Subcellular and Molecular Characterization of Salinity/Tolerance in Almonds with Novel Tools

Project No.: **15-HORT23-Drakakaki**

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Objectives:
1. Development of confocal based assays for sodium, chloride and potassium ion detection in almond cells and structural characterization of root cell morphology under salinity stress.
2. Conduct pilot screen of selected rootstocks.

Interpretive Summary:
In the first part of our three-year research plan the plant specific methodologies for the detection of the implicated ions in salinity stress, sodium, potassium and chloride have been established. Among our results are the first successful subcellular potassium and chloride imaging experiments in all plants. Distinct, genotype specific, subcellular accumulation patterns of sodium and potassium are observed in almond root cells. So far, the cumulative evidence suggest that two mechanisms contribute to halotolerance: I) sodium sequestration and II) potassium ion balancing.

California is experiencing increasing soil salinization, which is projected to accelerate in the current drought conditions due to the increased use of saline ground water (Letey 2000; Schoups et al., 2005). Almond plants, one of the most economically important crops in California and one with the highest expansion rate, are strongly sensitive to salt stress. Selection of elite rootstocks with improved salinity tolerance affords a way to ensure high yield production in this long term trend. The development of universal cellular and molecular methodologies towards identifying sodium uptake, ion sequestration and its effect on cellular morphology and viability for various rootstocks and rootstock/scion combinations is a hitherto unexplored approach.

Real time *in vivo* fluorescent microscopy affords localizing and evaluating saline induced structural and morphological changes in the cell and cell wall as a robust criterion for

determining halotolerance across various rootstocks. Towards the selection of elite genotypes, we develop confocal based assays for sodium, potassium and chloride detection. This will enable the quantitative correlation of salt tolerance with subcellular ion compartmentalization to efficiently characterize underlying mechanisms of tolerant genotypes.

Materials and Methods:

Almond rootstocks were provided by Sierra Gold Nurseries and were tissue culture propagated. Germinated seedlings were grown in ¼ MS media and salt stressed. Plant growth and shape were recorded weekly in the form of whole plant imaging.

Samples were collected for analysis of subcellular Na^+ and K^+ compartmentalization and analyzed for overall ion content and structural analysis as previously reported (Gonzalez et al., 2012; Le and Drakakaki 2013) over a period of 4 weeks.

Roots, were sectioned, incubated in osmolarity maintaining buffer to ensure tissue viability, and incubated with the respective dyes for ion and organelle localization as previously described (Lee and Drakakaki 2014). Micrographs were recorded on the ZEISS LSM, 700, 710 and the Leica SP8 MP microscopes as previously described (Lee and Drakakaki 2013; Park et al., 2014). Upon completion of treatments plant samples were collected and frozen for further analysis.

Results and Discussion:

Objective 1:

In the first part of our three-year research plan the methodologies for the live *in vivo* imaging of ions involved in salinity stress, Na^+, Cl^- and K^+ have been established, using a variety of preparation techniques and microscope modalities. Protocols are available for the research community and will be in published in detail. With no previous reports of chloride or potassium staining in plants existing, a highly customized staining process and imaging approach allowing the *in vivo* imaging at the cellular and subcellular level was developed.

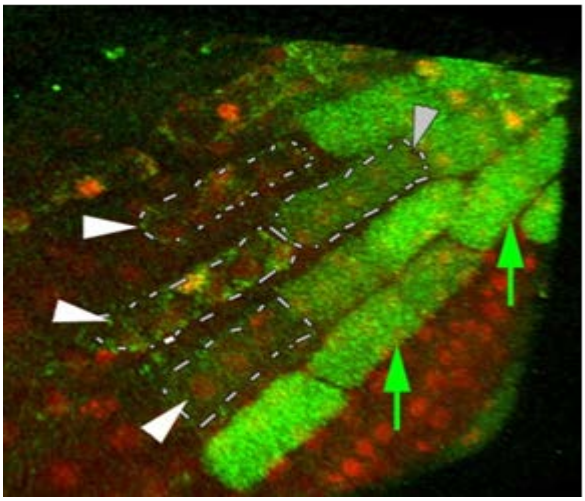


Figure 1. Chloride detection in plants. Chloride accumulation in salt stressed plants. Signal from the chloride specific dye, whose fluorescent emission is reduced when exposed to Cl^- (green, arrows) is indicating loading into the cell and arrowheads (darker regions) indicate elevated levels of chloride. Red indicates nucleus. Figure is a **3D** representation.

The challenge to identify specific chloride signal from plant auto-fluorescence and obtain confocal (i.e., blur free images) allowing 3D reconstruction was successfully addressed with the use of an advanced microscope modality (two photon microscopy), that has become available on campus in 2015. The established methodology allows quantitative determination of both uptake and sequestration of Cl^- in the plant vacuole **(Figure 1)**.

Objective 2:

With the extended scope of the experiments, we summarize below our preliminary findings and will [illegible] more

Pilot experiments were contacted on Empyrean 1, Hansen and Controller with rootstocks available through tissue culture from Sierra Gold Nurseries: Empyrean-1 showed superior tolerance among the rootstocks tested (**Figure 2 and Figure 3**)

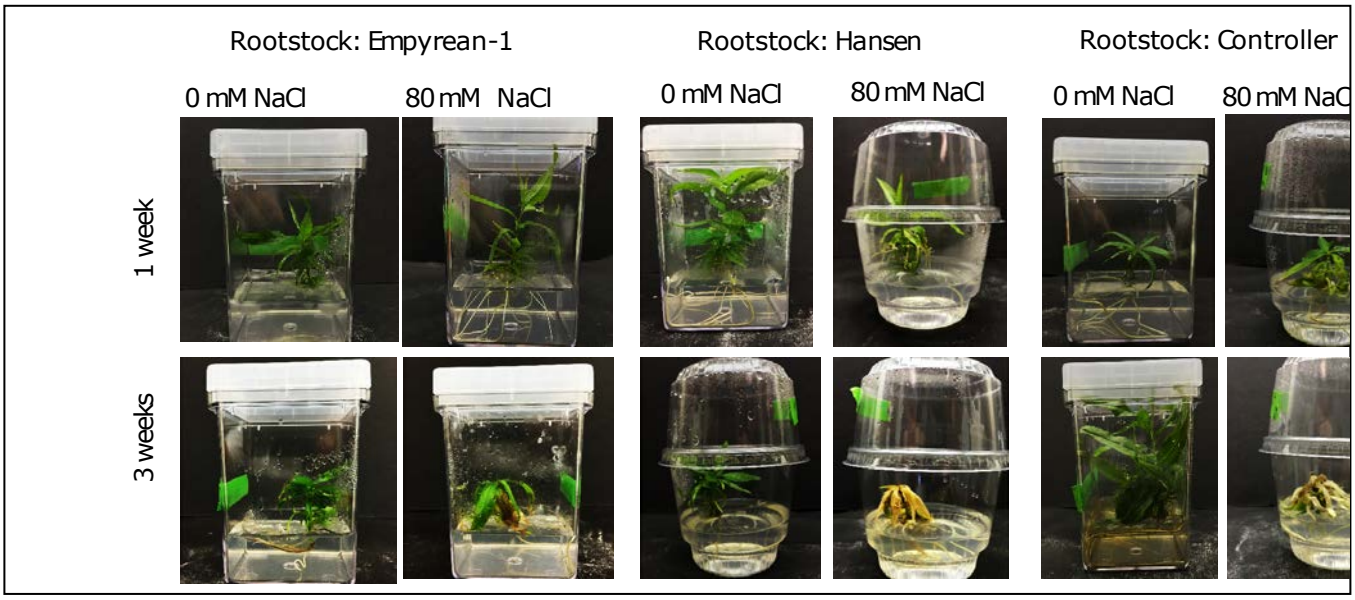
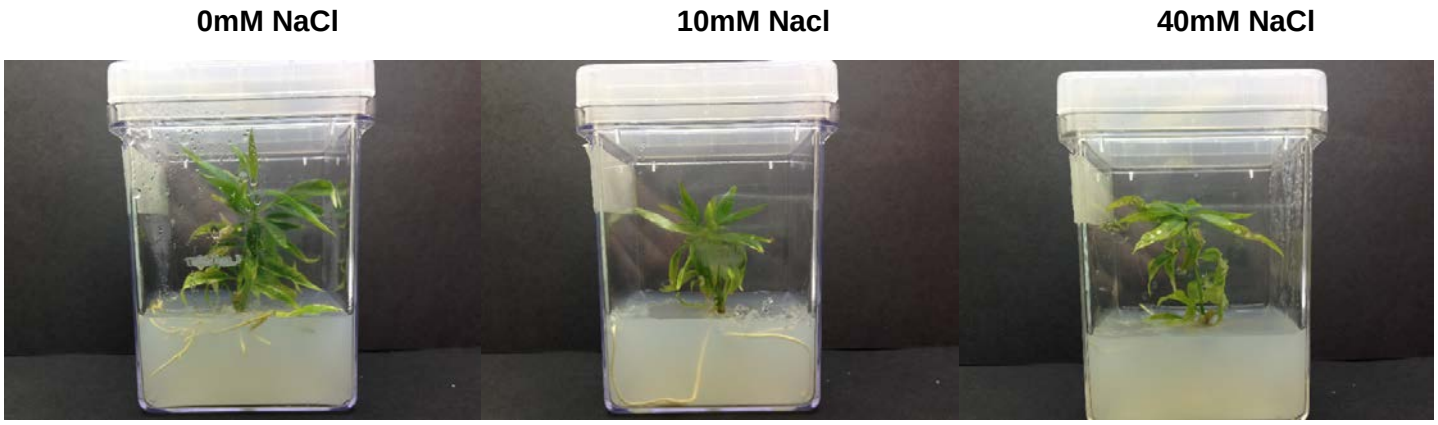


Figure 2. Response of selected rootstocks to salinity. Selected rootstocks (Empyrean-1, Hansen, Controller), were salt treated for 4 weeks. Representative images are shown. Empyrean 1 showed superior performance compared to Hansen and Controller upon 3 weeks of 80 mM NaCl treatment using phytogel as an agar source.

To verify the results above an independent study using Hansen and Empyrean-1 was conducted in which the solid growth medium was changed from phytogel to phytoagar. Consistent with our earlier observation Empyrean-1 showed superior performance over Hansen (**Figure 3**).

3 A) Hansen 2 Weeks

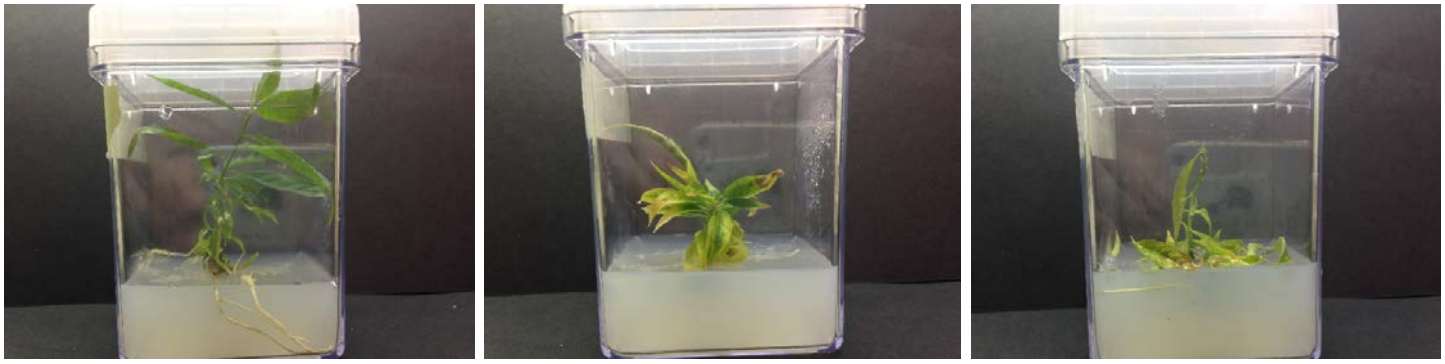


3B) Empyrean-1 2 weeks

0mM NaCl **10mM Nacl** **40mM NaCl**

3C) Hansen 3 weeks

0mM NaCl **10mM Nacl** **40mM NaCl**



3 D) Empyrean-1 3 weeks

0mM NaCl **10mM Nacl** **40mM NaCl**

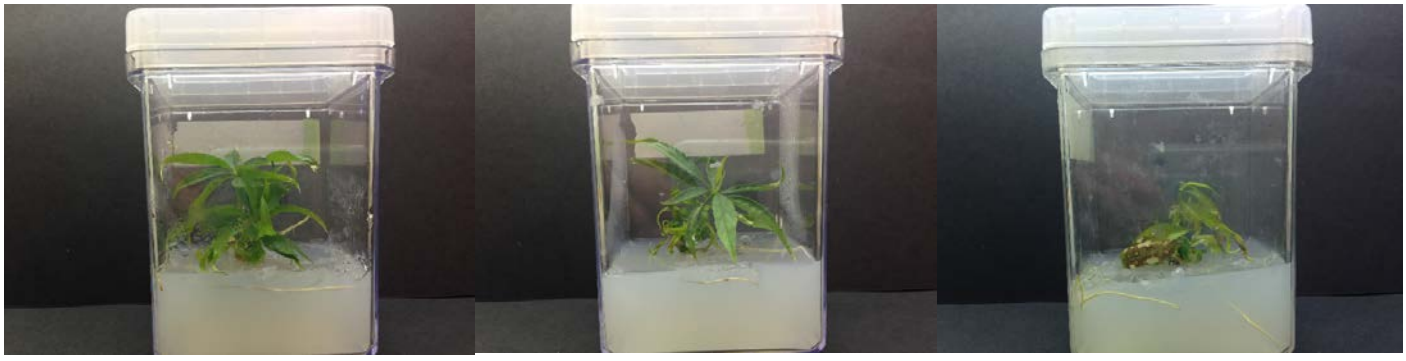


Figure 3. Response of selected rootstocks to salinity. Selected rootstocks (Empyrean-1, Hansen), were salt treated for 4 weeks. Represented images are shown. Empyrean-1 showed superior performance compared to Hansen under 3 weeks of 10 and 40 mM NaCl treatment using phytoagar as an agar source.

As a complementary criterion the leaf tissue discoloration, as an expression of plant health was evaluated by performing a red to green ratiometric measurement. As shown in **Figure 4**, Empyrean-1 maintains more of its original leaf color/spectrum as expressed by the red to green ratio while significant reduction is observed in Hansen, indicating advanced cell damage

for Hansen at concentration of 10 and 40mM., underscoring independently the superiority of Empyrean to salt stress.

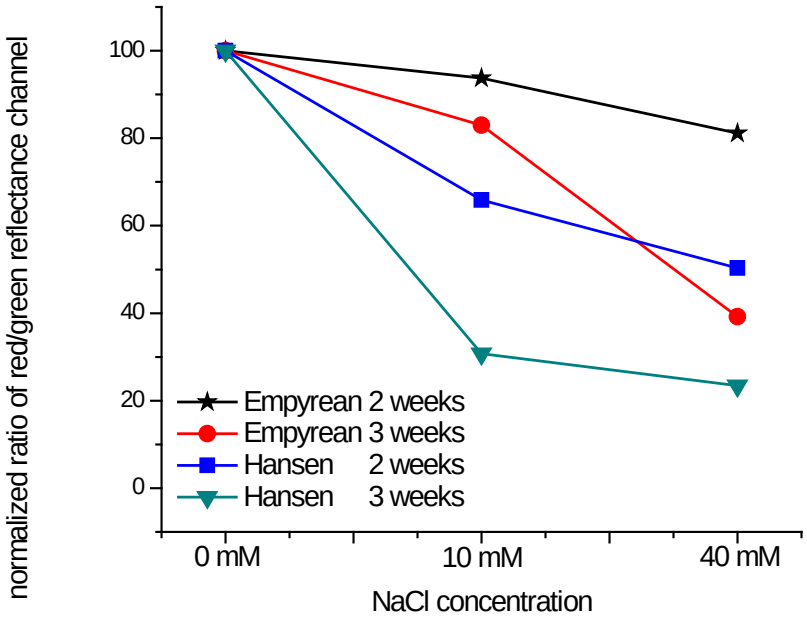


Figure 4. Leaf tissue discoloration of rootstocks under salt stress treatment**.**
Leaf tissue discoloration as means of plant viability, by red to green ratiometric measurement is shown for Empyrean-1 and Hansen after two and three week treatments. Empyrean-1 maintains higher ratio compared to Hansen for both 10mM and 40 mM NaCl treatments and at both time intervals.

Subcellular sequestration of sodium and potassium

The sequestration and overall cellular distribution of sodium and potassium was examined in detail in the two rootstocks Empyrean-1 and Hansen. Salinity treatment leads to a marked increase of the cellular localization of Na^+ and K^+ with a distinct pattern in Empyrean-1 roots (**Figure 5**). The staining pattern establishes both uptake and sequestration of sodium into parenchymatic root cells.

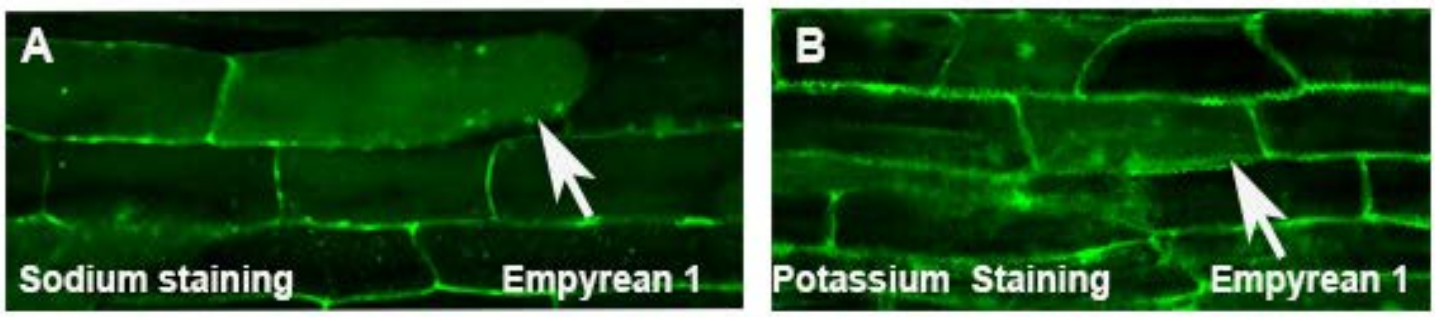


Figure. 5 Localization of sodium and potassium. In Empyrean-1 root cells, sodium accumulates dominantly in the cytoplasm and the plant vacuole (A), with potassium being localized in the cell wall and in the cytoplasm (B). Empyrean-1 plants were treated with 40 mM NaCl for 2 weeks and 100 μm root sections were imaged.

Further, salinity treatment leads to an increased cellular localization of Na^+ and K^+ in Hansen roots, albeit in a distinctly different pattern **(Figure 6)**.

A distinct localization pattern was not identifiable in the Hansen rootstock **(Figure 6)**. We hypothesize that the subcellular accumulation of Na^+ contributes to the increased tolerance observed in Empyrean-1 rootstocks under salinity treatments. The mechanism of the K^+ distribution is yet unclear, both redistribution or additional uptake are possible sources. *Our results so far show the feasibility of a comprehensive and simultaneous mapping of the various ion distributions in different genotypes towards identifying superiority among rootstock/scion combinations.*

In continuing experiments Empyrean-1 and Hansen, are subjected to a range NaCl concentrations and the subcellular distribution of sodium and potassium are mapped out using shorter time intervals of salinity treatments to avoid tissue damage prior to imaging. Additional rootstocks will be examined under the conditions established through our result from our initial studies. We hypothesize that a combination of ion uptake and redistribution including sodium

and potassium, alleviates cytotoxic effects of increased NaCl uptake by a mechanism related to ion balances that are not yet fully explored and understood. We will further explore if

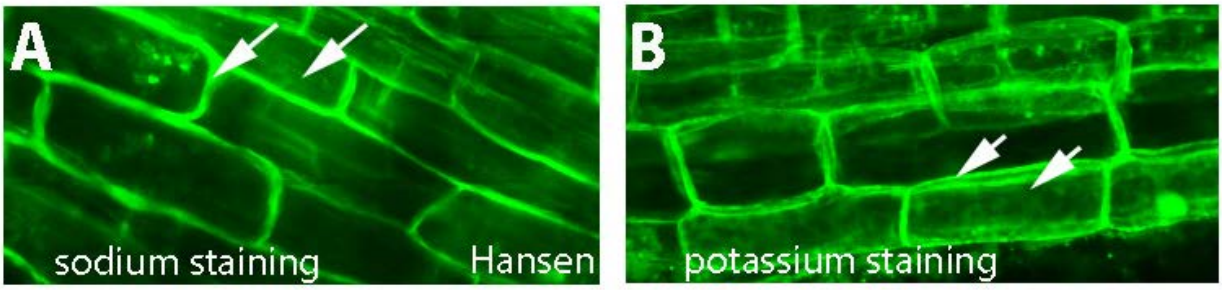


Figure 6 Localization of sodium and potassium. Hansen plants were treated with 40 mM NaCl for 2 weeks and 100 μm root sections were imaged. In Hansen root cells sodium (A) and potassium (B) accumulates at the apoplast and in the cytoplasm

addition of small amounts of KCl to the NaCl stress condition has a beneficial effect via ion balancing on NaCl resilience.

The established methodology will provide the flexibility for future extensions beyond salinity stress to assess cellular structural modifications in response to biotic and abiotic stresses, including that of various pathogens. We expect that dissemination of the developed methodology will spur rapid adaptation and follow up studies, in tandem with a proliferation of the use of advanced microscopy tools in almond research on the cellular level.

Research Effort Recent Publications:

Drakakaki G. Subcellular and Molecular Characterization of Salinity Tolerance in Almonds with Novel Tools. (2015). Annual Almond Conference, Sacramento California, USA. Oral and Poster presentations.

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