The role of WRKY15 in xylem development and salt response in *Arabidopsis thaliana*

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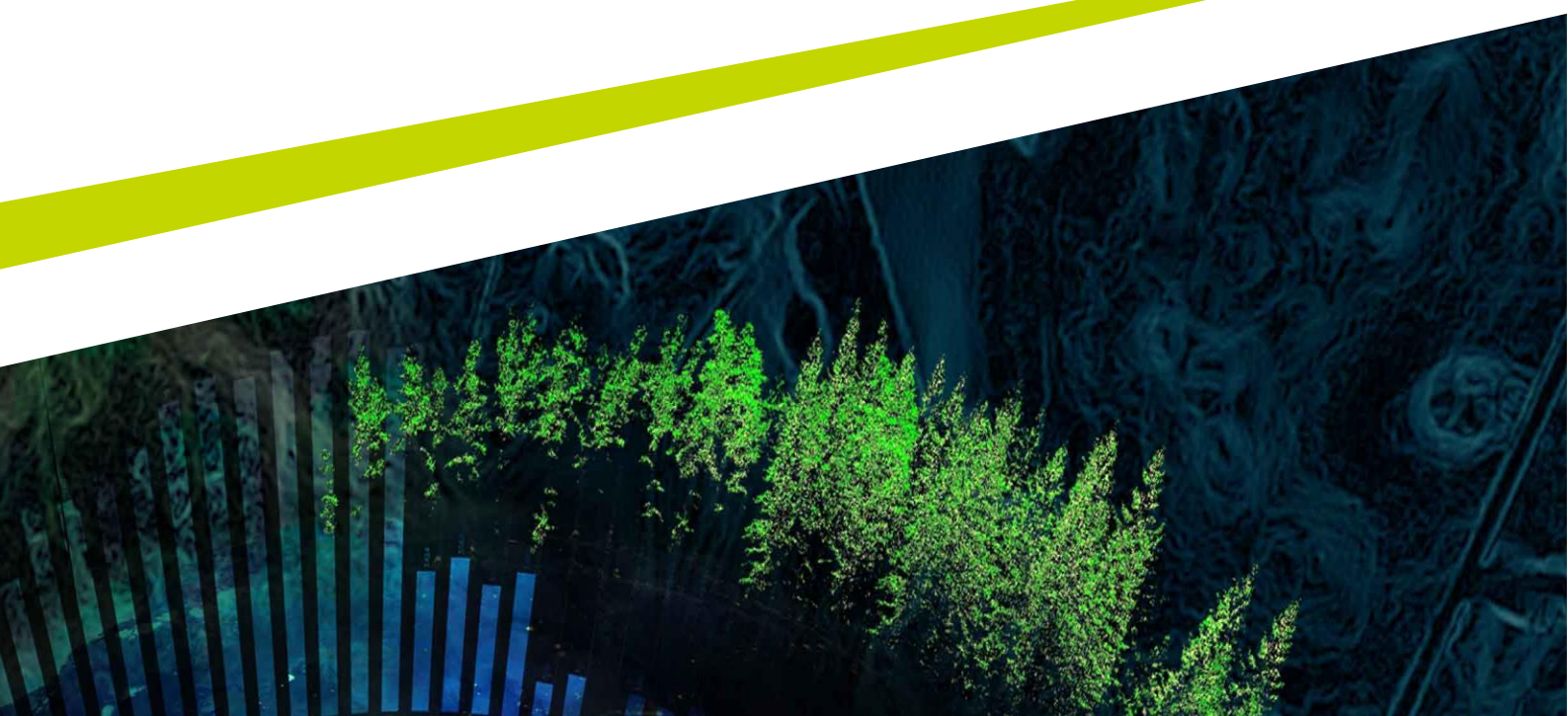

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Abstract

Xylem is a water-conducting tissue in vascular plants, that absorbs water from the roots and distributes it throughout the compartments, providing the plant with essential water and nutrients for its cellular processes. The xylem, divided into protoxylem and metaxylem, develops differently according to the various abiotic circumstances, in which transcription factors have a crucial role.

The transcription factor WRKY15 plays a part in the negative regulation of protoxylem differentiation and studies have shown that WRKY15 indirectly suppresses the master transcriptional regulator VND7 (VASCULAR RELATED NAC DOMAIN7), which has been observed to promote protoxylem differentiation. The transcription factor VND6 (VASCULAR RELATED NAC DOMAIN6) also plays a role in xylem differentiation, but instead as a master regulator in the metaxylem vessels.

The aim of this study is to investigate the role of WRKY15 transcription factor in xylem development and salt response in the model plant *Arabidopsis thaliana*. In this thesis, I am looking at the expression of VND6, VND7, and WRKY15 in plants subjected to high salinity and water deficiency, and the connection between them. In order to investigate this aspect, analysis was done using qPCR and GUS staining. Results from the experiments suggested that the increased expression of WRKY15 is an early response to salinity, and occurs in different locations in the root based on the environment.

Keywords: WRKY15, VND, xylem, salinity, water availability

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Abbreviations

ABA	Abscisic acid
SCW	Secondary cell wall
TE	Tracheary elements
VND1-6	Vascular-related NAC domain
WRKY15	WRKY- transcription factor

1. Water uptake

1.1 Structure and function of xylem

The main function of the xylem is to transport water and low-weight molecules such as minerals to different compartments, and is crucial for life on land for vascular plants (Kamon & Ohtani 2021). How the xylem is developed is a complex yet fascinating mechanism, and is influenced by many abiotic factors, such as gravity, salinity, water resources, nutrients, and access to light (Oda 2015). Roots anchored in the soil absorb available water, and the water-conducting tissue distributes it throughout the plant from the roots to the leaves. Water is necessary for photosynthesis and other cellular processes, making well functional xylem essential for plants to thrive.

Before looking into how the xylem responds to different abiotic circumstances, it is important to understand the process of how the xylem is developed in the plant. There are two types of xylem: 1) the primary xylem, derived from procambium cells that are generated in the apical meristem between the primary xylem and the primary phloem (Schuetz et al. 2013); and 2), the secondary xylem, produced in the lateral meristem, also known as cambium (Schuetz et al. 2013). The procambium, formed by parallel strands of procambial cells, gives rise to the cambium, at which the xylem precursor cells are formed from cambial stem cells (Schuetz et al. 2013). The xylem precursor cells undergo a cell fate, and differentiate into distinct xylem cell types; protoxylem tracheary elements, metaxylem tracheary elements, fibre or parenchyma cells (Schuetz et al. 2013). A crucial function of the vascular cambium is to generate differentiated cells from the meristematic cells, an essential process for the maturing of the plant (Schuetz et al. 2013).

The two types of xylem are composed of tracheary elements; hollow tubular vessels formed by dead cells (Ge et al. 2020) with the function to transport water and minerals. The formation process includes cell expansion, programmed cell death, and secondary cell wall formation (Kamon & Ohtani 2021), and is referred to as xylem differentiation. VND transcription factors are activated after cell elongation, and induce the process of programmed cell death and the formation of secondary cell walls (Kamon & Ohtani 2021).

The function of VND transcription factors is to regulate downstream genes and activate xylem-specific enzymes, for example nucleases and proteases. These enzymes play an important role in differentiation, as they are involved in programmed cell death (Kamon & Ohtani 2021). In this process, a perforation forms within the cell by intracellular degradation. The primary cell walls positioned between cells degrade, allowing water to flow throughout the vessels. Apart from this, secondary cell walls are developed as components associated with cortical microtubules forms an array across the primary cell wall, and have the function to synthesize the compounds lignin, cellulose and hemicellulose. Lignin has hydrophobic characteristics and is giving strength to the vessel, making lignin an incredibly important compound in xylem vessels (Kamon & Ohtani 2021).

The transdifferation of meristematic stem cells into protoxylem forms annular or spiral patterned secondary cell walls (Ge et al. 2020; Ramachandran et al. 2018). This type of secondary cell walls differs significantly from the metaxylem, which forms reticulate or pitted secondary cell walls (Ramachandran et al. 2018)

1.2 Water availability and salinity

During normal circumstances, water is transported from the soil, through the roots, and into the xylem of vascular plants, to be distributed in the plant. The water flow is driven by transpiration, as the plant opens its stomata and water releases from the leaves (Grip & Rodhe 2016). This process creates a water potential, which allows water to move from a high water content to a lower water content.

Water is bound to the soil both adsorptively and with capillary force (Grip & Rodhe 2016). A high amount of water in the soil means that there is a lot of available water to transport with the water potential. In conditions with limited water resources, the water molecules are bound more tightly to the soil, and roots may not absorb a sufficient amount of water.

The plants may temporarily adjust the transpiration at which water is released from the leaves, by closing its stomata (Grip & Rodhe 2016). This can only function to a certain extent, and the plant will be negatively affected if the transpiration is larger than the water uptake for a long period of time (Grip & Rodhe 2016).

In the qPCR experiment in this project, mannitol was used to simulate water stress as it lowers the water potential (Nikonorova et al. 2018), meaning plants are obstructed to take up water.

The osmotic effect of mannitol may affect the plant's availability to take up a sufficient amount of water. Likewise, salinity shares the osmotic characteristics with mannitol. The concentration of salt also takes part in this. A high concentration can lead to ion toxicity for the plant, resulting in negatively affected photosynthesis, respiration, ion uptake, and membrane integrity (Augstein & Carlsbecker 2022).

2. Xylem phenotypes upon salt and water stress

Looking at the tendencies of xylem development in saline environments suggests that roots, both primal and lateral, stop growing. It is also observed that the plants respond with adjustments, where roots continue to grow temporarily and mechanisms for salt tolerance are activated (Augstein & Carlsbecker 2022).

An effect of drought in plants with large-sized xylem vessels is embolism (Augstein 2022), air bubbles blocking the vessels. It is suggested that plants with fewer, but larger xylem vessels suffer a higher risk of drought-induced embolism than plants with a smaller xylem vessel diameter (Levionnois et al. 2021).
As plants are plastic, they are suggested to respond to low soil water availability with extra, smaller xylem strands to withstand the drought, and often results in thicker vessel walls (Augstein 2022).

Gaps in the protoxylem, also called discontinuous xylem, may occur as a response to salt and are suggested as a way for the plant to adapt to the environment (Augstein 2022). Observations have shown that the formation of gaps appears when the plant first is subjected to salt, and not later on, and that the rate of protoxylem gaps is concentration-dependent (Augstein 2022).

Duan et al. (2013) found that growth in lateral roots in *Arabidopsis thaliana* was negatively affected upon salt stress, suggesting that high salinity leads to a lower number of lateral roots. Results in this study indicate that the stress hormone abscisic acid functions as a suppressor of lateral root growth (Duan et al. 2013). The functions of ABA in xylem development were also studied by Ramachandran et al. (2018), showing that a lack of the hormone leads to discontinuous xylem strands, or a decrease in xylem strands.

3. The role of ABA and transcription factors in xylem development

3.1 ABA – the stress hormone

The plant hormone abscisic acid (ABA) is referred to as a stress hormone, for its crucial function of controlling the downstream stress responses (Augstein 2022). The hormone responds to both abiotic stresses, including drought, salinity, cold, and flooding, and biotic stresses, for example pathogens (Shu et al. 2018).

ABA plays an important role in how the xylem develops, and how plants handle water deficiency. In limited water resources, ABA signalling is important for sufficient water uptake as it modifies xylem development (Ramachandran et al. 2018).

The importance of ABA in plants with water stress has been studied, finding that endodermal ABA signalling increased the number of xylem strands in response to water stress (Ramachandran et al. 2018), by positively regulating genes responsible for VND transcription factors (Ramachandran et al. 2021). Plants lacking ABA signalling showed discontinuous xylem strands. Treating the plants with external ABA counteracted the effects and repaired the strands (Ramachandran et al. 2018).

Studies have shown that ABA has a positive effect on the differentiation of metaxylem to protoxylem, by inducing the expression of microRNA165 transcription factors (Augstein 2022). Activation of microRNA165 downregulates the HD-ZIPIII transcription factor (Ramachandran et al. 2021; Augstein 2022), which causes the shift in the xylem and forms extra strands of protoxylem (Augstein 2022).

3.2 Transcription factors

3.2.1 NAC and MYB

The formation of the secondary cell wall in xylem vessels is highly dependent on different transcription factors, such as members of the NAC transcription factor family and their downstream regulators, for example, members of the MYB transcription factor family (Ge et al. 2020). Within the NAC family, VND1-7 and NST1-3 are important transcription factors in the process of protoxylem differentiation (Ge et al. 2020).

The production of secondary cell walls is induced by VND transcription factors after cell elongation in protoxylem (Kamon & Ohtani 2021). Tracheary element differentiation is negatively regulated by the transcription factor WRKY15's indirect role on VND7, meaning overexpression of WRKY15 can suppress protoxylem differentiation (Ge et al. 2020).

Studies have shown that overexpression of both VND6 and VND7 induces the transdifferentiation of xylem vessels, as well as increases the thickness of the secondary cell walls (Yamaguchi et al. 2010). They both play a part in regulating the expression of genes that are involved in programmed cell death and the formation of secondary cell walls in xylem vessels (Yamaguchi et al. 2010).

Although VND6 and VND7 both share this function, they have some differences. VND6 acts as a master regulator in the metaxylem. VND7, on the other hand, is important for protoxylem development (Augstein 2022). While VND6 has been shown to induce differentiation into metaxylem-like vessels, VND7 induced differentiation into protoxylem-like vessels (Kubo et al. 2005). Based on observations that a lower amount of protoxylem gaps were formed in saline conditions compared to mock, Augstein and Carlsbecker (2022) suggested that VND6 is playing a part in forming protoxylem gaps in saline environments. However, it is suggested that other factors may also play a part in the formation of the gaps.

3.2.2 WRKY15

Plant growth, cell elongation, and lignification of secondary cell walls are highly dependent on the regulatory transcription factor WRKY15. An overexpression of WKRY15 possibly allows cells to elongate, as it negatively regulates the tracheary element differentiation of the cells (Ge et al. 2020). Studies have shown that overexpression of WRKY15 suppresses the protoxylem differentiation (Ge et al. 2020).

The protoxylem vessels have been observed to be discontinuous under WRKY15 overexpression, and additionally, the spiral-patterned secondary cell wall has been shown to be thinner (Ge et al. 2020).
Ge et al. (2020)'s studied the interplay between WRKY15 and VND7, where dominant-negative WRKY15-EAR expression induced extra protoxylem vessels and lignification of the secondary cell wall, and they suggested that WRKY15 has a role as a negative regulator of the transcription factor VND7. Moreover, protoxylem gaps were observed upon WRKY15 overexpression, due to the suppression of the tracheary elements (Ge et al. 2020).

4. WRKY15 and VND7 as a regulator of xylem development

The hypothesis in this study is that overexpression of WRKY15 leads to discontinuous protoxylem strands and the formation of protoxylem gaps. Moreover, overexpression of the VND7 transcription factor leads to an increased amount of protoxylem strands. WRKY15, along with VND6, is expected to have an early response to salt, while VND7 is expected to have a late salt response, inducing additional protoxylem gaps. Furthermore, protoxylem gaps are expected to occur upon expression of VND6 (Augstein & Carlsbecker 2022).

I propose that the correlation between WRKY15 and VND-transcription factors plays an essential role in controlling xylem development. Based on findings from Ge et. al (2020), WRKY15 and VND7 are expected to function as each other's contrasts.

Overall, I anticipate that this study will provide insights into the regulatory mechanisms, and the functional relationships between WRKY15, VND6, and VND7 and their role in the plant's response to water deficiency and salt.

5. Method

5.1 Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) Colombia-0 (Col-0) was used for qPCR analysis. For GUS staining, seeds from two separate lines of Arabidopsis (*Arabidopsis thaliana*) *pWRKY15:eYFP-GUS* (no. 3 and no. 4) were used.
Seeds were sterilized with 70% ethanol for 30 minutes, 95% ethanol for 2 minutes, and rinsed four times with sterile water for 2 minutes, then stored in the fridge up until use. For both qPCR and GUS staining, seedlings were cultivated vertically in a growth chamber in long-day conditions with cycles of 16 hours of light and 8 hours of darkness.

In the qPCR experiment, seeds were placed in agar plates containing ½ MS-medium (Murashige and Skoog) and 1% plant agar. After cultivating the seedlings in a growth chamber for 5 days, they were transferred using mesh, onto new agar plates prepared with MS-medium and supplemented treatment; mock (control), 140mM NaCl, or 280mM mannitol. The plates were then put in a growth chamber for 6 or 24 hours.

For the GUS staining experiment, agar plates were prepared with MS-medium and treatments; mock and 140mM NaCl. Sterilized seeds from *pWRY15:eYFP-GUS* (no. 3 and no. 4) were placed onto mesh on the solidified plates, and followed a program for four different treatments (Figure 1), and transferred two times.

5.2 qPCR analysis on Col-0

RNA extraction

To extract RNA, tubes were prepared with 10 glass beads each. A mix of 450 µl RLT-buffer-β-mercaptoethanol from RNeasy Plant Mini Kit from Qiagen was added to each tube. The tubes were stored in the fridge until use. Roots from each treatment were cut below the hypocotyl and collected into one tube each, and immediately frozen in liquid nitrogen before being stored in a -80°C freezer.

The samples were homogenized by first thawing on ice, then ground twice for 30 seconds at speed 30 with the TissueLyser. RNA was extracted from the lysate by following the RNAeasy Plant Mini Kit from the Qiagen protocol.

cDNA synthase for mRNA

To synthesize cDNA for mRNA, an iScript cDNA synthesis kit from BioRad was used. A total of 15 µl RNA and nuclease-free water were mixed with 5 µl master mix (iScript reaction mix and iScript reverse transcriptase) for a total reaction volume of 20 µl. The samples were incubated with a thermal cycler at 25℃ for 5 minutes, 42℃ for 30 minutes, 85℃ for 5 minutes, and then hold at 12℃.

qPCR analysis

Four qPCR 96-well plates were prepared: ATP1 (reference gene), WRKY15, VND6, and VND7. A master mix was made using 350 µl Maxima SYBR Green, 21 µl primer (containing 10 µl FP, 10 µl RP and 80 µl nuclease-free water), and 154 µl nuclease-free water. 2,5 µl of DNA template and 7,5 µl master mix were added into each well.

The samples were amplified. Analysis was done in Excel using the expression values yielded from the qPCR software, to calculate the amount of DNA in each sample.

Electrophoresis of RNA

To confirm the presence of RNA in the samples, electrophoresis was done using 1% agarose gel.

5.3 GUS staining

3-day-old seedlings placed on mesh were transferred from the four original mock plates to the second treatment (Figure 1). Mesh 1 and 2 were transferred to a new plate containing mock, and mesh 3 and 4 were transferred to a plate containing 140 mM NaCl, and then put to light for 3 days in a growth chamber.
When the seedlings were 6 days old, the mesh was transferred to the third treatment. Mesh 1 and 3 were transferred to mock. Mesh 2 and 4 were transferred to NaCl. The plates were then put to light for 4 days in a growth chamber.

Each plate was photographed for measuring the roots using Fiji/ImageJ software. The seedlings were collected from each plate, and incubated in 95% ethanol for 15 minutes on ice. The tissue was then rinsed with water. Substrate solution were added to the tissue. The samples were incubated at 37°C for 2 hours. The chlorophyll was removed with 95% ethanol. The ethanol was changed until no chlorophyll was visible and then rinsed and stored in water.
The seedlings were mounted on slides in chloralhydrate solution, and then visually analyzed in an axioscope. The number of seedlings with no expression was counted, and xylem phenotypes were noted for further analysis.
Pictures were taken near the hypocotyl, on the maturation zone, and on the root tip.

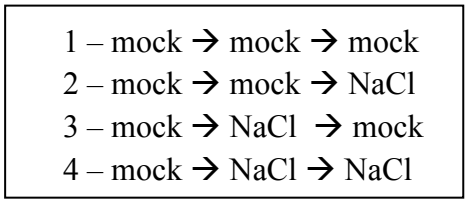

Figure 1 *Program for the four different treatments. Mesh was transferred between treatments following the program. The 3-day-old seedlings were transferred to the first treatment. The seedlings were transferred to the second treatment as 6-day-old.*

6. Results

6.1 qPCR analysis on Col-0

To assess the expression of the *WRKY15* transcription factor under varied conditions, a qPCR was conducted on 5-day-old Arabidopsis roots subjected to two different treatments: 140 mM NaCl and 280 mM mannitol. To confirm the presence of RNA, electrophoresis was done on an agarose gel. Bands were observed for all samples, proving a successful RNA extraction (Figure 2).

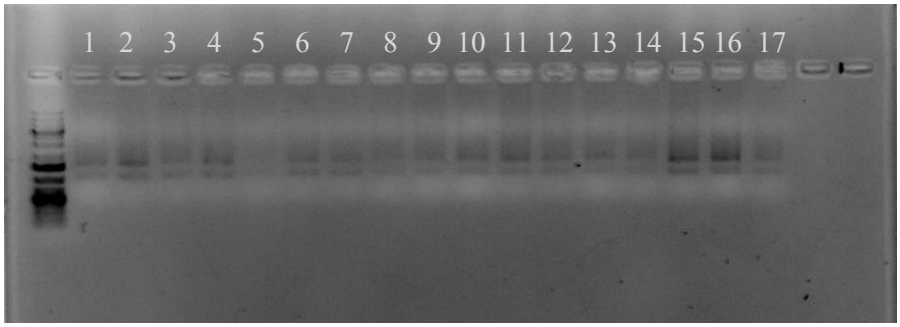

Figure 2 *Electrophoresis of RNA on agarose gel. Samples of RNA used in electrophoresis are the same as those used in qPCR. For 6h treatment, well 1-3 represents mock. Well 4-5 respresents NaCl. Well 6-8 represents mannitol. For 24h treatment, well 9-11 represents mock. Well 12-14 represents NaCl. Well 15-17 represents mannitol.*

In this experiment, I found that *WRKY15* expression was vastly upregulated in both NaCl and mannitol 6h treatments compared to mock, and results for the 24h treatments showed similar expression as mock (Figure 3). This suggests that *WRKY15* is regulated by both salt and water deficiency and has an early response to salt.

For *VND6* and *VND7*, the expression was upregulated in NaCl treatments, mostly in the 6h treatment. In mannitol treatment, *VND6* had little upregulation. For *VND7*, the expression was upregulated in both 6h and 24h mannitol treatments. Overall, the qPCR experiment showed dynamical changes in *VND6*, *VND7* and *WRKY15* expression upon salt and mannitol treatment.

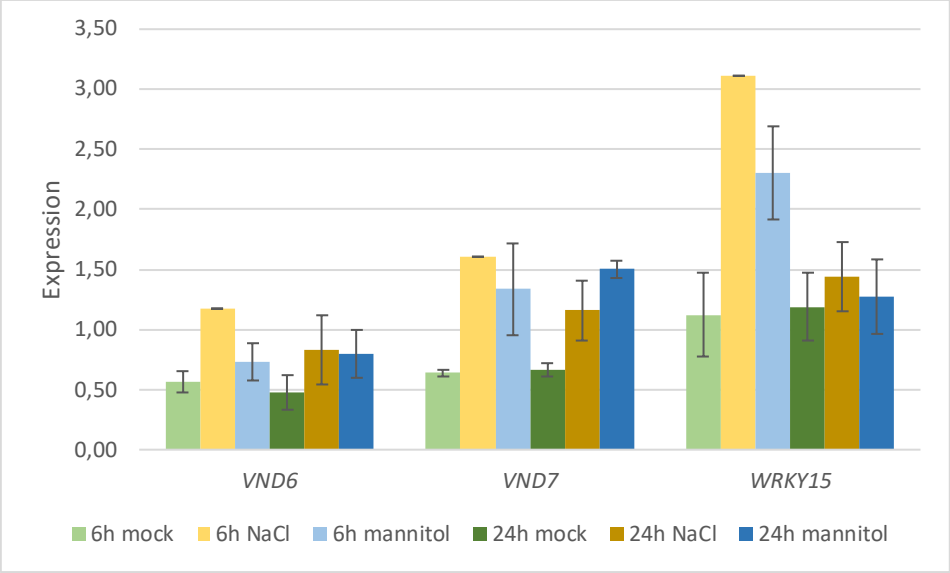

Figure 3 *Gene expression overview for VND6, VND7 and WRKY15 in 5-day-old Col-0, Arabidopsis thaliana, treated for 6 or 24 hours in mock, 140mM NaCl, or 280mM mannitol.*

6.2 GUS staining

In addition to looking at the expression values using qPCR, it is of interest to see where in the root the expression is located. Using the GUS staining method gives an idea of where *WRKY15* has a higher concentration and makes it possible to interpret the effects of the treatments. Two lines of Arabidopsis (*Arabidopsis thaliana*) *pWRKY15:eYFP-GUS* (line 3 and line 4) were used for this experiment, cultivated in four different treatments containing mock or salt (Figure 1). Blue stain appeared in the seedlings indicating an expression of the transcription factor *WRKY15*.

Measuring of root length showed clear similarities between the two lines (Figure 4). The mock-mock treated plants showed an average root length of about 3 cm. The mock-NaCl treated plants exhibited an average root length between 1,5 and 2 cm. NaCl-mock-treated plants had an average root length of about 2 cm. NaCl-NaCl treated plants had an average root length of about 1,5 cm. In general, salt-treated plants had limited root growth compared to mock, suggesting that salt may have a restricting effect on the elongating process of the cells.

Based on the similarities comparing the two lines, a conclusion can be drawn that the seedlings from both lines have responded equally to the treatments.

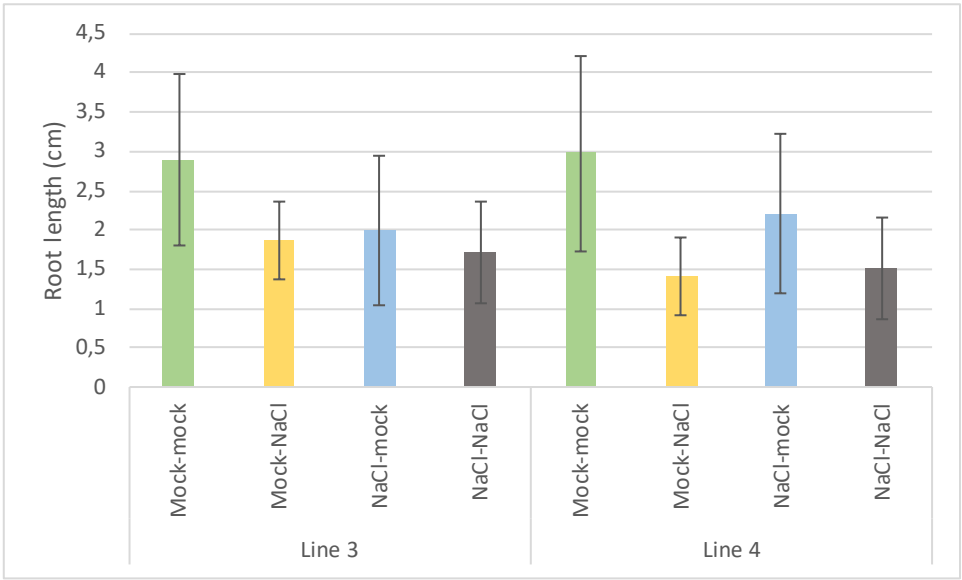

Figure 4 *Mean root length (cm) for each treatment and line, in Arabidopsis thaliana, pWRKY15:eYFP-GUS no.3 and no.4. Root length were measured using Fiji/ImageJ software on all seedlings on each plate, and a mean value was calculated.*

Looking at where the expression was positioned, both line 3 and line 4 showed similar results in expression in every treatment. The treatments had clear differences regarding expression.
Mock-mock treated plants had for the most a consistent expression in both lines, although the expression closer to the hypocotyl was shown in the center part of the xylem, and further down the maturation zone, the expression shifted to the outer part. Between the locations of expression, the xylem did not show expression.
Mock-NaCl treated plants showed expression inconsistently and mostly in lateral roots, where line 4 showed somewhat weaker staining than line 3.
NaCl-mock treated plants showed expression inconsistently in protoxylem.
NaCl-NaCl treatment showed some expression below the hypocotyl, and along the root, protoxylem gaps were visual. In general, salt-treated plants tended to have discontinuous xylem in the root. Additionally, they tended to have more prominent staining in the lateral roots.

In line 3 (Figure 5), Mock-mock treated plants (A, E, I) stained strongly below the hypocotyl (A), and had a constant decrease in color in the maturation zone (E). Root tip (I) had no expression.
Mock-NaCl treated plants (B, F, J) had weak coloring below the hypocotyl (B), strong expression in lateral roots (F), and some expression in the root tip (J).
NaCl-mock treated plants (C, G, K) had weak expression in the hypocotyl, mostly in lateral roots (C). Inconsistently expressed genes in metaxylem (G) and no expression whatsoever in root tips (K).
NaCl-NaCl treated plants (D, H, L) showed no expression in the hypocotyl (D), inconsistent staining (H), and patches of stain in the root tips (L).

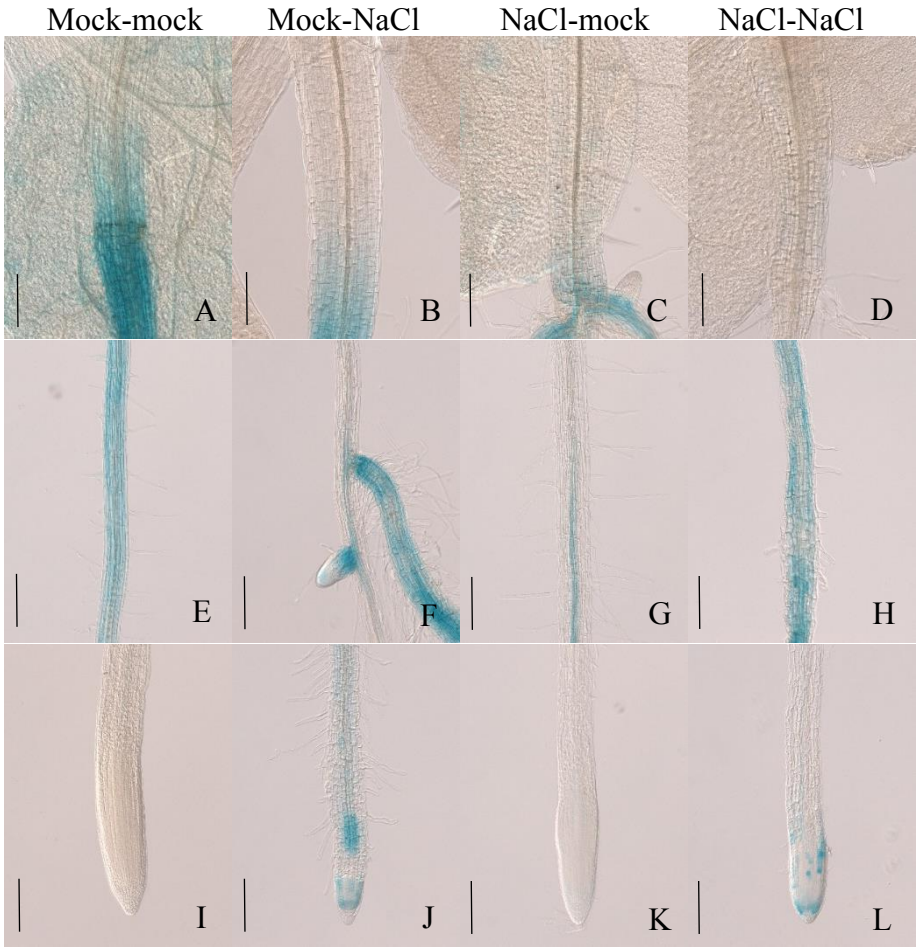

Figure 5 *Expression of pWRKY15:eYFP-GUS line 3, in 10-days-old Arabidopsis. The scale bar shows 100 µm. All samples were first treated in mock for 3 days, then transferred to treatment (mock or NaCl) for 3 days. The samples were transferred again to treatment (mock or NaCl) for additional 4 days. Seedlings were GUS stained and the samples were analyzed with an axioscope. Pictures show near hypocotyl (A, B, C, D), maturation zone (E, F, G, H), and meristematic zone (I, J, K, L).*

In line 4 (Figure 6), mock-mock treated plants' (M, Q, U) expression of *WRKY15* showed in the hypocotyl (M) and continued consistently along the maturation zone (Q). Expression was shown in the metaxylem in the upper part of the maturation zone and shifted to the protoxylem in the lower part. The two parts were separated with a gap without expression. No root tips were stained (U).
Mock-NaCl (N, R, V) treated plants showed expression in lateral roots (N, R) and root tips (V).
NaCl-mock treated plants (O, S, W) showed weak expression in the hypocotyl (O), inconsistent along the root (S), and no expression in root tips (W).
NaCl-NaCl treated plants (P, T, X) showed weak expression in the hypocotyl (P). The expression was patchy along the root (T). Some expressions could be seen in root tips (X).

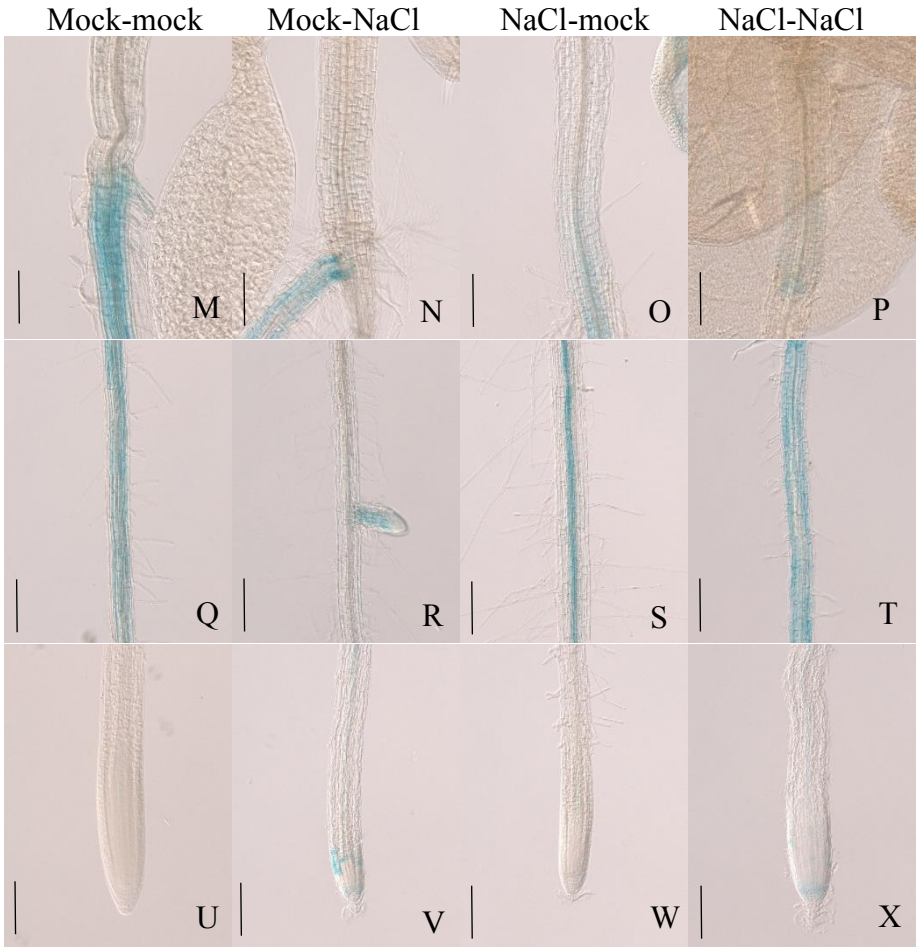

Figure 6 *Expression of pWRKY15:eYFP-GUS line 4, in 10-days-old Arabidopsis. The scale bar shows 100 µm. All samples were first treated in mock for 3 days, then transferred to treatment (mock or NaCl) for 3 days. The samples were transferred again to treatment (mock or NaCl) for additional 4 days. Seedlings were GUS stained and the samples were analyzed with an axioscope. Pictures show near hypocotyl (M, N, O, P), maturation zone (Q, R, S, T), and meristematic zone (U, V, W, X).*

In line 4, 40% of the seedlings in mock-NaCl treatment did not show any expression, and 5% for both NaCl-mock and NaCl-NaCl treatment (Figure 7). In line 3, the number of unstained plants was more evenly spread out amongst the treatments. For this line, 13% of mock-NaCl treated plants did not show expression. As for NaCl-mock-treated plants, 17% showed no expression, and in NaCl-NaCl-treated plants, 18% showed no expression.

For both treatments, no mock-mock treated plants remained unstained. This suggests that NaCl may inhibit the expression of *WRKY15*, as treatments containing salt had unstained seedlings in both lines.

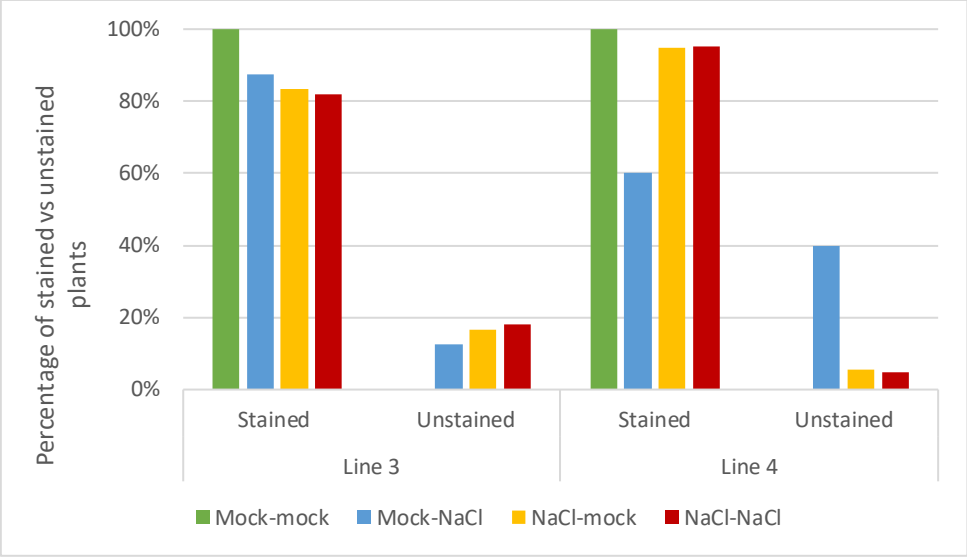

Figure 7 *Percentage of plants that stained, versus remained unstained during the GUS procedure in Arabidopsis thaliana, pWRKY15:eYFP-GUS no.3 and no.4.*

7. Discussion

This study has investigated the expression of *WRKY15*, *VND6*, and *VND7* in *Arabidopsis thaliana* using various treatments simulating a saline environment and water deficiency. As mentioned in earlier sections, plants have a way of adjusting to their environment as the xylem develops differently based on the surroundings, caused by responding transcription factors.

The hypothesis suggested that overexpression of WRKY15 leads to the formation of protoxylem gaps. The suggestion of WRKY15 as an early response to salt indicates that gaps might be a result of the saline environments. A clear observation using the GUS staining method is that plants treated with salt, mostly showed WRKY15 expression in lateral shoots, and in patches along the root. These plants also showed expression in the root tip. As the expression was present where new cells are formed – the meristem, indicates that protoxylem gaps would be more frequent in plants exposed to salinity. In contrast, mock-treated plants showed a more even expression throughout the root, and no expression in the root tip, suggesting that no protoxylem is formed. An interesting take from this could be that the expression in salt-treated plants is concentrated in shoots and patches, but the overall expression might be equal to the mock-treated plants. However, to confirm this, further investigation is needed. Furthermore, for more insight into this topic, expression patterns could be researched aiming to see how it changes over time.

When using the GUS staining method, some plants remained unstained and did not show expression. A possible explanation could be that the lines might be hemizygous and lack the promotor construct for WRKY15, or that the construct got silenced. Another possibility is that the seedling could have been affected by the salt, resulting in a fatal reaction to the treatment before responding to the staining.

Although results for the GUS staining experiment suggest that *WKRY15* is inhibited by NaCl, the results from qPCR analysis indicate that *WRKY15* was upregulated in both 6h and 24h NaCl treatments compared to mock (Figure 3). This correlates to the hypothesis that WRKY15 responds quickly to salt stress, and induces protoxylem gaps to counteract the salt.

Additionally, Ge et. Al (2020) proposed that WRKY15 functions as an indirect suppressor of VND7. Results from the qPCR analysis did not confirm this hypothesis, as the expression of *VND7* was unexpectedly high in 6-hour treatments.

However, studies have shown that WRKY15 and VND7 function indirectly as each other's contrasts. Overexpression of VND7 promotes protoxylem transdifferation, whereas WRKY15 suppresses the transdifferation (Ge et al. 2020).

Based on earlier research from Augstein and Carlsbecker (2022), VND6 may have a role in forming protoxylem gaps. Results for the qPCR in this study show an upregulation of VND6 under salt, suggesting that protoxylem gaps may form more frequently. As expected, VND6 was more upregulated in 6-hour treatment than in 24-hour treatment, supporting the hypothesis of an early response to salt.

Overexpression of WRKY15 could also be seen in 6h mannitol treatment, suggesting that a water deficiency could lead to a higher expression of WRKY15 to allow cells to elongate. VND6 and VND7 were more upregulated in 24h mannitol than 6h mannitol. Like WKRY15, both VND6 and VND7 were more upregulated in 6h salt treatment than in 24h.

Measuring the root length showed that roots in mock displayed a greater elongation than roots in salt, (Figure 4), suggesting that root growth was inhibited by salt. Augstein and Carlsbecker (2022) observed that roots treated with 200mM NaCl impeded growth significantly, with a conclusion that xylem growth is negatively affected by salinity (Augstein 2022).

In summary, I found that upon salt stress, WRKY15 is expressed in the roots as an early response. A possible effect of WRKY15 is formation of protoxylem gaps. Likewise, VND6 has a similar effect regarding protoxylem gaps. WRKY15 is revealed to suppress the tracheary element differentiation, while VND7 promotes it (Ge et al. 2020). The connection between WRKY15 and VND7 is suggested to be that WRKY15 suppresses VND7 indirectly (Ge et al. 2020).
Although research has yielded valuable knowledge and insights into xylem development and plants' response to salt, the interconnection between transcription factors and hormones is a topic for future research.

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