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Lessons from Nature: Studies on Mangrove Trees and Biotechnology

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Summary

Salinity is an abiotic stress that reduces the growth and productivity of crop plants worldwide. Mangrove trees such as *Avicennia officinalis* exhibit remarkable ability to grow in saline environment by means of various adaptations. Such adaptations include secretion of excess salt from the leaf surface via specialized salt glands, as well as the ability to exclude ~95% salt from seawater due to enhanced hydrophobic root barriers (suberin lamellae) in their roots. Certain cytochrome P450 enzymes play a key role in biosynthesis of suberin precursors. Knowledge gained by studying mangroves can be used for biotechnological applications. Thus, we identified several *CYTOCHROME P450* (*CYP*) genes that were induced by salt treatment in *A. officinalis* roots. Using appropriate *Arabidopsis* mutants, such as *atcyp94b1*, we characterized the function of *CYP94B1* gene in regulating suberin biosynthesis. The *atcyp94b1* mutant seedlings showed salt sensitivity with reduction in root elongation. When treated with salt, their roots exhibited reduced suberin lamellae and Casparian bands. Heterologous expression of the coding sequence of *A. officinalis CYP94B1* in *atcyp94b1* resulted in rescuing of the salt sensitive phenotypes, indicating the involvement of CYP94B1 enzyme in suberin biosynthesis. Additionally, we have expressed selected genes from the mangrove in rice, and the transgenic rice plants acquired higher salinity tolerance. These findings opened up additional strategies for salinity tolerant crop plants in the future. Our current efforts in these projects will be discussed.

INTRODUCTION

This is the text version of an invited talk delivered online at the IPPS Western Region, USA, 27-29 October 2020. I thank the organizers for giving me an opportunity to share some of our research findings at this forum. Our major research interests can be summarized under three categories: 1) Plant hormone signal transduction and control of plant development, with the focus on cytokinin and gibberellin signaling (Ravindran *et al.*, 2017). 2) Mechanism of salt tolerance in the mangrove tree species, namely, *Avicennia officinalis*. This project includes the mechanism of ultrafiltration of salt at the roots and salt secretion at the leaf via salt glands (Tan *et al.*, 2013). 3) The findings from the above studies are being applied for devising crop improvement strategies (biotechnological and molecular) using rice as the crop model. Also, other findings such as using molecular biology knowledge such as water and ion transporters from the mangrove for recombinant protein production and preparing biomimetic membranes incorporating aquaporin protein for water purification in the long-term. This talk will cover some of our findings from the second and third research areas of interest.

According to an estimate ~2,000 hectares/day of irrigated land is degraded by increased soil salinity across 75 countries. Every week, an area larger than Manhattan is lost due to salinity. Thus, currently an estimated ~62 million hectares (which corresponds to 20%) of the world's irrigated lands are affected by high salinity problems (up from 45 million hectares in 1990s). The corresponding lost crop value/year is $27 billion annually. According to the United Nations University Institute for Water, Environment and Health, "We can't afford not to restore the productivity of salt-affected lands". It is well established that salinity stress adversely affects plants in multiple fronts, e.g., genetic, physiological and morphological status of crop plants, ultimately leading to major losses in crop yield. Therefore, it is important to develop stress-tolerant varieties to ensure future food security.

One of the strategies to develop stress tolerance in plants is pathway engineering, which may be accomplished by biotechnology or by molecular breeding techniques. Specific plant hormone signaling pathways are important to alter the survival, growth and yield of plants. Cells perceive the stress-signals from the soil and environment and various endogenous factors then react in order to attempt to suitably modify the growth and physiology of plants (Munns and Tester, 2008). For example, salinity and drought stresses upset ion balance in the cells. One will need to use as much data as possible to attempt to modify the multitude of endogenous factors to remediate the stress. Among the possible strategies an important one is to try and modify genes that affect tolerance, e.g., using genomic breeding approaches. Thus, the use of 'big data' can help to derive strategies for breeding crops having higher tolerance to stresses in the years to come. Where will we be able to obtain such big data? Various omics data sets

(e.g., proteomics, genomics, transcriptomics, metabolomics) are becoming available for crop plants. Such information from naturally salt tolerant plants (e.g., mangroves) can be useful to confer stress tolerance to crop plants. As a brief background for the mangrove species, they naturally grow right in the seawater. They are able to thrive in such high salinity that would not permit growth of crop plants, because mangroves possess multiple adaptations, including propagules that germinate while still attached to the parent plants so that when they are shed, they can readily establish themselves as seedling on the muddy floor, or will be carried by ocean currents to long distances before establishing themselves on distant shores. Seedlings grown from propagules in the greenhouse are shown in Figure 1.

Figure 1. Greenhouse-grown seedlings of *Avicennia officinalis*. Propagules collected from the trees were sown in the soil and allowed to grow for about 2 months.

Mangroves are highly recalcitrant species and thus far they have not been successfully propagated by tissue culture. Natural propagation occurs by means of fruits/propagules, which are usually viviparous (germinate on the mother plant as mentioned above). One of our earlier studies had revealed that several species of mangroves have propagules that can float and they are transported to long distances in sea currents.

Desalination in mangroves via ultrafiltration at the roots

Plants inhabiting a saline environment literally lead a 'Life in a pickle'! They are under constant stress of high solute (salt) soil environment and have to deal with a resultant water-deficit condition. This limits the growth and survival of most plants, but mangroves (that are categorized as halophytes) thrive in marine and estuarine shorelines. They accomplish this because of their special adaptations, including, 1) Their roots carry out ultrafiltration of salt (prevent salt uptake); and 2) some mangroves can secrete excess salts via specialized salt glands on leaves (Figure 2). These represent a form of natural desalination process at work! Salt glands are microscopic structures located mainly on leaf surfaces. And, the roots of mangroves are able to carry out ultrafiltration of salt. Therefore, we are interested in understanding how salt glands and roots work. These insights can help in developing stress tolerant plants in the long-term.

Figure 2. Two-month-old *Avicennia officinalis* seedlings were transferred to pots with sand and allowed to adapt for two days before treating with 500 mM NaCl. Salt secretion could be visualized as white salt crystals on the leaves after two weeks of salt treatment.

Ultrafiltration at the mangrove roots represents the first physiological defense for plants growing in saline soil. This refers to exclusion of salt prior to uptake of water into the xylem. Our experimental results have shown that *Avicennia* roots are able to exclude ~95% of the salt from seawater because of the presence of physical barriers, namely, suberin lamellae in specific root cell layers (e.g., endodermis, exodermis). We have published several papers reporting that increased suberization in response to salt treatment helps in efficient root filtration of salt, and that a series of genes are differentially expressed in response to salt (Krishnamurthy *et al.*, 2014a; Krishnamurthy *et al.*, 2017; Krishnamurthy *et al.*, 2014b).

Unravelling the mechanism underlying salt uptake at the roots of mangroves

Control of growth needs plant water relations to be properly regulated. Ion transporters are involved in regulating water relations as well as uptake of nutrients (e.g., K^+, NO_3^-). Other genes that were identified include those that encode membrane proteins, such as aquaporins and ion channels involved in salt uptake and secretion. We are studying several genes in this category, and some of these genes were used in our biotechnological efforts for generating salt tolerant plants

(Krishnamurthy et al., 2019; Rajappa *et al.*, 2020). However, due to time constraints, these findings will not be discussed further in this talk.

Regulation of apoplastic barrier formation and identification of the molecular mechanism

We will discuss some details from our attempts at understanding the mechanism of salt ultrafiltration. We discovered several genes encoding for Cytochrome P450 monooxygenases as being specifically induced by salt based on a differential gene expression analysis using transcriptomics, followed by gene function analysis.

When we subjected our RNAseq data (transcriptomics) to functional analysis using the KEGG pathway, we discovered that several genes for suberin biosynthesis are differentially regulated by salt treatment in the mangrove roots. It was previously known that enzymes, such as, CYP94A1, CYP86B1 are required for biosynthesis of suberin precursors.

We carried out most of the molecular biological and transgenic plant studies using *Arabidopsis thaliana* as the experimental species. This is due to the fact that: 1) homologs of most of these genes can be identified in *Arabidopsis*, 2) relevant mutants in the genes of interest are available in this species, and 3) mangrove trees cannot be subjected to transgenic plant production and genetic analyses. Therefore, other than simple gene expression analysis in *Avicennia*, the bulk of the results are from *Arabidopsis* studies. The key findings are presented in summary form in the sections below. Details have just been published (Krishnamurthy et al., 2020) and readers may wish to refer to this publication.

Our results showed that *CYP94B1* gene is consistently upregulated by salt treatment, especially in the roots of *Avicennia* and *Arabidopsis*. The enzyme encoded by this gene, namely, CYP94B1 catalyzes synthesis of omega-hydroxy fatty acids, which are suberin monomers. Detailed gene expression analysis showed that *AtCYP94B1* is preferentially expressed in the endodermis under salt treatment. We obtained the relevant mutant *atcyp94b1* from the stock center. This mutant exhibits salt sensitive phenotype, as illustrated by inhibition of seedling root elongation when grown on nutrient medium with 75 mM NaCl. This phenotype could be rescued by genetic complementation accomplished by trans-genically expressing either *AoCYP94B1* or *AtCYP94B1* gene. Transgenic lines expressing mangrove CYP exhibit better growth under NaCl conditions.

Because of the involvement of the gene in regulating suberin biosynthesis, we examined suberin content in the various seedlings using confocal laser scanning microscopy. Suberin deposition was significantly reduced in the mutants. But, this was restored to normal levels when the *CYP* gene was expressed in the roots of *atcyp94b1* mutant. We showed that the reduced suberin in the roots of *cyp94b1* causes more uptake of salt as indicated by the loading of the tracer apoplastic dye (FDA) to pass through the endodermis and enter the pericycle cells. The transgenic lines showed that along with the restoration of suberin in the endodermis cells, the uptake of the tracer (indicative of salt up-take) was also significantly reduced. This is

the ultrafiltration mechanism that confers enhanced salt tolerance to the plants.

Can such salt tolerance be transferred to crop plants such as rice?

We were interested to test if a similar salt tolerance mechanism can be conferred to rice seedlings by introducing the *CYP* genes. Accordingly, we generated transgenic rice plants expressing *AoCYP94B1* gene and our results showed that these plants have increased salt tolerance as evidenced by good growth under 100 mM NaCl treatment. We also showed that such salt treatment to normal wild type plants leads to stunted growth of seedlings. The transgenic rice seedlings exhibited better seedling height and better recovery growth after 3 weeks of salt treatment.

One-month-old *pUbi::AoCYP94B1* rice seedlings were treated with salt for 21 days and then allowed to recover by removing the salt in the water (Figure 3 and Table 1).

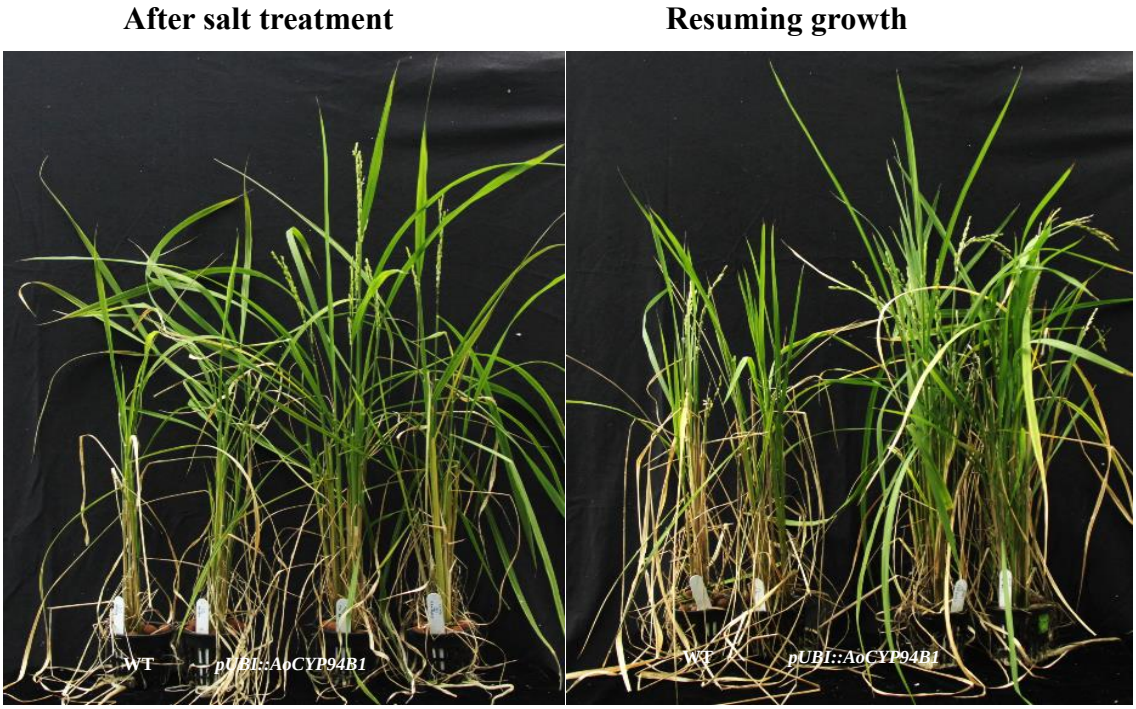

Figure 3. Four-week-old hydroponically grown wild-type and transgenic *pUBI::AoCYP94B1* rice plants after 21 days of 100 mM NaCl treatment and an additional 10 days of recovery growth without NaCl.

Table 1. Survival rates of wild-type and transgenic rice plants (from Fig 3) after salt treatment and recovery.

Genotype	Survival rate (%) ± SD
WT	36.7 ± 6.3
pUBI::AoCYP94B1	74.9 ± 11.6

More importantly, we were able to show that the transgenic rice roots expressing the mangrove *CYP* gene (*AoCYP94B1*) have increased suberin deposition in both endo-dermis and exodermis. Therefore, the higher salinity tolerance of these transgenic plants correlates with increased suberin deposition in their roots, similar to our observations in *Arabidopsis*. These results collectively show that salt tolerance trait can be conferred to rice and other plant species by introducing *CYP94B1*.

What is the molecular regulatory factor of AtCYP94B1 gene in Arabidopsis?

Lastly, we attempted to determine how *AtCYP94B1* gene is regulated in *Arabidopsis*. A bioinformatic analysis showed that *AtCYP94B1* promoter contains several stress related transcription factor (TF) binding sites, such as WRKY. We found that WRKY33 is involved in regulating the gene expression. Coincidentally, salt induces *WRKY33* gene similar to the induction of *CYP94B1* gene. Also, we obtained data to show that *AtCYP94B1* expression is suppressed in *atwrky33* mutants, and that AtWRKY33 directly binds to *AtCYP94B1* gene promoter and induces its expression (Y1H, ChIP, Luciferase assays) confirming this molecular regulation process.

CONCLUSION

- Ultrafiltration in the mangrove *Avicennia* roots helps to exclude ~95% salt from seawater.
- Endodermis and exodermis show increased suberization in response to salt treatment, which help in ultrafiltration of salt at the roots of the mangrove tree.
- Several *Cytochrome P450* (regulating biosynthesis of ω-hydroxylases involved in suberin biosynthesis) and *WRKY* genes are upregulated by salt (transcriptomics data).
- *AoCYP94B1* (also *AtCYP94B1*) rescues salt sensitive and reduced suberin phenotypes of the *Arabidopsis atcyp94b1* mutant roots.
- WRKY33 transcription factor that is co-induced by salt, helps to regulate *AtCYP94B1* gene.
- Transgenic rice plants expressing mangrove At*CYP94B1* exhibit increased suberin deposition and enhanced salinity tolerance.
- Understanding the physiological and molecular mechanisms of stress tolerance in mangroves (that have special adaptations to survive in the saline environment) may help to generate future crop plant varieties with higher abiotic stress tolerance.
- We need to combine gene technologies with novel propagation and crop management techniques to enable plants to grow well with reduced inputs and tolerate serious abiotic stresses.

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