Annual Report

Cellular, subcellular and molecular characterization of salinity tolerance in pistachio with novel tools

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Summary:

Understanding the mechanism of sodium uptake, transport and sequestration at the cellular and molecular level is valuable in assessing mechanisms of salinity tolerance in pistachio rootstocks. In this project we develop the tools for the detection of sodium, potassium and chloride for a detailed cellular characterization of rootstocks towards identifying superior salinity tolerance. We established novel methodologies, for the detection of sodium, potassium and chloride ions in plants at the cellular level. Distinct accumulation pattern of sodium and potassium were observed at the subcellular level in UCB1 root cells. The overall results suggest that both sodium sequestration and potassium ion balancing are mechanisms contributing to salinity tolerance. Using advanced microscopy, we are first to detect chloride and potassium localization in plant cells at the subcellular level using live imaging.

Background and Significance:

Soil salinization in California is increasing. Pistachio plants with relative high saline tolerance afford the possibility to use marginal land and improve yield on current acreage. So far little is known about the cellular uptake and translocation of nutrients and salts throughout the pistachio plant under salt stress, and more importantly how to systematically and economically select for elite cultivars.

Pistachio trees are strongly contributing to California's economy. Their relative saline tolerance (Picchioni et al., 1990; Ferguson et al., 2002) provides great potential for future expansion into marginal land with elevated salt levels (Sanden et al., 2006). Understanding the mechanism of sodium uptake, transport and sequestration at the cellular and molecular level is valuable and can provide a convenient and economical way of identifying desired plant "characteristics" to be selected for in rootstocks, scions or their combinations in order to achieve optimal plant performance and composition. Insights gained will be assisting in the development of better agricultural practices under saline conditions.

Given the damaging effects of salinity on crop growth and productivity, application of genetic resources to the breeding of salt tolerant genotypes is a valuable tool for the development of sustainable agriculture. So far, reports on species propagated by grafting often ascribe salinity tolerance to the rootstock (Okubo et al., 2000; Rivera et al., 2003; Huang et al., 2010). Roots play a key role in the salt tolerance of plants, for they represent the first organs to control the uptake and translocation of nutrients and salts. Accumulation of Na^+ in the roots is an adaptive response used by several woody species to minimize its toxicological effects on shoots (Walker et al., 1987; Picchioni et al., 1990; Gucci and Tattini, 1997). Accordingly, the control of the root-to-shoot transport of salt can serve as a criterion for tolerance (Chelli-Chaabouni et al., 2010).

Our work aims at the establishment of cellular and molecular methodologies to identify sodium, potassium and chloride uptake, ion sequestration and its effect on cellular morphology and viability for various rootstock scion combinations. Our working hypothesis is that sodium and chloride sequestration in pistachio cells is an important and identifiable trait for salinity tolerance and that it is mediated by the activity of specific transporters. By observing sodium, potassium and chloride localization in live plants at the subcellular level with non-invasive fluorescence microscopy and saline induced structural/morphological cell and cell wall changes, we are aim in the in depth characterization of salinity tolerance. Thus, understanding the cellular sequestration of Na^+ and Cl^- and K^+ in a quantitative manner can provide evaluation criteria for the identification of most suitable rootstocks.

Specific aims

Develop and optimize approaches of imaging sodium, potassium and chloride subcellular localization in pistachio.

With these tools examine pistachio rootstocks with promising improved salt tolerance.

Experimental procedures

Pistachio seedling propagation and salinity treatments: Pistachio seeds were surface sterilized using sodium hypochloride and stratified at 4 °C. Germinated seedlings were grown in ¼ MS media and salt stressed. Samples were collected for analysis of subcellular Na^+ and K^+ compartmentalization and analyzed for overall ion content and structural analysis (Gonzalez et al., 2012) for citrus and our previews report (Le and Drakakaki 2013) over a period of 4-8 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 LSM710 and the Leica SP8 MP microscopes as previously described (Lee and Drakakaki 2014; Park et al., 2014).

Results

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. The developed approaches allow live *in vivo* imaging at the cellular and subcellular level. In order for the indicator dyes to be effective, a highly customized staining process had to be developed, since no previous report of chloride or potassium staining in plants exists. The challenge to identify specific chloride signal from plant auto-fluorescence was successfully addressed with the use of an advanced microscope modality that has become available on campus in 2015 (two photon microscopy). Both uptake and sequestration of Cl^- in the plant vacuole **(Fig.1)** can be determined in a quantitative manner.

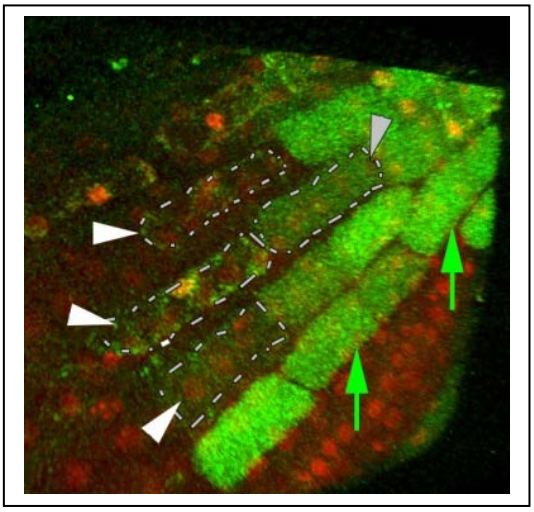

Fig. 1 Chloride accumulation in salt stressed plants. Signal from the chloride specific dye (green, arrows) is indicating loading into the cell and arrowheads (darker regions) indicate elevated levels of chloride. Red indicates nucleus. Fig. is a **3D** representation.

With the extended scope of the experiments, we summarize below our preliminary findings so far and will extend the findings in more detail in the upcoming reports. The salinity treatment leads to a marked increase of the cellular localization of sodium, and somewhat surprisingly to and increased concentrated localization of potassium in UCB1 rootstocks compared to non salt stressed plants.

In detail, the distinct cellular and subcellular localization patterns of the metal ions is best described by characteristic sodium localization in both the cytoplasm and the plant vacuole, with a higher concentration in the vacuole, and dominant potassium localization in both the cytoplasm

and in the apoplast. At low NaCl (~20 mM) concentrations, well defined localization of Na in the plant vacuole is observed, however this definition deteriorates at high NaCl (50 mM) concentrations when treatment is carried out for extended periods of time. Characteristic cell damage is observed and sodium appears to be dispersed throughout the cell. Interestingly, for the higher NaCl concentrations increased fluorescence of the potassium specific stain is observed and well defined, with strong levels at the apoplast **(Fig.2).** The origin of the K^+, whether through redistribution or addition uptake, is yet unclear.

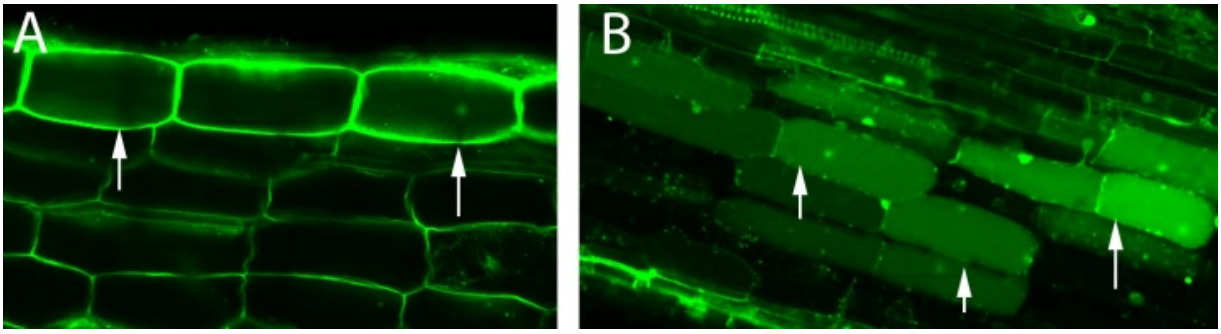

Fig. 2 Localization of potassium and sodium. UCB1 plants were treated with 20 mM NaCl for 8 weeks and 100 μm root sections were cut. Potassium staining is detected at the cell wall and in the cytoplasm (A) with sodium being dominantly found in plant vacuole (B) and the cytoplasm.

On the basis of this ion localization pattern, we hypothesize that potassium uptake via the roots and its subsequent specific distribution, mediates cytotoxic effects of increased NaCl uptake by mechanism related to ion balances that are not yet fully explored and understood.

In ongoing experimental studies, UCB1, *P. atlantica*, *P. integerrima* seedlings are subjected to a range of 0- 100 mM NaCl treatments and the subcellular distribution of sodium and potassium are examined. The mapping out of chloride in the salt treated population is in progress.

Our results so far show the feasibility of a comprehensive and simultaneous cellular mapping of the various ion distributions in different genotypes such as UCB1, P. atlantica, P. integerrima and others.

Conclusions and Practical Application

The overall objective of the project is to develop and establish tools for the detection of sodium, potassium and chloride for an efficient assessment of mechanisms

contributing to salinity tolerance in pistachio rootstocks and ultimately enable more straightforward screening of rootstocks leading to the identification of halotolerant elite cultivars. In the first part of our three year research plan the methodologies for the detection of the implicated ions in salinity stress, sodium, potassium and chloride have been established. Our results are the first successful reported potassium and chloride imaging experiments in plant cells. Distinct accumulation patterns of sodium and potassium are found at the subcellular level in UCB1 root cells.

The established methodologies, soon to be made available through publication, open new avenues towards understanding cellular and subcellular mechanisms contributing to salinity tolerance. On the basis of our results we have formed the hypothesis that distinct sequestration of sodium and chloride into subcellular compartments and the uptake of potassium with specific distribution, in which it forms a counter ion balance, both contribute to the salinity tolerance in pistachio. Application of these methods in larger genotype screening efforts affords a unique opportunity to assess rootstocks and elite cultivars.

Continuing with our now established methodologies, we will conclude the evaluation of different rootstock genotypes in order to dissect the mechanism contributing to salinity tolerance. Selected genotypes, with desired phenotypic characteristics, will be further examined at the genetic level for the evaluation of increased gene expression, allowing the identification of specific molecular markers for salinity tolerance.

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