CHARACTERIZATION OF THE ROLE OF PIC30 PROTEIN IN PLANT IMMUNE RESPONSE

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DEDICATION

I would like to dedicate this thesis to my cousin Adelaida “Yaya” Tello.

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Abstract

Plants have developed several defense mechanisms to cope with biotic and abiotic stresses, including hypersensitive response (HR), an apoptotic response of plant tissues to infecting microbes leading to systemic acquired resistance (SAR). Salicylic acid plays an important role in plants when sending signals from the sites of HR to healthy plant organs during the development of SAR. PIC30, a protein previously characterized as a transporter protein belonging to the Major Facilitator Superfamily (MFS) in the model plant *Arabidopsis thaliana*, transports nitrate ions and the synthetic auxin, picloram into cells. A transgenic reporter line *PIC30::PIC30-GUS* displayed increased expression of the fusion protein when plants were exposed to salicylic acid. When salicylic acid binds to its receptor, NPR1, copper must also bind to NPR1 to facilitate the interaction. Therefore, copper is required for the activation of defense genes during salicylic acid-mediated SAR. *PIC30::PIC3-GUS* transgenic line also displayed a response to salicylic acid and copper. Primary root elongation of wild-type plants was inhibited while the roots of *pic30* knockout mutant displayed slight resistance to high salicylic acid and copper concentrations. Overexpression lines of *pic30* displayed high inhibition suggesting a possible role of PIC30 in the transport of salicylic acid and copper ions during SAR in plants.

Introduction

Plants have developed mechanisms to defend themselves against pathogens. One such is called hypersensitive response (HR). When an avirulent pathogen infects a leaf, HR occurs that causes all the plant cells immediately surrounding the infected area to program their death, a process known as apoptosis. This defense response is an adaptation to prevent further spread of the pathogen by isolating it (M'etraux, 2001). The areas on the plant where the cells died create lesions, which are known as HR regions. In the cells surrounding HR regions there is an accumulation of salicylic acid (SA). It serves as a long-distance signaling agent in the plant to alert other leaves to develop immunity against the pathogen (Wu et al., 2012). Traces of SA have been detected in distant healthy plant cells not affected by the pathogen, leading to the idea that SA is involved in signaling pathogen infections to healthy plant cells (M'etraux, 2001). Once this pathway is activated, several signaling processes lead to an overall resistance of the plant against the pathogen, the process known as systemic acquired resistance (SAR) (Ryals et al., 1996).

How fast the SAR activation takes place determines whether the plant will be able to become resistant against the pathogen (Bashir et al., 2013). SAR includes the activation of pathogenesis-related (*PR*) genes which, function in mechanisms leading to defense against the pathogen attack. Of these *PR-1* gene is highly induced by salicylic acid. During *PR-1* gene activation, SA binds to its receptor NPR1 (NON-EXPRESSOR OF PR-1), and copper (Cu^{2+}) is an essential cofactor for the interaction between NPR1 and SA for activation of *PR-1* gene (Wu et al., 2012).

How salicylic acid and Cu^{2+} are transported to the target cells during the process of SAR is a matter of interest. PIC30 is a transport protein that belongs to the major facilitator superfamily (MFS) that has been previously discovered in the Dharmasiri lab (Kathare et. al., 2019). In this study, three allelic Arabidopsis mutants of *PIC30* gene (*pic30-1*, *pic30-2*, *pic30-3*) that are resistant to picloram have been identified. The *pic30-3* mutant has an early stop codon in the *PIC30* gene, and its transcript level is much lower than the wild-type indicating it is a knockout mutant. This makes the *pic30-3* mutant an excellent choice for conducting experiments to compare the effect of the absence of the PIC30 protein in plants compared to the wild-type plants. Further studies in Dharmasiri lab have shown that that PIC30 transports picloram as well as nitrate ions into the cell. The plasma membrane located MFS proteins are general transporters for various substrates into or out of the cell (Remy et al., 2013). Since PIC30 can transport picloram and nitrate ions, there is a possibility that it can also transport other organic and inorganic molecules.

During preliminary experiments done in Dharmasiri lab using a transgenic line expressing PIC30 protein with GUS reporter gene driven by PIC30 promoter (*PIC30::PIC30-GUS*), it had been discovered that the GUS reporter protein level increased when plants were exposed to salicylic acid, copper ions, and bacterial infections. This raised the possibility that PIC30 protein may be involved in plant responses to pathogenesis. Based on the preliminary observations, we proposed the following hypothesis;

PIC30 protein may serve as a transporter of salicylic acid and copper into *Arabidopsis* cells.

The work presented here tests this hypothesis to determine if PIC30 protein is involved in the transport of either SA or copper during the development of SAR. The results suggest that PIC30 may be a transporter of copper ions and SA, thus, may be involved during plant immune responses.

Materials and Methods

Plant material and growth conditions

All wild-type, mutant, and overexpression lines of *Arabidopsis thaliana* used in this study are in Col-0 ecotype. *PIC30::PIC30-GUS* reporter line has been previously described (Kathare et.al., 2019). Plants were either grown on ATS media (*Arabidopsis thaliana* medium with 1% sucrose) in a controlled environment growth chamber at 22ºC in continuous light, or in soil.

Root growth assay on salicylic acid (SA)

Wild-type (Col-0) and *pic30* knockout mutant (*pic30-3*) were grown on unsupplemented ATS media or ATS media containing 20 μM salicylic acid. The plates were placed vertically in 22ºC growth chamber for 6 days, and root lengths of seedlings were measured. The experiment was repeated twice.

Root growth assay on copper sulfate (CuSO4)

Wild-type (Col-0), *pic30* knockout mutant (*pic30-3*), *PIC30* overexpression line 25 (OX25), and *PIC30* overexpression line 29 (OX29) were grown on unsupplemented ATS media or ATS media containing 30 μM or 40 μM copper sulfate. The plates were

placed vertically in 22ºC growth chamber for 6 days, and the root lengths of seedlings were measured.

Histochemical staining of *PIC30::PIC30-GUS* seedlings

Three solutions with concentrations of 0 µM, 5 µM, and 20 µM SA were prepared from a 100 mM stock solution of SA. Five-day-old *PIC30::PIC30-GUS* seedlings, grown on ATS, were placed in a SA solution for 24 hours. After the seedlings were removed from the SA solutions and rinsed in liquid AT media (Similar to ATS, but without 1% sucrose), 3 mL of GUS fixer solution (0.5 M Mannitol, 100 mM MES pH 5.6, 37% Formaldehyde, and Milli Q. H_2O) was added to each sample. The samples were vacuum infiltrated for 5 minutes and then placed on the shaker for 40 minutes. The samples were then washed 3 times for 5 minutes each with 3 mL of 100 mM Na_2HPO_4 (pH 7.4). After washing, the samples were vacuum infiltrated for 20 minutes in 1 mL of GUS staining solution (0.5 M Na_2HPO_4 pH 7.4, 100 mM potassium ferricyanide, 100 mM potassium ferrocyanide, 0.5 M Na_2EDTA pH 8, 20% Triton X-100, Milli Q. H_2O, and 5 mg of 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid, cyclohexylammonium salt (X-Gluc) dissolved in 50 µL of N, N, DMF) overnight at 37ºC. The next day, the staining solution was removed, and 10% ethanol was added to the samples to preserve and remove chlorophyll from the tissues.

The same protocol was followed for five-day old *PIC30::PIC30-GUS* seedlings exposed to $CuSO_4$ with the exception of method of exposure. The seedlings were grown on 50 µM $CuSO_4$ plates for 1, 3, 6, 18, and 24 hours. Seedlings were removed from the plate at designated time intervals and were rinsed in liquid AT media. Staining for GUS activity was done as described above (Histochemical staining).

Quantitative MUG assay

PIC30::PIC30-GUS transgenic seedlings were grown on ATS media for 4 days. Then, twenty seedlings were transferred to liquid AT media containing different concentrations (0, 5, 10, and 20 μM) of SA for 24 hours. Total proteins were extracted from treated seedlings, and 50 μg equivalent of total protein from each sample was used to estimate the activity of GUS enzyme in fluorogenic MUG assay as described previously (Jefferson, 1987). Each time point was repeated for 3 times, and fluorescence was measured using a fluorometer (Turner Biosystems, model #9200-002).

RNA extraction and Quantification

Five-day-old wild-type (Col-0) seedlings grown on vertical agar plates were incubated in ATS media with or without 20 μM SA for 0, 3, 6, and 24 hours. Total RNA was extracted using TRI reagent (Sigma, MA). RNA extraction was performed as previously described (Kathare et. al., 2019). Total RNA was quantified using a NanoDrop 1000 (Thermo Scientific).

RNA gel electrophoresis

A 1.5% agarose gel was prepared for RNA gel electrophoresis as previously described (Kathare et. al., 2019). RNA samples were prepared by adding 2 μL of 3X RNA Dye + EtBr, 3 μL of DEPC H_2O, to 1 μL of total RNA. RNA samples were heated at 65ºC for 7 minutes, and then loaded on the gel. The gel was run in 1X MOPS buffer at 100 V.

Reverse transcription

An RNase-free DNase treatment was conducted on the total RNA to eliminate contaminating DNA. Reverse transcription was performed to synthesize cDNA from 5 µg of total RNA following the manufacturer's protocol (Invitrogen, CA).

Reverse transcription-PCR

Polymerase chain reaction (PCR) was used to check cDNA. A master mix containing 10X Taq buffer, 10 mM dNTPs, DNA polymerase, Milli Q H_2O, and the corresponding primers were added to 1 µL of cDNA. The PCR was performed at 95ºC for 2 minutes, 95ºC for 20 seconds, 63ºC for 20 seconds, 72ºC for 45 seconds, and 72ºC for 5 minutes for 30 cycles. PCR products were separated on a 1% agarose gel with 1X TAE buffer.

qRT-PCR for native PIC30 gene expression

cDNA prepared using 5 µg of total RNA was used for Quantitative RT-PCR. Quantitative RT-PCR was performed using *PIC30* specific primers (Kathare et. al., 2019) and UBA standard housekeeping gene primers. Data was analyzed as previously described (Kathare et. al., 2019). Each time point was repeated three times.

Results

***pic30-3* knockout mutant seedlings are resistant to SA**

The effect of SA on PIC30 was examined by growing wild-type and mutant *pic30* seedlings on 20 µM SA and measuring SA effect on the primary root length. Data were presented as percentage inhibition of primary root growth by SA relative to the control

media. Results show that the *pic30-3* knockout mutant seedlings display resistance to the 20 µM SA compared to wild-type Col-0 seedlings (Figure 1).

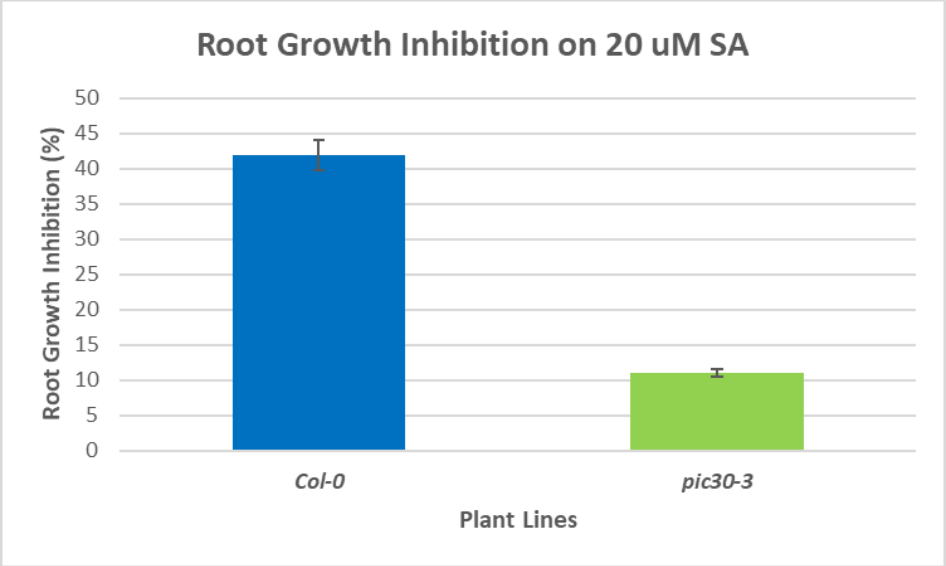

Figure 1. The *pic30-3* knockout mutant seedlings demonstrate resistance to SA, while wild-type Col-0 seedlings' root length was significantly inhibited (P value: < .00001; analyzed by one-way ANOVA).

PIC30 overexpression lines were highly inhibited by copper

The response of wild-type and mutant *pic30-3* seedlings was also observed with copper. Seeds were germinated on ATS or ATS media containing 30 µM and 40 µM $CuSO_4$ for 6 days, then the length of primary root was measured an expressed as percentage inhibition compared to the control. Wild-type Col-0, *pic30-3* knockout mutant, and two *PIC30* overexpression lines were grown on control ATS media or on ATS with varying concentrations of $CuSO_4$. The overexpression lines, OX25 and OX29, were significantly inhibited when exposed to both 30 µM and 40 µM $CuSO_4$

concentrations while the knockout mutant, *pic30-3*, displayed slight resistance at 30 µM $CuSO_4$ (Figure 2).

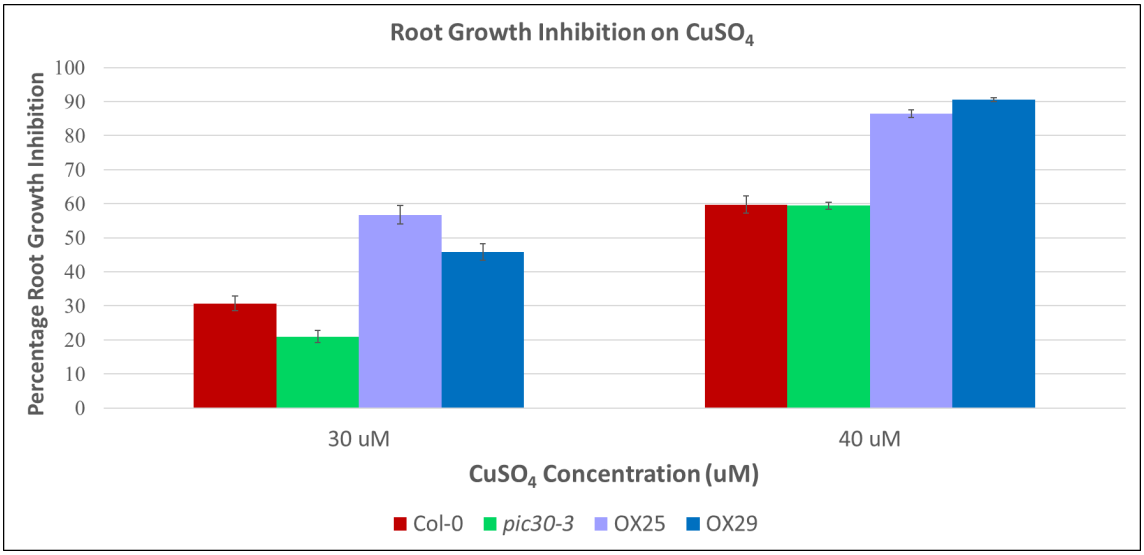

Figure 2. Percentage primary root growth inhibition of seedlings exposed to 30 µM and 40 µM $CuSO_4$ (P value: < .00001; analyzed by one-way ANOVA).

Col-0 = wild-type, *pic30-3* = knockout mutant, OX25 = *PIC30::PIC30-GFP line 25, OX29 = PIC30::PIC30-GFP line 29*

***PIC30::PIC30-GUS* expression was increased in response to SA treatment**

Transgenic plants carrying the *PIC30::PIC30-GUS* used reporter gene construct were used to observe the induction of *PIC30* expression when exposed to salicylic acid. Five-day-old seedlings were incubated in the media containing varying concentrations of SA for 24 hours, and expression was detected as blue color development due to GUS enzyme activity. After preserving and removing chlorophyll from seedlings, it was

observed that GUS activity in *PIC30::PIC30-GUS* seedlings increased in response to SA in a concentration dependent manner (Figure 3).

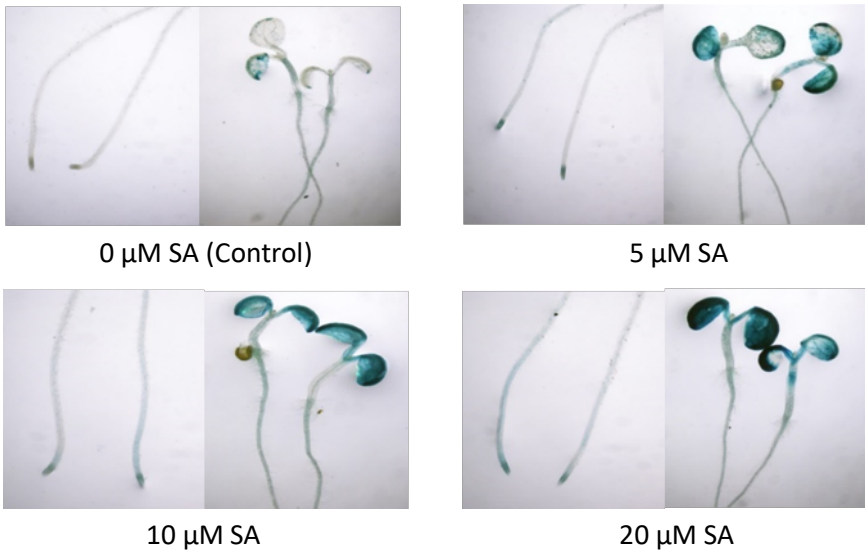

Figure 3. Expression of transgenic PIC30-GUS fusion protein increases in response to SA treatment compared to control.

PIC30::PIC30-GUS **expression in roots decreases in response to long-term exposure to $CuSO_4$**

Transgenic plants carrying *PIC30::PIC30-GUS* fused reporter gene construct were also used to observe the induction of PIC30 expression in response to copper. Seedlings exposed to copper were grown on 50 µM $CuSO_4$ plates for 1, 3, 6, 18, and 24 hours and the expression of PIC30-GUS was detected as blue color development due to GUS enzyme activity. GUS activity in *PIC30::PIC30-GUS* seedlings exposed to 50 µM $CuSO_4$ was steady for up to 6 hours, but then decreased after 18 hours in the roots (Figure 4).

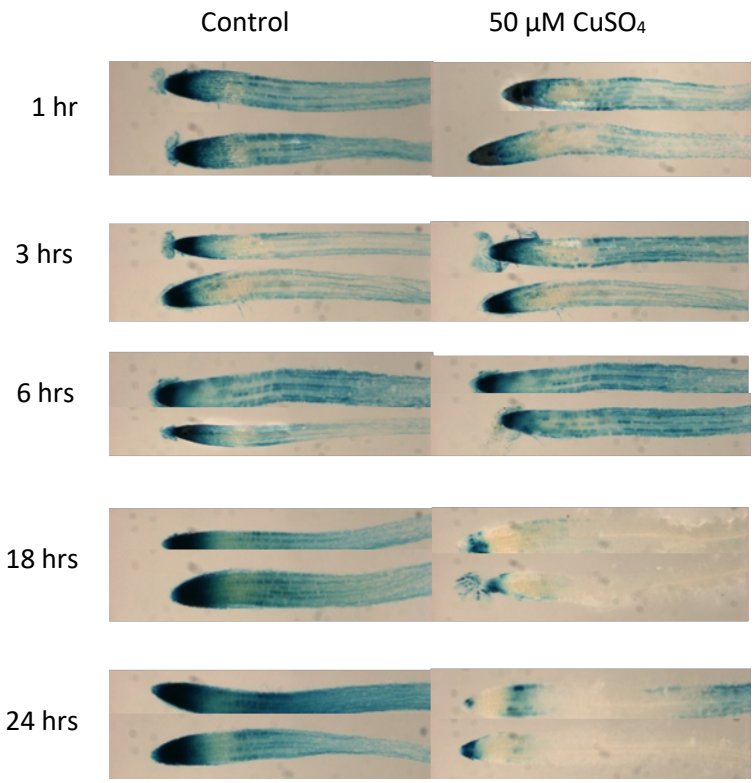

Figure 4. In roots, the expression of transgenic PIC30-GUS fusion protein decreases after long exposure to $CuSO_4$.

PIC30::PIC30-GUS expression increases in shoots in response to copper treatment

When the same transgenic plants carrying the *PIC30::PIC30-GUS* fused reporter gene construct were exposed to 50 µM $CuSO_4$ plates for 1, 3, 6, 18, and 24 hours, expression of the PIC30-GUS fusion protein increased in the shoots. This increase of, GUS activity was observed up to 24 hours (Figure 5).

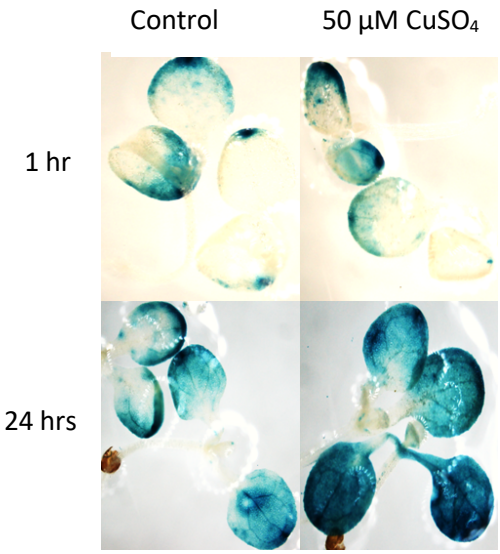

Figure 5. Expression of *PIC30::PIC30-GUS* gene in shoots increases in response to copper treatment.

Quantitative GUS assay confirms increased *PIC30* expression in response to SA

Results of histochemical staining of *PIC30::PIC30-GUS* seedings give only qualitative differences in *PIC30* expression. Therefore, a quantitative MUG assay was used to quantify the expression of PIC30-GUS fusion protein in response to SA. Transgenic plants carrying *PIC30::PIC30-GUS* reporter gene construct were exposed to 10 µM SA at different time intervals and GUS activity was estimated as the rate of conversion of MUG into a fluorogenic product, MU. A fluorometer was used to detect the production of MU to quantify GUS activity. Quantitative MUG assay exhibited an increase in GUS activity over time up to 24 hours when exposed to 10 µM SA (Figure 6).

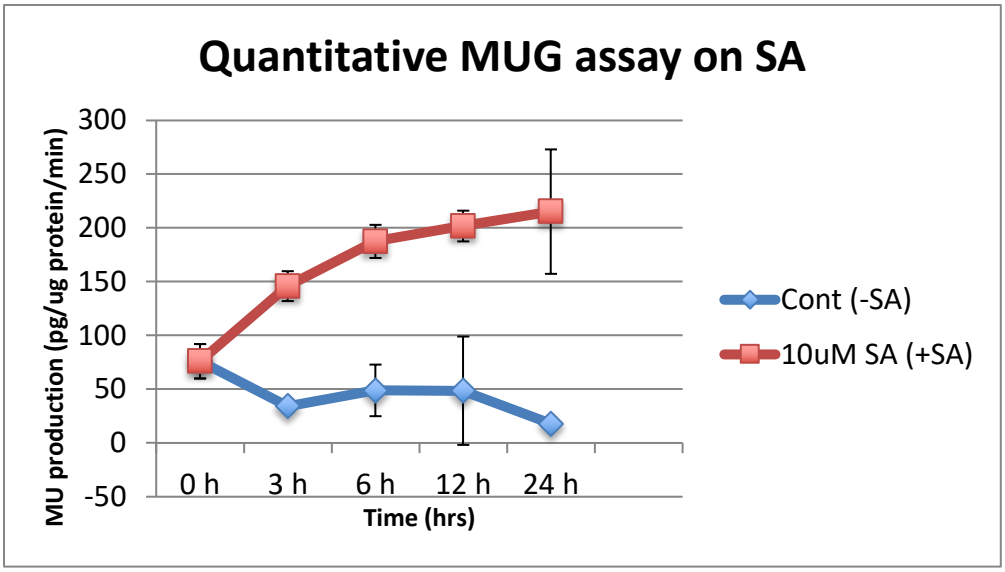

Figure 6. *PIC30::PIC30-GUS* gene expression is enhanced in response to SA treatment.

Expression of native *PIC30* gene increases in response to SA treatment

RNA isolated from five-day-old wild-type Col-0 plants grown in ATS media with or without 20 µM SA was quantified, separated on a 1.5% agarose gel (figure 7), and then used to synthesize cDNA. The cDNA was amplified by PCR to confirm the presence of the cDNA. PCR products were separated on a 1% agarose gel (figure 8). After successful completion of the previous steps, diluted cDNA was used to perform qRT-PCR. Relative expression was normalized to the expression level of PIC30 in untreated (0 hr) sample. Ubiquitin associated protein (UBA), a housekeeping protein, transcript was used as the standard to normalize transcript levels. Native *PIC30* gene expression of five-day-old wild-type (Col-0) seedlings exposed to SA was shown to increase with time (Figure 9).

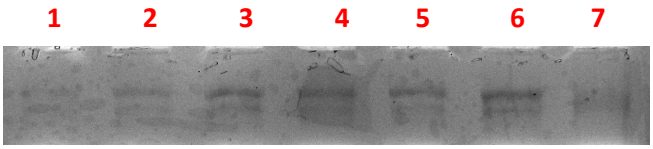

Figure 7. RNA gel (1.5% agarose gel) shows ribosomal bands, indicating presence of total RNA in each sample.

1 = 0 hr (Control), 2 = 3 hrs w/o SA, 3 = 3 hrs w/ SA, 4 = 6 hrs w/o SA,

5 = 6 hrs w/ SA, 6 = 24 hrs w/o SA, 7 = 24 hrs w/ SA

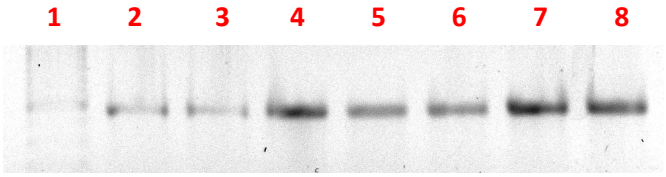

Figure 8. DNA gel (1% agarose gel) exhibits PCR amlified bands, indicating the presence of cDNA.

1 = 100 bp DNA ladder, 2 = 0 hr (Control), 3 = 3 hrs w/o SA, 4 = 3 hrs w/ SA,

5 = 6 hrs w/o SA, 6 = 6 hrs w/ SA, 7 = 24 hrs w/o SA, 8 = 24 hrs w/ SA

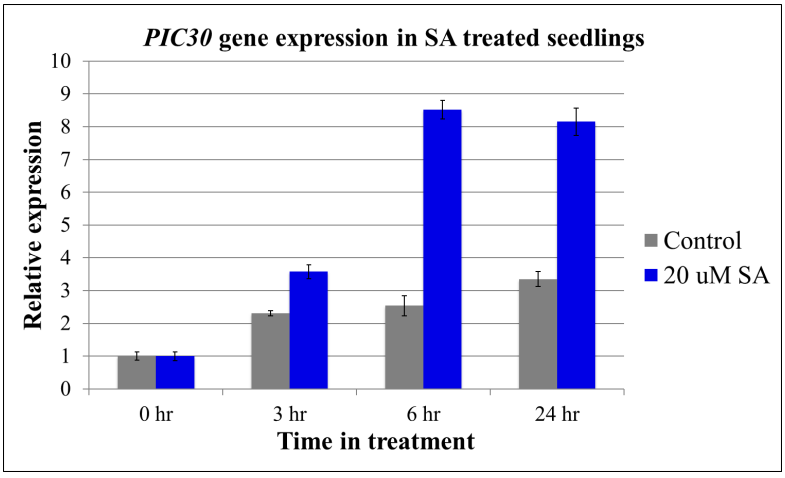

Figure 9. Expression of native *PIC30* gene increases in response to 20 µM SA treatment.

Discussion

As a member of the MFS transporter proteins, PIC30 may have the potential to transport a variety of molecules, although it has only been confirmed experimentally to transport picloram and nitrate ions. We hypothesized that PIC30 protein may serve as a transporter of salicylic acid and copper into *Arabidopsis* cells. A transporter of salicylic acid and copper would facilitate systemic acquired resistance (SAR) in plant immune responses, since both salicylic acid and copper are required for SAR. During SAR, salicylic acid is transported from the initial infection site to the rest of the healthy plant tissues. An accumulation of salicylic acid is present at the HR regions where the initial infection takes place, but there is no known mechanism of how SA is transported from the HR regions to healthy parts of the plants (M'etraux, 2001). SA acts as a secondary signaling molecule during activation of pathogenesis-related (PR) genes (Wu et al., 2012). PR genes are usually activated during an infection by a pathogen in order to counteract or defend against the pathogen (Wu et al., 2012). SA activates the expression of *PR-1* gene during SAR by interacting with its receptor, NPR1. However, in order for SA to bind to NPR1, copper is a required as a cofactor (Wu et al,, 2012). Thus, a plasma membrane transporter must be involved in transporting copper and SA into cells during SAR.

It is logical to assume that the abundance of a transporter protein may increase when cells are required to transport a certain substance. Our results indicate that *PIC30* gene expression increases when seedlings were exposed to both SA and copper, suggesting a strong correlation between PIC30 protein function and transport of SA and copper. *PIC30* gene expression increased in both roots and shoots during 24 hours after

initial exposure to SA. Interestingly, *PIC30* gene expression showed a different pattern in response to copper, where it continued to increase expression in shoots, but decreased in roots after 18 hours. This suggests a possibility of an internal mechanism within roots to avoid copper toxicity by preventing the absorption of copper through roots. However, the continuous *PIC30* gene expression in shoots even at 24 hours after exposure to $CuSO_4$ suggest that PIC30 protein may still be involved in copper transport in shoots. SAR occurs in plant shoots (M'etraux, 2001), thus it would be expected to see higher amounts of transporters present in these regions of the plants. SAR does not occur in the roots (M'etraux, 2001), but the roots are responsible for the uptake of various essential micronutrients, including copper. The roots have a major role in controlling the absorption of copper from the environment into the plant cells (Andrés-Colás et al., 2010). Thus, it would not be surprising to see a decrease in expression of PIC30 in the roots, when copper concentrations are high in the medium for prolonged periods, since plant cells would try to avoid absorbing copper to minimize damage.

The results presented here are encouraging, but not conclusive. More work needs to be done to confirm the involvement of PIC30 protein in transport of SA and copper into cells, preferably using a heterologous system such as frog oocytes expressing PIC30 protein. Frog oocytes do not normally express PIC30, therefore, changes in concentration of SA or copper could be monitored with oocytes expressing PIC30. Expression of PIC30 protein could also be observed in plants induced into SAR. This would require growing 3-week-old plants and inoculating healthy plant leaves with SA to induce a simulated SAR response. Both *PIC30* and *PR-1* gene transcript levels could be quantified using qRT-PCR to monitor the expression levels of *PIC30* and track induction of SAR through

PR-1 transcript levels. This experiment could be repeated with the exception of inoculating healthy plant leaves with a *Pseudomonas syringae* instead of SA to induce a true SAR response. Both *PIC30* and *PR-1* gene transcript levels could be monitored in future studies using qRT-PCR. The results of simulated and true SAR responses could then be compared and if similar expression patterns are observed in both, then there could be more clarity in the relationship between SAR induction and PIC30 protein.

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