Characterization of root anatomy and plasticity in almond rootstocks for improved nutrient uptake and stress response

Project No.: **HORT23A.Drakakaki**

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A. Summary

California Central Valley undergoes increasing soil salinization, which is estimated to accelerate in the current climate conditions due to the increased use of saline groundwater. Almond plants are crops of economic importance and high expansion rate in California, and they are strongly sensitive to salt stress. Selecting rootstocks with improved salinity tolerance will help to maintain high yield production of almonds in this long term trend. However, no existing technology can efficiently sort salt-resistance rootstocks during their seedling stage. Gene expression analysis upon salt stress were performed on genes of cation transporters, hormone-responsive genes, and biopolymer biosynthesis in rootstocks of extreme behaviors, and expression markers were identified for application. Based on cellular and molecular evidences, we found the rootstock Empyrean-1 (compared to other rootstocks) has higher capacity to exclude sodium from the root using a combination of upregulating ion transporter expression and enhancing polymer deposition in cellular barriers like suberin, in response to salt stress. Gene expression analysis was well correlated with cellular phenotypes and indicated candidate genes for marker analysis. This study reveals mechanisms of salinity tolerance in superior rootstocks and provides insights and tools for further screening of rootstocks resistant to salt stress. We will use our microscopy-based methodologies to validate the selected premier traits at cellular level, including high sodium exclusion and suberin accumulation upon salt stress, in combination with our gene expression markers to facilitate the high throughput selection of salt tolerant rootstocks.

B. Objectives

Aim I: Characterization of root anatomy and architecture to identify root developmental traits that account for root adaptability in abiotic stress with emphasis in salinity. Determine changes in root architecture and specifically endodermis that contribute to stress adaptability in selected rootstocks that are superior in performance to salinity and other abiotic stresses. Examine deposition of cell wall in the root vasculature and determine cell wall changes that can provide structural barriers for ion exclusion and reduction of water loss. Compare changes in root anatomy in laboratory grown plants and field grown plants.

Aim II) Expression analysis toward the identification of ion transporters that lead to the observed differential ion accumulation.

Aim III) Initiate molecular marker analysis for root structure and development that lead to root plasticity and adaptability. Given that many genes that are involved in endodermis development for suberin, lignin and cell wall in other species, determine differentially expressed genes that may contribute to root adaptability and abiotic stress response.

C. Results

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

Result 1 is the evidence of realization of **Aim I**. **Result 2** realizes **Aim II and III**, and will be expanded into additional 10-20 rootstocks to demonstrate the power of our established system corroborating cellular and molecular tools to facilitate the high throughput selection of high salt tolerance rootstocks.

Result 1: Analyses of sodium accumulation and biopolymer deposition patterns at cellular level reveals a physiological mechanism of the rootstock vulnerability or tolerance towards salt stress.

We analyzed sodium staining of root cross-sections along the developmental gradient as the textbook (Taiz and Zeiger, 2010), and quantified the signal intensity of all samples, in order to quantitatively analyze the salt accumulation difference between rootstocks or across a root developmental gradient within single rootstock upon salt stress. Both meristematic zone and elongation zone of Controller-5 show high vacuole sequestration of sodium, compared to Empyrean-1, while Empyrean-1 is capable of excluding sodium at exodermis layer of the root (**Figure 1**). Suberin is a carbon-rich biopolymer associated with the root robustness, as it increases the water impermeability of endodermis, protecting the plants from the salt stress. Suberin staining indicates that higher suberin deposition occurs at the meristematic zone of salt-treated samples in both rootstocks, compared to the untreated control (**Figure 2**). However, upon salt stress, Empyrean-1 is capable of accumulating more suberin at both elongation maturation zone, than under control condition (**Figure 2**). Controller-5 shows the opposite pattern of suberin deposition as Empyrean-1 at the elongation and maturation zone, as salt stress induces less suberin deposition in those two zones in Controller-5 (**Figure 2**). Lignin staining indicates both rootstocks accumulate more lignin upon salt stress than under untreated condition, while Empyrean-1 accumulates significantly more lignin at the endodermis layer than Controller-5 in the meristematic zone (**Figure 3**), suggesting the earlier maturation of the root in Empyrean-1. The result suggests Empyrean-1, capable of excluding sodium at the outer layer of root, also actively synthesize high amount of biopolymer, especially suberin, to convey the salt tolerance towards the salt treatment. **These results indicate we have established microscopy-based methodologies to validate the selected premier rootstock traits at cellular level, including high sodium exclusion and cellular barrier differentiation by suberin accumulation upon salt stress.**

Figure 1. Sodium accumulation of three rootstocks across the root developmental gradient. After transferred to soil for two months adaptation, Controller-5 and Empyrean-1 were treated by 150 mM NaCl solution, and the sodium staining by CoroNa-Green of live cells was performed. Intensity of sodium staining is quantified by ImageJ. ANOVA analysis (Tukey's test) was performed for the meristematic zone data. Different letters indicate significant difference ($p < 0.05$).

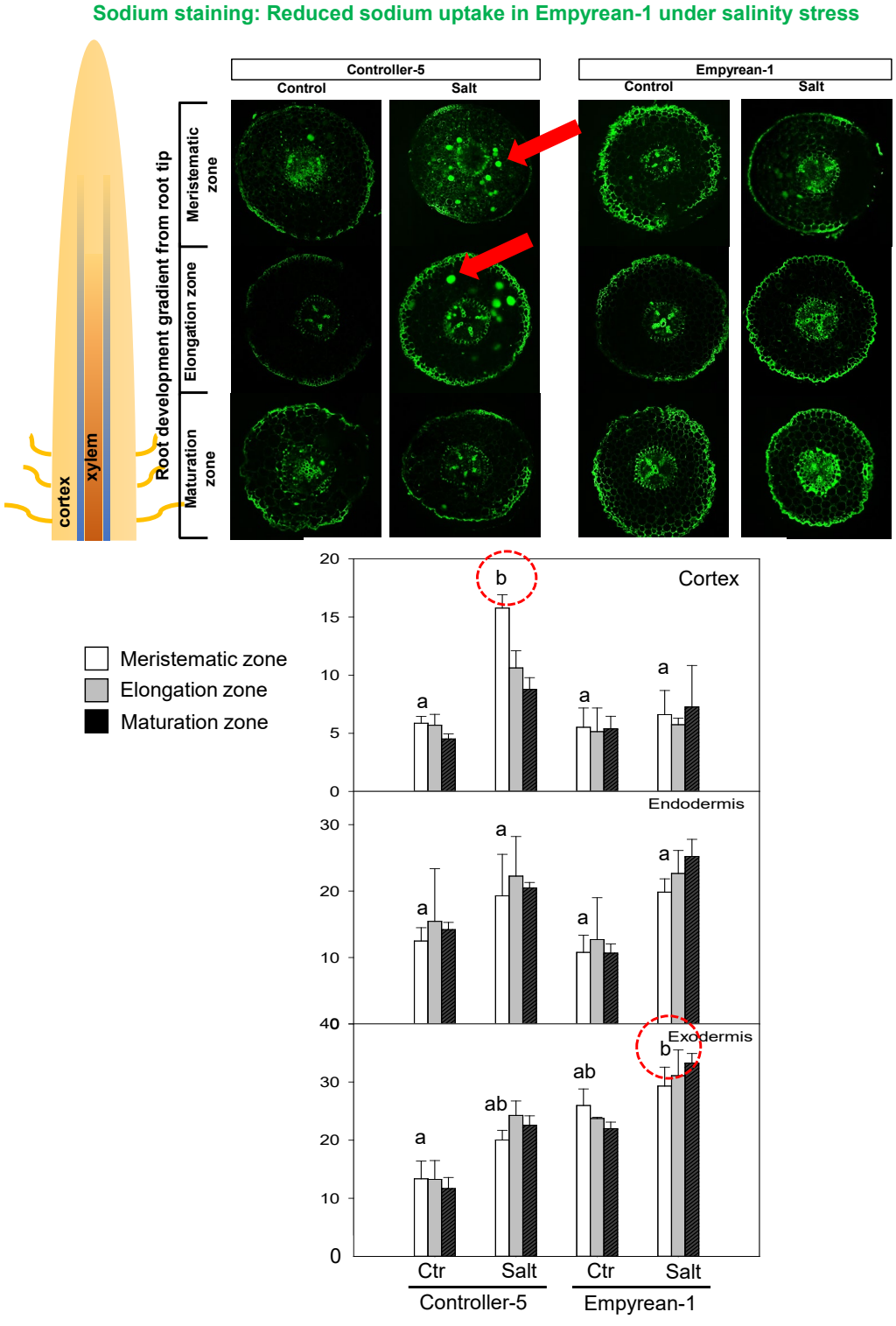


Figure 2. Suberin deposition across the developmental gradient in Controller-5 and Empyrean-1 with or without salt stress. After transferred to soil for two months adaptation, Controller-5 and Empyrean-1 were treated by 150 mM NaCl solution, and the suberin staining by Nile Red was performed. Intensity of suberin staining is quantified by ImageJ. Cross-sections of 1, 2, 3 cm from the root tips matching the developmental zone were selected. t-test is used to compare salt-treated samples versus control, and asterisk indicate significant difference ($p < 0.05$).

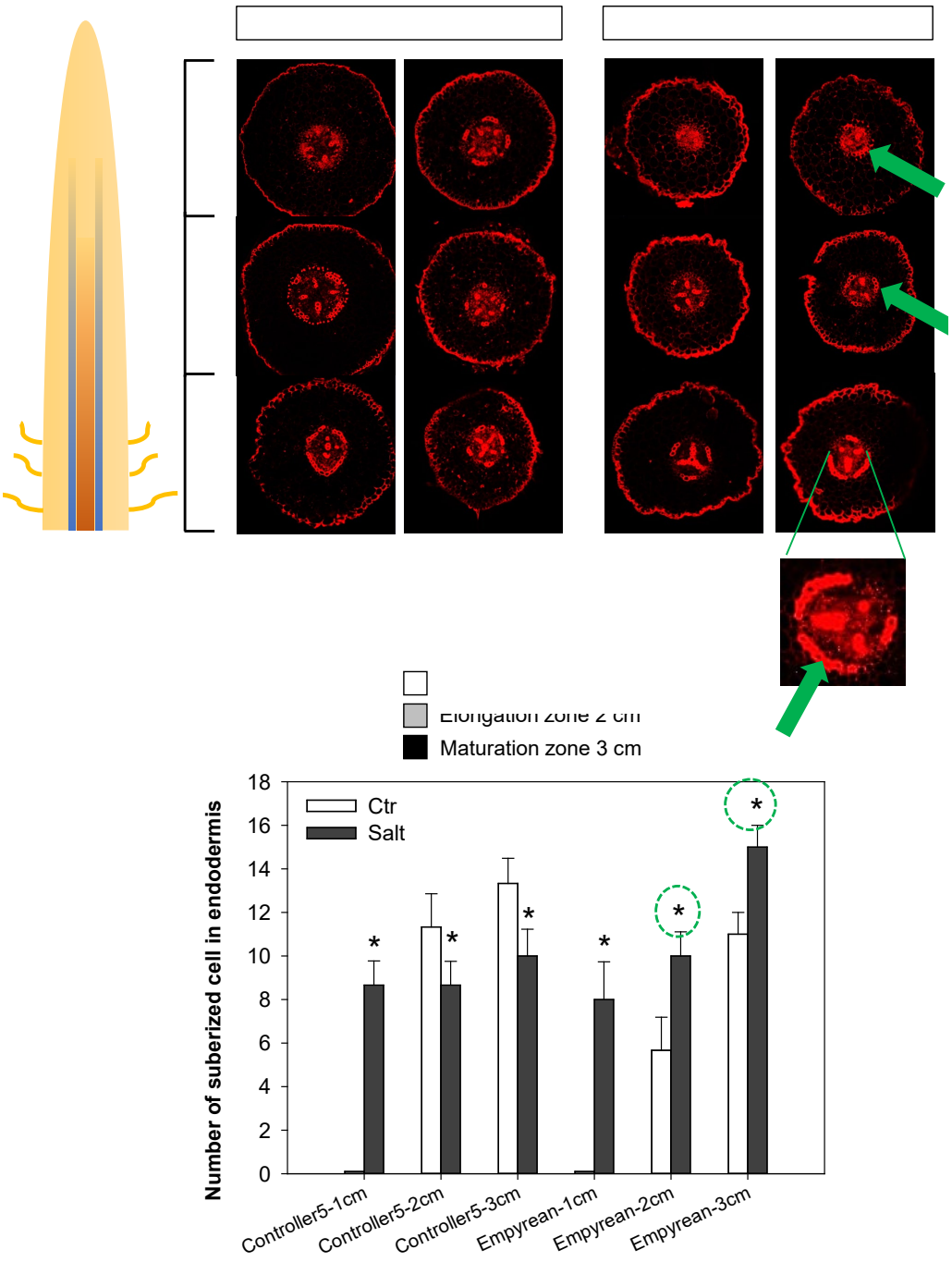


Figure 3. Lignin accumulation in response to salt treatment in Controller 5 and Empyrean-1. After transferred to soil for two months adaptation, rootstocks were treated by 150 mM NaCl solution or control half Hoagland solution, and the lignin staining by Fuchsin was performed. Intensity of Fuchsin staining is quantified by ImageJ. ANOVA analysis (Tukey's test) was performed for the salt-treated meristematic zone data of two rootstocks. Different letters indicate significant difference ($p < 0.05$).

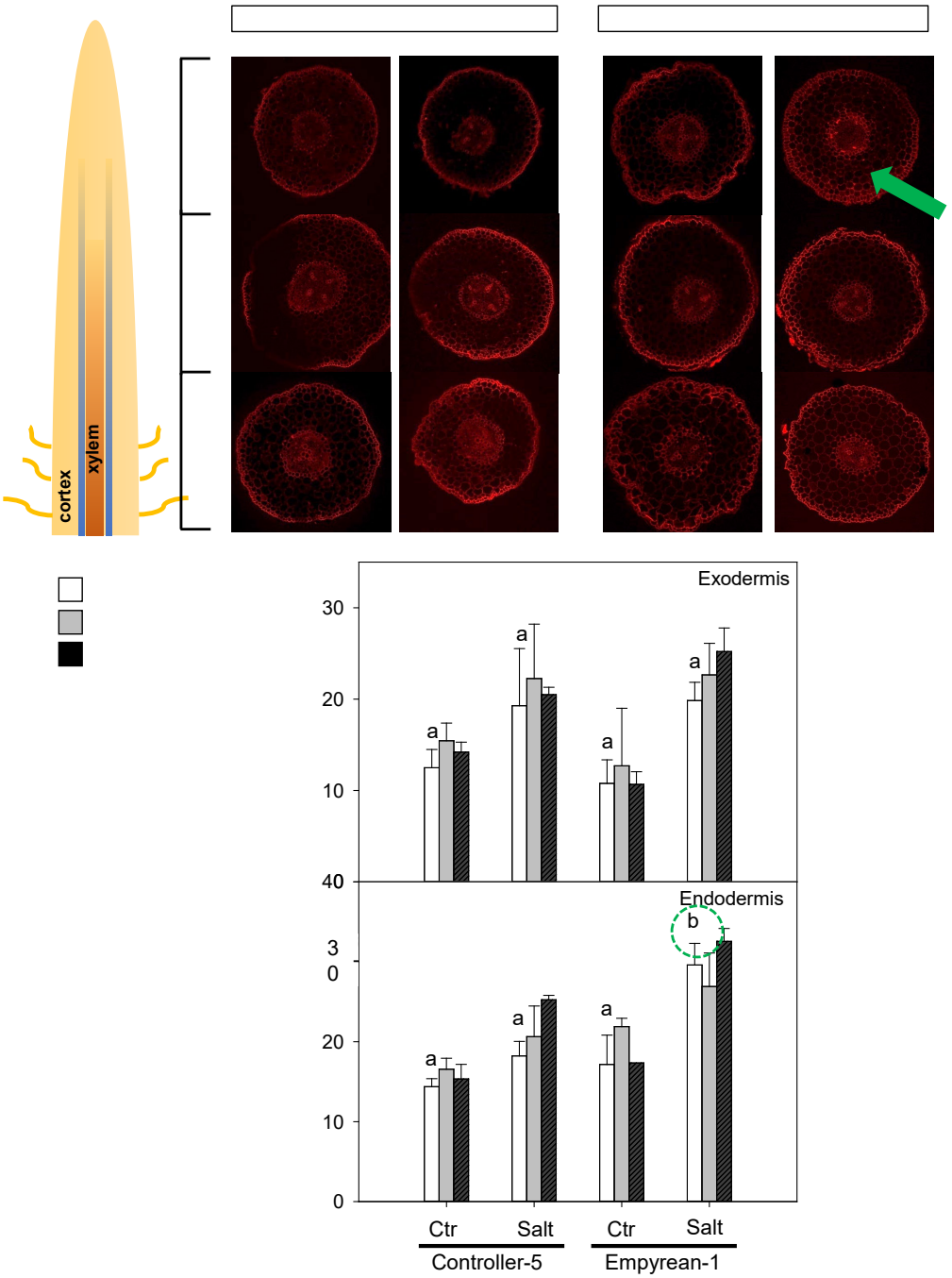
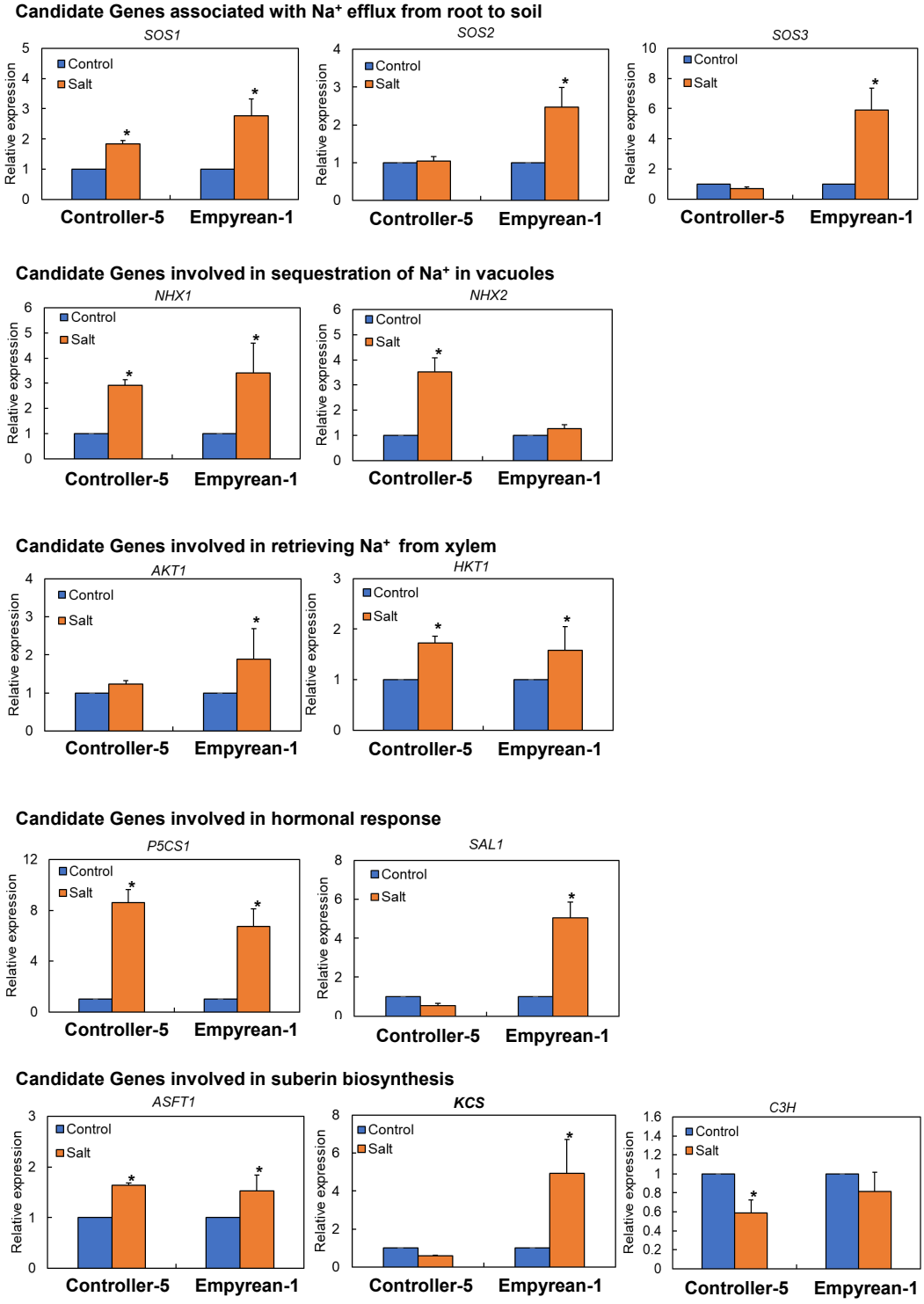


Figure 4. Candidate gene expression markers of rootstock salt tolerance tested in Controller-5 and Empyrean-1. After transferred to soil for two months, rootstocks were treated by 150 mM NaCl solution or water control, and RNA was extracted from root tips of one cm length for expression analysis. Asterisks indicate gene expression levels are significantly different in salt-treated samples and control ($p < 0.05$, n = 3).

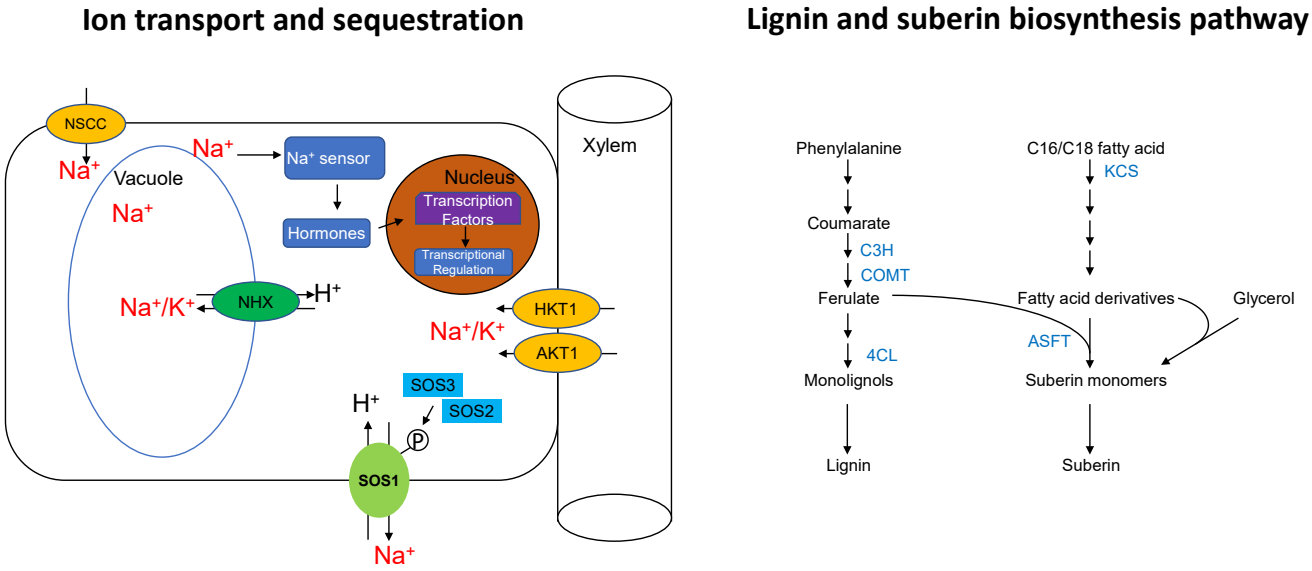


Result 2. Expression analysis of marker genes in pathways involved in cation transport, hormonal response and suberin deposition were altered differentially in the salt-tolerant genotypes.

Genes involved in *ion transport, hormonal response, and biopolymer biosynthesis* are recruited as candidate gene expression markers to identify the high salt tolerance rootstocks. Controller-5 and Empyrean-1, two rootstocks with extreme salt tolerance behaviors upon salt stress, are utilized to validate the efficiency of these candidate markers. After transferred to soil from root medium for two months, rootstocks were treated by 150 mM NaCl solution or water control for 14 days, and then root tips are washed and collected, and candidate gene expression levels in the meristematic zone of root is determined by qPCR (**Figure 4**). The functions of all candidate genes are briefly described here, and summarized in **Figure 5**. SOS1, SOS2, SOS3 (SALT OVERLY SENSITIVE 1/2/3) were identified from mutants of the model plant Arabidopsis by forward genetics. SOS1 encodes is a Na+/H+ antiporter responsible for extruding salt from cytoplasm to the apoplast, and SOS1 activity is induced by salt stress, mediated by the calcium-activated SOS2-SOS3 protein kinase complex (Shi et al., 2000; Qiu et al., 2002). Vacuolar NHX1 and NHX2 are also ion transporters, and they maintain the turgor pressure of plant cells through uptake of potassium into vacuoles (Barragán et al., 2012; Bassil et al., 2011). AKT and HKT, cation transporters both capable of exchanging H^+ with Na^+ or K^+, help to maintain plant ion homeostasis by controlling sodium entry into plant roots by selectively transport K^+ under salt stress (Rus et al., 2001; Apse and Blumwald, 2007). P5CS1 is delta1-pyrroline-5-carboxylate synthase that catalyzes the rate-limiting enzyme in the biosynthesis of proline, whose cellular levels is associated with osmotic homeostasis (Maghsoudi et al., 2018), and SAL1 encodes a 3'(2'),5'-bisphosphate nucleotidase involved in hormone signaling pathway responsive to abiotic stress like drought (Estavillo et al., 2011). P5CS1 and SAL1 are used as marker genes of hormone-responsive genes under abiotic stress.

Expression levels of lignin and suberin biosynthesis genes (C3H, ASFT1, KCS1, Rains et al. (2018)) are also investigated in this study, and the previous report indicates theses three genes are highly upregulated in root suberin biosynthesis in responsive to osmotic stress (Kreszies et al., 2019). KCS1 is involved in the synthesis of the fatty acid monomer of suberin, while ASFT1 catalyzes the transferring of the phenolic head towards the acyl-chain of suberin monomer (Fleck et al., 2011). C3H catalyzes the synthesis monolignol precursor as indicated in **Figure 5**. Our results indicated both *SOS1, NHX1, HKT1* are upregulated upon salt stress in Controller-5 and Empyrean-1, while *SOS2-SOS3* and *AKT1* are differentially upregulated only in Empyrean-1, which entails the cellular trait that Empyrean-1 has higher exclusion of salt, as higher expression of *SOS2-SOS3* implies higher activity of SOS1 to transport salt towards the outside of the cell. *NHX2*, the vacuolar antiporter responsible of sodium sequestration into the vacuole in exchange with potassium, is only upregulated in Controller-5, also matching the cellular trait of Controller-5 that high sodium sequestration into the vacuole is observed upon salt treatment. *SAL1* is only upregulated in Empyrean-1, while *P5CS1* is upregulated in both rootstocks, implying *SAL1* is likely associated with the sodium exclusion pathway. *C3H* does not significantly change upon salt treatment in Empyrean-1, which is similarly observed in Arabidopsis treated by salt stress (Chun et al., 2019). We have also recruited COMT1 and 4CL to further screen gene expression markers for genes involved in lignin biosynthesis. **Altogether, we have identified representative candidate genes contributing to salinity tolerance for almond rootstocks, that corroborate cellular and physiological behavior and will use them as expression markers to identify the elite almond rootstocks with both high salt tolerance and improved performance upon abiotic stress.**

Figure 5. Illustration of ion transport and sequestration in a root cell, and general biopolymer (suberin and lignin) biosynthesis pathway. Candidate genes illustrated in this figure are used in our gene expression marker screening.



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

Using almond rootstocks with extreme behavior towards salt stress, for example, Controller-5 and Empyrean-1, we can demonstrate the potency of our established system combining cellular and molecular approaches to evaluate salinity tolerance. Microscopy analysis of root cross-sections across a developmental gradient revealed distinct cellular and structural responses towards the salt stress (**Figure 1-3**). Our results show that **(I)** Increased sodium uptake is observed in the parenchymatic cells of salt-sensitive genotypes (such as Controller-5, **Figure 1**, **red arrow**). In contrast, salt-tolerant genotypes such as Empyrean-1 likely exclude sodium, as observed by reduced sodium detection in the cortex and enhanced staining in the epidermal layers. **(II)** Importantly, cellular layers such as the endodermis are reinforced with suberin to further exclude sodium from being transported to the vasculature. Furthermore, higher suberin deposition is observed in the endodermis layer of Empyrean-1 than Controller-5 upon salt stress (**Figure 2**, **green arrow**), pointing to a mechanism of salinity tolerance in Empyrean-1. **(III)** Expression analysis of marker genes in pathways involved in ion transport, hormonal response and suberin deposition were altered differentially in the salt-tolerant genotypes. (**Figure. 4**). The results suggest our **(I & II)** cellular/structural analyses combined with **(III)** expression analysis can qualitatively and quantitatively identify salt-resistant rootstocks at both cellular and molecular levels. We are currently summarizing our findings into a manuscript that will be submitted within the next two months for publication.

E. Materials and Methods

Plant Materials

Controller-5 (*P. salicina x P. persica*), and Empyrean-1 (*P. persica x P. davidiana*), are kindly provided by the Sierra Gold Nursery. 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. One cm section from root tips was used for RNA extraction, while 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.

Gene expression analysis

RNA was extracted from salt-treated and untreated root tips by Qiagen RNeasy kit (Qiagen, Hilden, Germany), and transcribed into cDNA by High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA), according to manufacturer's instructions. 2000 ng total RNA is used as starting material for cDNA synthesis, and SYBR™ Green PCR Master Mix (Applied Biosystem) was mixed with primers and cDNAs. Monitoring of SYBR green signal and analysis of the signals were performed on ABI 7300 Real-Time PCR System (Applied Biosystem). Relative quantification method using *ACTIN2* of *Prunus* as a reference gene was utilized for transcript abundance comparison.

Unforeseen development and challenges

Vacuole sequestration of sodium is observed in Controller-5 upon salt treatment, and Controller-5 is selected as one of the most vulnerable rootstocks toward salt stress. Vacuole sequestration is generally considered as a cellular trait associated with salt tolerance in the studies of annual plants (Apse and Blumwald, 2007; Yamaguchi and Blumwald, 2005). Mangrove, the famous coastal woody plants adapted to the saline conditions, is also found to have the cellular trait of vacuole sequestration of salt. However, mangrove has the feature of quickly expanding the size of the vacuole together with length of the root (Mimura et al., 2003), and implying the explanation of mangrove salt resistance mechanism cannot be easily applied to land plants facing the severe drought more frequently due to the climate change. Vacuoles of various plant species have different capacity of salt sequestration (Munns, 2005), and our result implies woody crop plants need more attention for their salt tolerant mechanism. Whether high levels of vacuole salt sequestration is associated with woody crops salt resistance needs to be investigated in more detail at both molecular level and physiological aspect. While our results at first appeared unexpected opened the discovery of new mechanisms that collectively contribute to salinity stress tolerance that are likely dominant in woody plant species. This opens avenues for application of those newly discovered correlations between cellular traits and salinity tolerance in rootstock breeding programs.

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 into a manuscript that will be submitted within the next 2 months for publication.

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