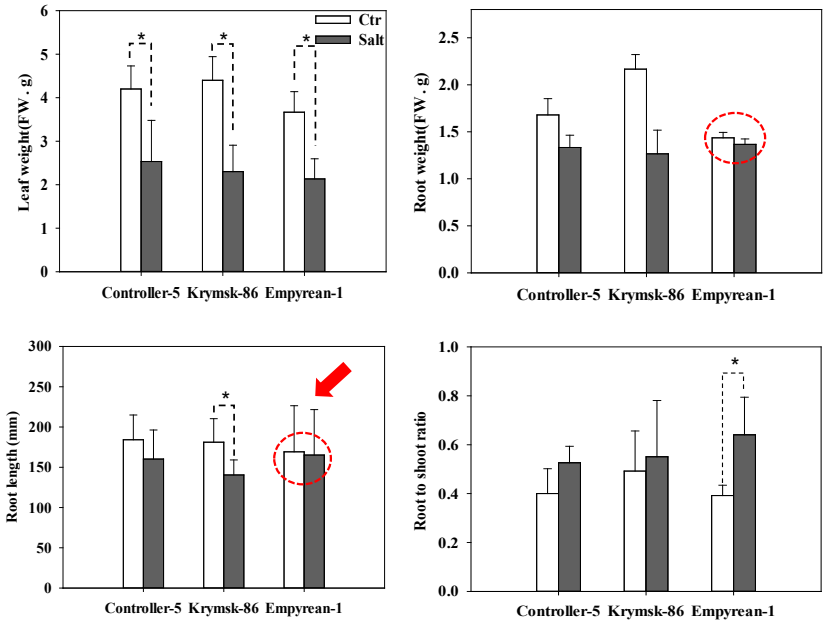
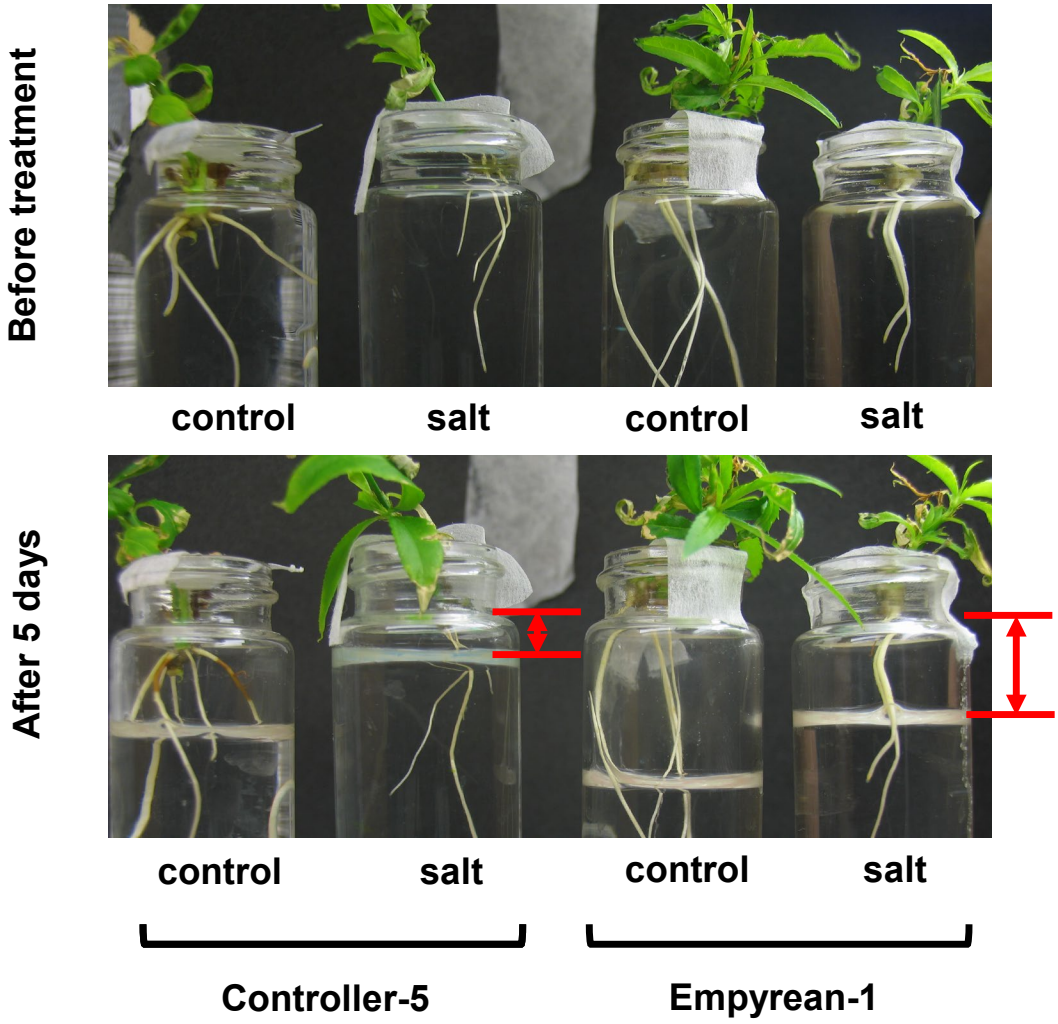


Figure 2. Phenotypic observation of three selected rootstocks upon salinity stress. After transferred from rooting medium to soil for three months, rootstocks were watered by 150 mM NaCl solution and photos were taken after two weeks. (A) Three rootstocks with distinct and contrasting phenotypes were selected for further repetitions at a more mature stage of growth, for plants adapted in the soil for three months. Empyrean-1 roots in salt treatment did not show visual difference from the untreated control. (B) Quantification of leaf weight, root weight, root length, and root to leaf ratio.

Figure 3. Water uptake of Controller-5 and Empyrean-1 in control and salt-stressed condition in a hydroponic system. Half Hoagland solution was used as control solution, and 50 mM NaCl added to half Hoagland solution was used as salt stress solution. *Time-lapse movie of the whole water uptake process of five days is available upon request.*



Result 2: We established a hydroponic system coupled with video recording to monitor the water uptake and phenotypic changes during the salt treatment.
We tested various hydroponic conditions, including nutrient components and salt concentrations, to demonstrate the water uptake capacity of various rootstocks under salinity conditions. After multiple tests, we determined that half Hoagland solution with 50 mM NaCl can support the growth and distinguish the two rootstocks (Controller-5 and Empyrean-1) of distinct salt tolerance. **Empyrean-1, treated in salt solution, showed more water uptake in hydroponic conditions than controller-5 (Figure. 3, indicated by the length of red bars).** Higher water uptake in salt solution is an important trait for rootstock salt resistance. Absorbing water actively in salt stress is not only a trait related to rootstock salt resistance, but also a trait of high economic value, as it helps the transpiration of scions, under salt stress, and should be considered as an important feature of robust salt-resistant rootstock.

Result 3: Live cell sodium staining protocol is established to indicate the cellular trait of various rootstocks.

Three days of 150 mM NaCl treatment was applied to total of eight different rootstocks, and sodium accumulation was monitored across root development gradient as previously described (Taiz and Zeiger, 2010). The cellular trait can be widely categorized into three different types, and **three distinct cellular localizations of sodium accumulation were observed in eight different rootstocks** (**Figure 4**). Empyrean-1 likely excludes salt at the outer layer of root, with less accumulation of sodium at the cortex as compared to the other seven rootstocks tested; Controller-5 and Bright Hybrid-5 sequester the salt into the vacuole (**Figure 4**, **red arrows**); and Nemagaurd, Hansen-536 showed increased levels of sodium in the cortex. Titan, and Viking showed variable levels of salt at the cortex between different root developmental layers and require further examination. Relating to **Result 1** and **Figure 1**, rootstocks capable of excluding sodium at the exodermis or epidermis, such as Empyrean-1, survived in the long-term salt treatment best. There is a clear association between the sodium accumulation mode at cellular level in **Figure 4** and the rootstock macrophenotype observed in **Figure 1**.

Result 4. The temporal change of sodium accumulation at the root meristematic zone is observed in three rootstocks.

As we found the cellular trait appears as early as three days after salt treatment, while the macrophenotype is not clear until 10 – 14 days after treatment, we selected three time points (3, 5, and 14 days) of salt treatment length to observe the cellular sodium accumulation changes during the macrophenotype development period (**Figure. 5**). **During 14 days of salt treatment, Empyrean-1 showed less sodium staining compared to other phenotypes. In contrast, Controller-5 showed the highest accumulation of sodium in cortex cells** (**Figure. 5**)**.** Notably, no difference can be observed across genotypes after 14 days of salt treatment, suggesting the death of root meristematic zone cells in all rootstocks after 14 days of salt treatment, as our sodium staining method requires active live cells for analysis.

Result 5: Prolonged salinity stress-induced the death of root tip cells.

In order to find the appropriate time window to observe the salinity response and explain why 14 days of salt treatment lead to lower signals compared to 3 and 5 days treated plants (**Figure 5)**, we performed fluorescein diacetate (FDA) staining of root tip cells treated by 150 mM NaCl for 3, 5 and 14 days, versus control, which only labels the viable cells (**Figure 6**). The FDA staining indicated non cell viability even over 50% of cells at the root meristematic zone after 3 days of salt treatment, while after 14 days of salt treatment, the majority of the meristematic zone cells were not viable. Notably, Empyrean-1 showed the highest number of viable cells compared to the other two rootstocks, especially compared to Controller-5, after 3 days of salt treatment (**Figure 6, red arrow**). Based on this result, we decided to set 3 days of salt treatment as the time point to observe the sodium accumulation pattern at cellular level in various rootstocks, in order to capture the difference visible cellular traits underlying the salt resistance mechanism in various rootstocks.

Figure 4. Sodium accumulation along the developmental gradient of eight rootstocks under salt stress. After transferred to soil for three weeks, rootstocks were treated by 150 mM NaCl solution, and sodium staining of live cells was performed in roots after three days of salt stress. Sodium staining of root cross-sections along the developmental gradient is observed by confocal microscopy, and rootstock response at the cellular level was categorized into three different classes.

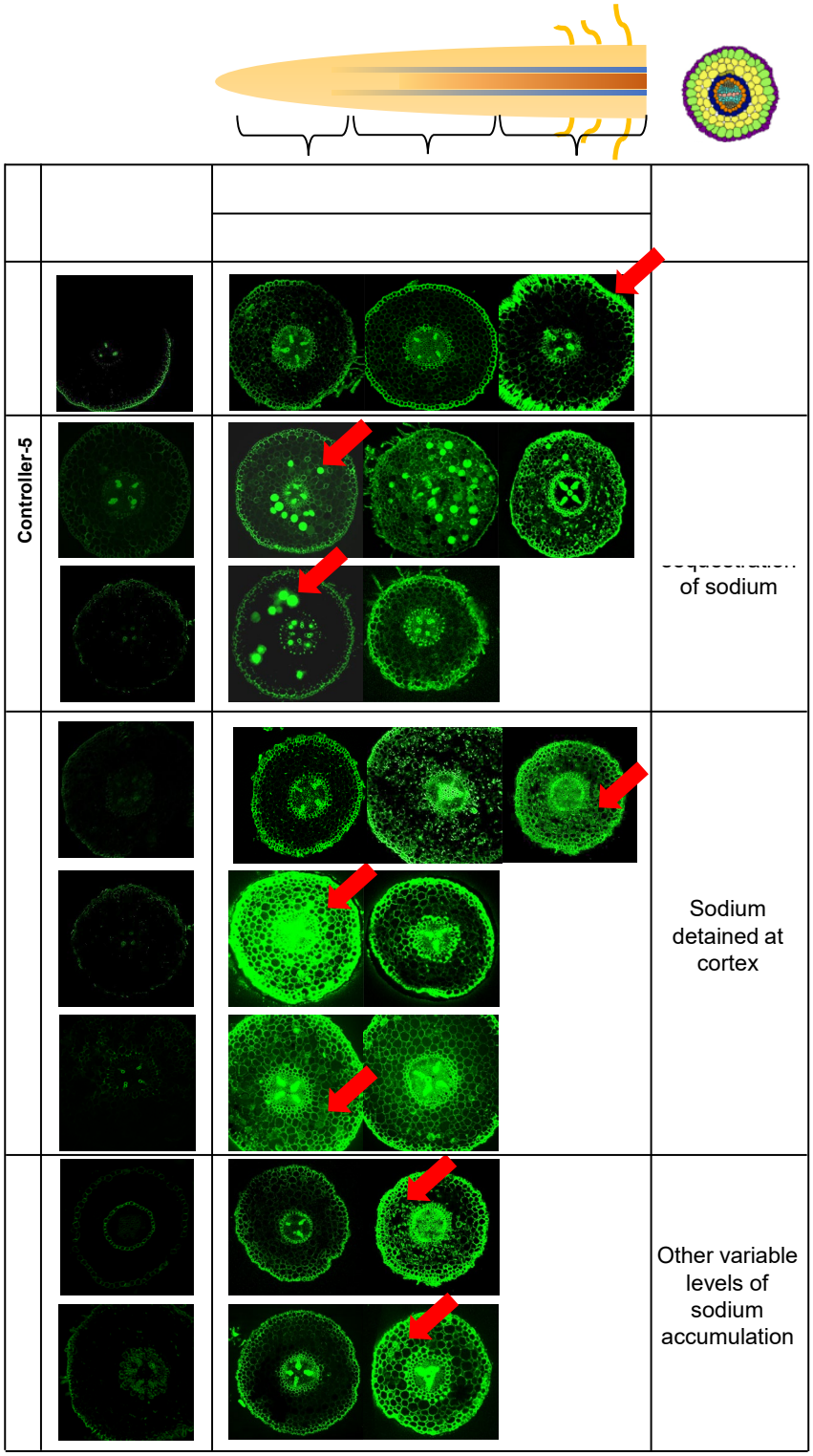


Figure 5. Sodium staining of root meristematic zone after 3, 5, and 14 days of salt treatment. Seedlings adapted in soil for two months were treated by 150 mM NaCl for three days. Red arrow on Controller-5 at 3 days points to sodium signal of vacuole sequestration of salt. Arrows on Controller-5 and Krymsk-86 at 5 days show sodium accumulation in the cortex layer.

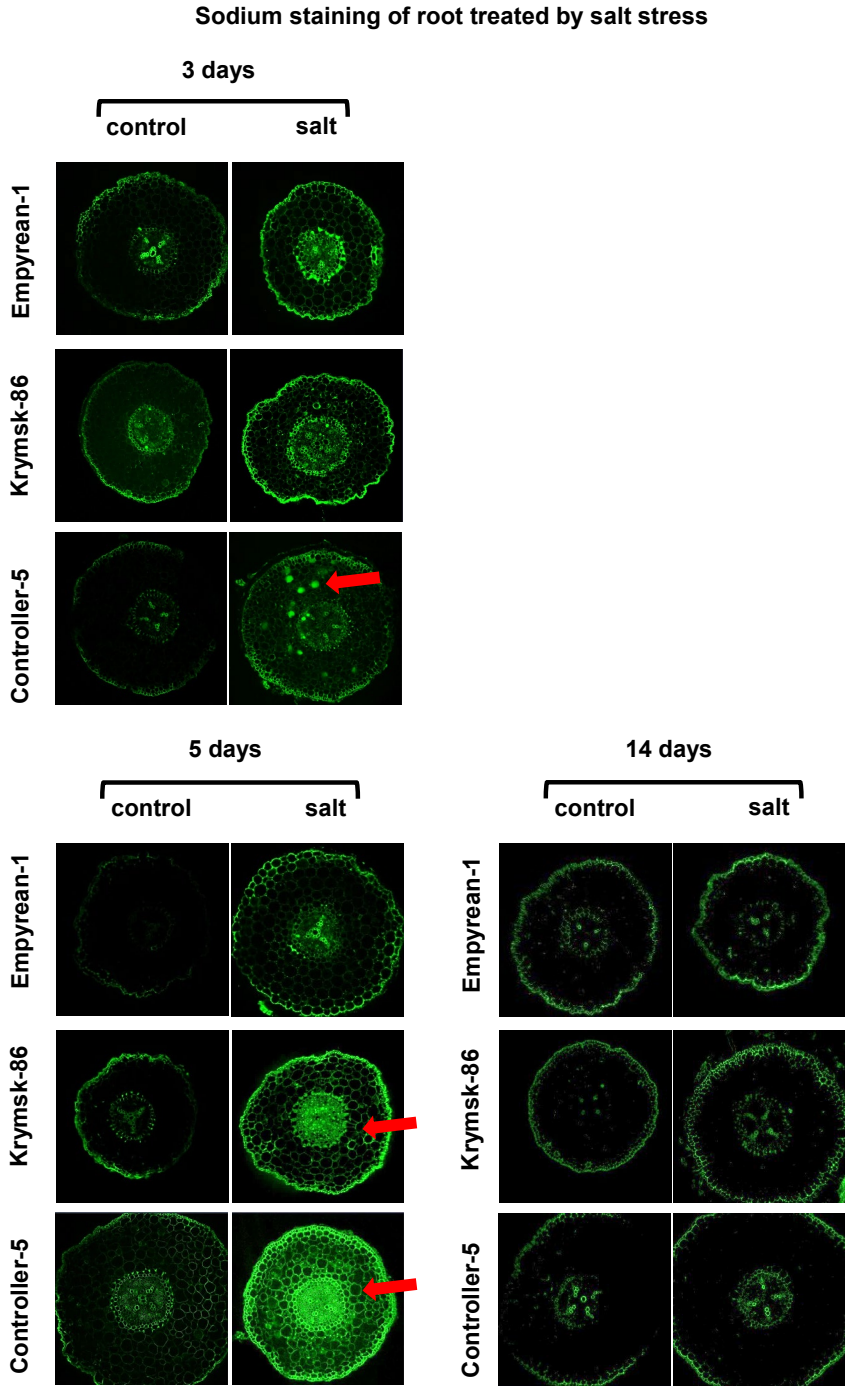
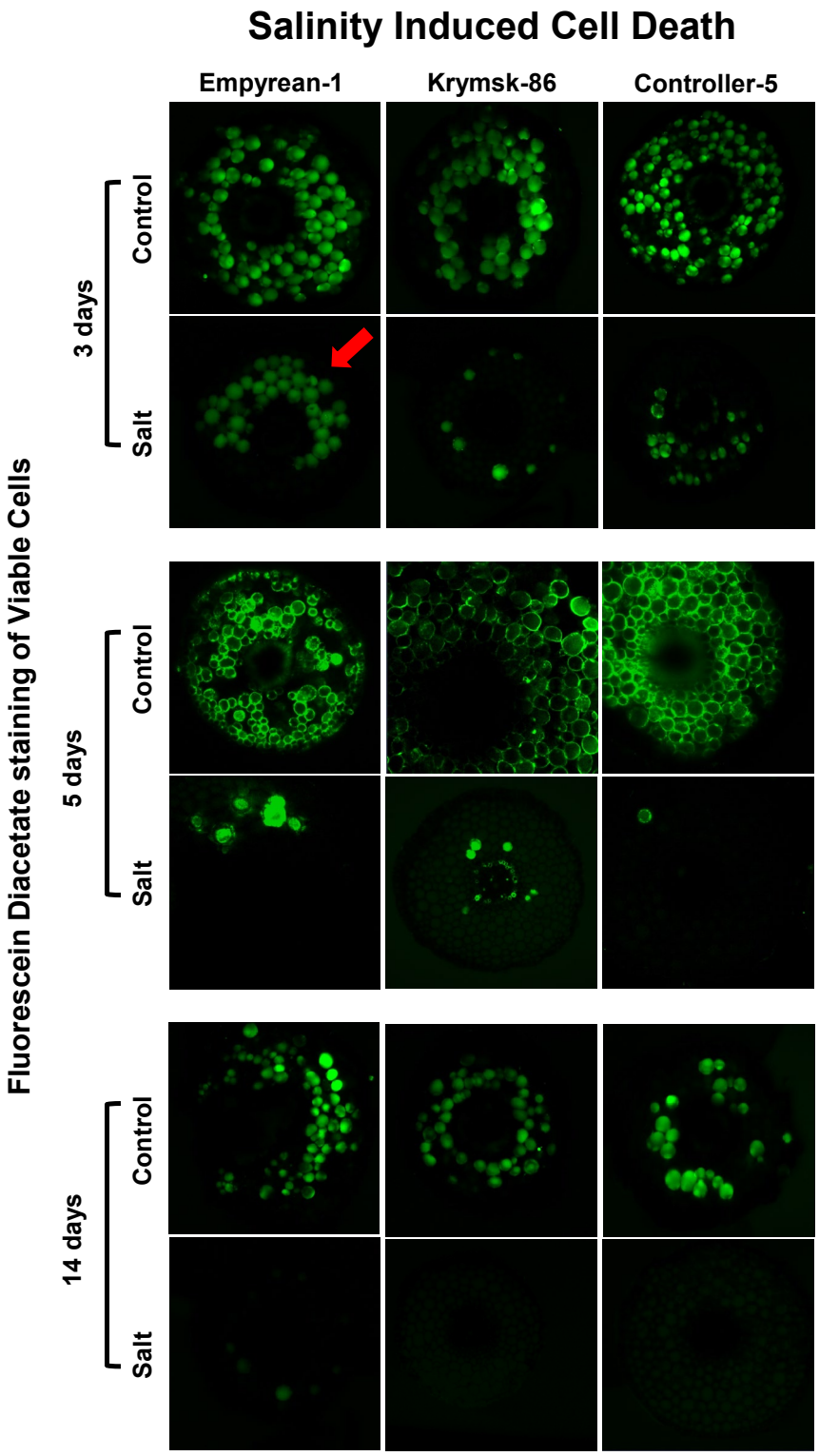


Figure 6. Cell viability staining (FDA staining) of the root meristematic cells of three rootstocks upon salt treatment. Fluorescein diacetate staining indicates live cells under salt treatment time points. Seedlings adapted in soil for two months were treated by 150 mM NaCl for three days. Red arrow indicates the high percentage of viable cells in Empyrean-1 after 3 days of salt treatment.



D. Discussion and Conclusions

We began the screening of total eight rootstocks for their salt tolerance towards a strong salt stress of 150 mM NaCl (**Figure 1**). A large scale screening at the cellular level indicated that genotypes with a cellular response of excluding salt at the epidermis or exodermis have the highest tolerance towards salt stress (**Figure 1** and **4**). Vacuole sequestration of salt is generally considered as a salt tolerance trait at the cellular level in annual model plants (Apse and Blumwald, 2007). However, our result suggests that vacuole sequestration alone, as shown in Controller-5, is not consistently associated with salt tolerance. Thus additional mechanisms that exist in woody crops contribute to salt tolerance.

We have established a hydroponics system coupled with video imaging to monitor the water uptake of rootstocks under salinity stress (**Result 2** and **Figure 3**). This system highlighted the volume of water uptake in salt-tolerant rootstocks under salinity stress. This should be considered as important trait in the screening of salt-tolerant rootstock, as grafting and growth of high economic value of scion under the stress conditions present in the current climate (Rivero et al., 2003). The stomata opening and closure should be further monitored in resistant lines, as stomata closure under drought is associated with the hormone levels responsive to salt stress and subsequently the decrease of crop yield (Peleg and Blumwald, 2011). Our result suggest that the degree of leaf stomata closure in Empyrean-1 upon salt treatment may be relatively low, and the percentage of stomata opening in a salt-tolerant rootstock can also be considered as important trait in rootstock selection.

Upon Na+ treatment, less decrease of root growth was observed in Empyrean-1, compared to the Controller-5 and Krymsk-86, suggesting that Empyrean-1 has higher ability of salinity tolerance. Upon Na+ treatment, less Na+ accumulated in the Empyrean-1 roots than other genotype, while Controller-5 absorbed Na+ into the both cortex and vacuoles (**Figure 5**). Empyrean-1 maintained more viable cells than other genotypes after 3 days of salt treatment (**Figure 6**). No significant decrease of root growth was observed in Empyrean-1 upon salt treatment, corroborating the salt tolerance of Empyrean-1 compared to the other genotypes.

High soil salinity is a major abiotic stress that affects plant growth and development, and it reduces the yield of almonds in central valley (Schoups et al., 2005). In this study, we utilized eight almond rootstocks of different genetic background, and observed different distinct salinity tolerance among the rootstocks. Controller-5, Krymsk-86, and Empyrean-1, are further selected to study their capacity and mechanism of salinity tolerance at cellular levels because they represent a wide spectrum. To investigate the Na^+ accumulation and cell viability in the root, short and long periods of salt treatments were applied to these rootstocks. Our cell biology studies showed that Controller-5 can accumulate salt into the vacuole, while Empyrean-1 has the capacity to exclude salts from the root, and is more tolerant to salt stress. Prolonged Na^+ treatment induced the root tip cell death of all genotypes. **This study provides the first layer of a mechanism for salinity tolerance at a cellular level and insights to investigate and screen for salt tolerance rootstocks with superior behavior such as Empyrean-1.**

E. Materials and Methods

Plant Materials

Salt stress response of total eight *Prunus* rootstocks were investigated in this work. They are Controller-5 (*P. salicina x P. persica*), Krymsk-86 (*P. cerasifera x P. persica*), Empyrean-1 (*P. persica x P. davidiana*), Bright's Hybrid-5 (*P. amygdalus x P. persica*), Nemaguard (*P. persica* × *P. davidiana*), Titan (*P. dulcis*), Hansen-536 (*P. dulcis x P. persica*), and Viking (*P. persica* × *[P. dulcis* × *(P.davidiana* × *P. mume)]*). Treated plants were washed and weighed for biomass analysis. Shoots of micropropagated rootstocks were transferred to rooting medium for 2 weeks under sterile tissue culture conditions for root emergence. Then the rootstock seedlings were transferred to soil for a two-month adaptation. Total three cm section was used in microscopy-based structural analysis.

Confocal microscopy analysis of cellular traits of rootstocks upon salt stress

The developmental gradient of the root was investigated under confocal microscopy according to the degree of xylem maturation. Roots were sectioned, incubated in osmolarity maintaining buffer to ensure tissue viability and/or will be fixed in 4% paraformaldehyde (Park et al., 2014; Drakakaki et al., 2006) further characterization. CoroNa-Green was used for sodium staining; Fuchsin was used for lignin staining (Kapp et al., 2015); Fluorol-Yellow or Nile Red for Suberin (Naseer et al., 2012). Micrographs were recorded on the ZEISS LSM/ 700 710 and the Leica SP8 MP microscopes.

Unforeseen development and challenges

Rootstocks of woody crops often entail the hybrid vigor of various species, and the situation also brings challenges toward the analysis of genetic cause of the phenotype. Interestingly, the rootstocks Empyrean-1 and Nemaguard have the same genetic background as *P. persica x P. davidiana*, but do not show similarities in our cellular and phenotypical analysis of their salt tolerance. The result suggests the epigenetic adaptation likely occurred during the breeding process (Thiebaut et al., 2019), and marker expression analysis of beneficial trait related genes under stress is the shortcut to the high throughput screening for plant molecular breeding.

F. Publications that emerged from this work

This report contains unpublished data of our work, and figures will be directly used in the publication of a peer-reviewed journal. We are currently summarizing our findings of this work into a manuscript that will be submitted within the next 2 months for publication.

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