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The Effects of Cytokinin on the Transcriptional Regulation of PIN Expression in Arabidopsis thaliana

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Abstract

The processes of cell division and differentiation are critical to the development of any multicellular organism. During the formation of plant roots these processes take place at a region of the root tip called the meristem. Cytokinin and auxin are two plant growth hormones that influence this process. Although these two growth hormones are both necessary they also appear in many ways to have an antagonistic relationship. As meristematic root cells undergo differentiation they cease dividing. It has been proposed that the size of the root meristem and thus the overall rate of root growth are determined by the balance between the rate of cell division, determined by auxin, and the rate of cell differentiation, determined by cytokinin. One of the ways cytokinin may have an antagonistic influence on auxin regulation is by limiting auxin transport. Directed auxin transport from one part of a plant to another is controlled by a family of auxin efflux proteins called PIN proteins. To date, studies reported in the literate are inconsistent as to whether and how cytokinin influences PIN protein production at the transcriptional and/or translational level. This study was undertaken to explore the effect of cytokinin signaling on PIN gene expression using real-time quantitative PCR.

Background

The object of this study was to examine the relationship between two plant growth hormones, auxin and cytokinin, in *Arabidopsis thaliana*. The effect of cytokinin signaling on auxin transport in the root-tip was explored by looking at modifications of PIN proteins at the transcriptional level.

Arabidopsis thaliana is a model organism used in plant biology research. It is a small flowering plant and has a short life cycle of about 6 weeks from germination to mature seed. *A. thaliana* has 5 chromosomes which have been physically and genetically mapped and there are a large number of mutant lines available for use by researchers making it ideal for research. This plant is a member of the Brassicaceae family, along with familiar cultivated plants like cabbage and broccoli. Although the *A. thaliana* genome has been sequenced a lot is still unknown about the function of individual gene products, how their protein products are made, and how these proteins interact. Researchers are still in the midst of figuring out the roles, regulation, and interactions between different parts of a cell in order to be able to understand the workings of the plant.

One process that *Arabidopsis thaliana* can be used to model is root growth. Growth of the root occurs in the meristem located at the root-tip, where cells are actively dividing and elongating during the formation of new tissues. Root growth is dependent on the creation of new cells and their transition into differentiated and elongated cells (Ruzicka et al, 2009). Cell division is initiated at a region near the root-tip called the stem cell niche and continues through the meristematic zone of the root. Stem cells are cells that have not differentiated to assume a specific function. Division of the stem cells continues up through the meristematic zone until

eventually, cell division ceases as elongation and differentiation commence at the transition zone. Beyond the transition zone cells acquire characteristics that are tissue specific. The overall rate of root growth is determined by the balance between the rate of cell division in the meristematic zone and the rate of cell differentiation in the transition zone (Perilli, Moubayidin, & Sabatini, 2010). It has been proposed that this balance is influenced by the interaction between the plant growth hormones cytokinin and auxin (Moubayidin et al., 2010).

Cytokinins are a class of phytohormones, chemical messengers produced by plants that regulate physiological and developmental processes. It is an important director of shoot and root architecture, it regulates seed development and abiotic stress, and is also involved in root growth (Pernisova et al, 2009). Some evidence suggests that cytokinins act to promote the rate of cell differentiation in the root meristem, which reduces the amount of time cells spend dividing in the division zone (Perilli, Moubayidin, & Sabatini, 2010). It seems that cytokinin primarily affects the meristematic cell differentiation rate, which results in shortening of the meristematic zone and therefore shortening of the root (Dello Ioio et al, 2007). *Arabidopsis* responds to cytokinins through a phosphorelay pathway, a multi-stage process of signal transduction which involves the movement of phosphate groups from the cytokinin receptors to down-stream targets. This pathway is similar to that of bacterial two-component receptors and includes a receptor with a histadine kinase domain (His Kinase) that initiates the phosphate transfer. Ultimately, the phosphate is delivered to the receiver domain of a down-stream response-regulator (Taiz & Zeiger, 2006).

The Mathews lab has been studying the effects of mutations in a group of transcription factors (proteins that bind to DNA and play a role in regulating gene expression) that are the down-stream targets of this cytokinin signaling pathway called *Arabidopsis* response regulators (ARR).

There are two classes of ARRs, type-A ARRs, whose products comprise only the receiver domain, and type-B ARRs, whose products include both a transcription factor domain as well as a receiver domain (Taiz & Zeiger, 2006). The expression of type-A ARRs is a primary response to cytokinin signaling. Type-A ARRs compete with type-B ARRs for the activating phosphate groups and therefore act as negative feedback regulators of cytokinin. Their expression is directly promoted by the activation of type-B ARRs. (Hwang, Sheen, & Muller, 2012). Type-B ARRs are positive regulators of cytokinin. Unlike in type-A ARRs, loss of function mutants in certain type-B ARRs are insensitive to cytokinin. This study focused on two type-B ARRs, ARR1 and ARR12, which are known to have a role in root formation. The double arr1, arr12 loss-of-function mutant is particularly insensitive to cytokinin. Root growth of wild-type plants is normally inhibited by cytokinin treatment but the arr1, arr12 mutants are much less sensitive.

Another important phytohormone is auxin. It has been demonstrated that auxin plays a role in the process of root organogenesis, specifically with the initiation of the root pole in embryos, determining the position and forming of the stem cell niche, and maintaining cell division in the proximal meristem (Ruzicka et al, 2009). It is the high concentration of auxin at the root tip that is thought to be primarily responsible for initiating and maintaining cell division in the root meristem (Perilli, Moubayidin, & Sabatini, 2010). Auxin exhibits polar transport so most of the auxin is produced in the shoot apical meristem and is transported to the root-tip (Taiz & Zeiger, 2006). The proteins responsible for this transport are a collection of auxin-specific influx and efflux proteins. The directional transport of auxin is controlled by certain efflux proteins referred to as PIN proteins. PIN proteins are a family of integral membrane proteins aligned with the direction of auxin transport; different PINs are responsible for distributing auxin to specific areas of the root (Taiz & Zeiger, 2006). Several PIN genes have been modified by

attaching the coding sequence for the green fluorescence protein (GFP) as a translational fusion to the PIN coding sequence. These GFP reporter constructs have been introduced into *Arabidopsis thaliana* lines, which allow us to get a picture of exactly which PINs are present in which part of the meristem. The PIN proteins that have been shown to be involved in directed transport of auxin in the root are, PIN1, PIN2, PIN3, PIN4, and PIN7.

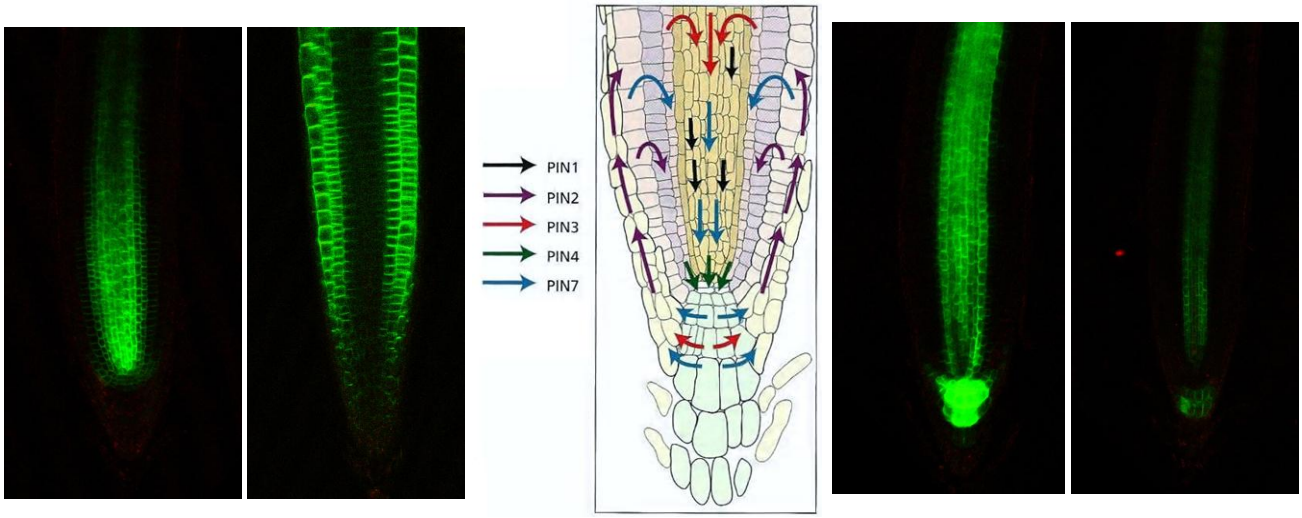

Figure 1: *Arabidopsis* root-tips showing the cells that contain the different PIN proteins. The middle figure depicts the regions where each PIN is thought to play a role in auxin efflux. The surrounding figures are green fluorescence images that indicate where the PIN proteins are present. PIN1 (left) appears down the center of the root; PIN2 (middle left) appears in the outside layers of the root; PIN3 (middle right) appears down the center of the root as well as in the quiescent center; PIN7 (right) appears faintly down the center of the root and at the quiescent center as well. (GFP images taken by Mckenzie Shaw)

In many aspects of plant growth and development auxin and cytokinin have an antagonistic relationship. A few theories exist about this relationship, for example some evidence suggests that a protein called SHY2 is critical to the interaction of auxin and cytokinin. SHY2 is part of the AUX/IAA family of proteins that act to inhibit the function of transcription factors called auxin response factors (ARFs) by binding to them. While these two proteins are bound the

ARF protein cannot function to regulate the transcription of auxin response genes (Perilli, Di Mambro & Sabitini, 2011). Auxin has been shown to target AUX/IAA proteins for degradation thus freeing the ARF transcription factors to regulate auxin response genes. It has been shown that cytokinin can promote expression of the SHY2 AUX/IAA protein and it has been proposed that this results in the negative regulation of PIN1, PIN3, and PIN7 expression in the transition zone (Dello Ioio, 2008). This limits auxin transport, which reduces the auxin concentration in the root meristem. The reduced auxin concentration promotes cell differentiation over cell division, which then reduces the overall root meristem size. Auxin signaling, on the other hand, results in degradation of SHY2 and other AUX/IAA proteins, which maintains PIN production and auxin transport. The resulting high concentration of auxin in the root tip promotes cell division, increasing growth and meristem size. This balance between cell differentiation and cell division is thought to determine the size of the root meristem and the overall size of the root.

Recent literature exploring the effect of cytokinin on the transcriptional regulation of PIN genes has been inconsistent. It was originally proposed that cytokinin affects auxin signaling at the transition zone of the root-tip by limiting auxin transport through the reduction of PIN gene expression (Dello Ioio, 2008). Being the first publication with few attempts to confirm the results this idea has been generally accepted by the scientific community. However, more recent publications have presented conflicting data. The goal of this study was to explore this relationship between cytokinin signaling and PIN transcript levels using quantitative real-time PCR.

Methods

Seed Sterilization and Growth

The two seeds types used were wildtype (WT) which came from the Columbia ecotype and an arr1,12 mutant. Seeds were sterilized using ethanol and plated onto either 1X Murashige and Skoog media with 1% sucrose (MS) or MS media with added benzyl adenine (BA), a synthetic cytokinin, at a concentration of 1 uM. Two rows of seeds were placed on each plate. Each plate was placed in the fridge for cold treatment for 2-3 days to ensure that the seeds would germinate at the same time. After two days, the seeds were placed in a growth chamber with continuous light set to 22º C for five days.

RNA Isolation

After the five day growth period the terminal 1-2 millimeters of each root-tip was collected and stored in Qiagen "RNA later®" solution. Using the Qiagen "RNeasy® plant mini kit" the RNA was isolated from the root-tips using RLT buffer in our isolation. The final product was eluted from the filter with 50 ul of water and was then used to re-elute any remaining product in order to increase concentration.

RNA Quantification

The isolated RNA from each treatment group was quantified using the NanoDrop Spectrophotometer. The setting used for the measurement was nucleic acid - "RNA-40". To ensure sample purity, overall spectral quality was assessed and the ratio between wavelengths of 260/280 nm and 230/260 nm were checked to be near 1.8 and 2.0 respectively.

cDNA Synthesis

cDNA was synthesized from RNA isolated from each treatment. A maximum of 2.5 ug RNA can be used in the SuperScript® Vilo™ cDNA synthesis kit by Invitrogen. The reaction volume was 20 ul with up to 14 ul of RNA. The treatment with the lowest concentration of RNA (determined with the Nanodrop) was used at the maximum volume (14 ul). The volume for the other samples differed depending on their concentration, so that all contained an equal amount of RNA going into the cDNA reaction. After the reaction, the cDNA was diluted to a concentration of 10 ng/ul.

Measure of Gene Expression

PIN gene expression was quantified using the 'Stratagene Mx3000P' real-time quantitative PCR by Agilent Technologies. ROX and SYBR were the two dyes used with ROX being the reference dye. The conditions for the qrt PCR were divided into three segments: segments one - one cycle at 95º C for 10 minutes, segment two - forty cycles of 95º C for 15 seconds, 55º C for 15 seconds, and 72º C for 1 minute, and finally segment three - one cycle of 95º C for 1 minute, 55º C for 30 seconds, and 95º C for 30 seconds. The reaction volume was 20 ul and contained 2 ul each of 5' and 3' primer at a final concentration of 200 uM, 10 ul of 2X Syber® Select MasterMix, and 10 ng of cDNA. Each reaction was run in triplicate and averaged together when calculating the Ct value. The primers used were created with the "PrimerSelect" program. The un-translated regions of the PIN gene sequences were where we looked first because the PIN genes have very similar translated regions. The primers were selected as to generate relatively short amplification products (between 100 - 150 basepairs), and the primer melting points were between 50º and 60º C. Attempts were made to optimize primers using primer efficiency tests and temperature gradients. Each primer pair was also checked for effectiveness using agarose gel electrophoresis to confirm product size and to make sure there

were no primer dimmers or mis-primed products being amplified instead the desired gene product. Several primers were designed over the course of this project in an attempt to get the best possible amplification efficiency. Our current primers were designed in an attempt to have melting temperatures between 59º and 60º C. The primers used to generate the data reported in this study are listed below.

Primer sequences:

ACT: Upper Primer 5' - TCCTCATGCCATCCTCCGTCTT - 3'

Lower Primer 5' - TTCCCGCTCTGCTGTTGTGGT - 3'

ARR5: Upper Primer 5' - TCGACGGTTACTCAGAGTCTATCTT - 3'

Lower primer 5' - TCCCAGGCATAGAGTAATCCGTCATT - 3'

SHY2: Upper Primer 5' - TTCTCGGGCAAGATCTATGTTCATTG - 3'

Lower Primer 5' - GGACAACCTTTTGCCTGTTTCTG - 3'

PIN1: Upper Primer 5' - GCTTTTGCGGCGGCTATGAG - 3'

Lower Primer 5' - TGGCAAACACAAACGGTACTATTCCTT - 3'

PIN2: Upper Primer 5' - TATCCACCACCCTAAAGTTTCTATTCCT - 3'

Lower Primer 5' - ACTTTCCCCCGTTATTACCGTCTTG - 3'

PIN4: Upper primer 5' - GAACACATATGCCGCCGACAAG - 3'

Lower Primer 5' - AACAAGAGCCCATATGAGACCGATTAG - 3'

PIN7: Upper Primer 5' - AAAGAAGCTATAGAAACGGGTGAAACTGA - 3'

Lower Primer 5' - AAGAGCCCAAATGAGACCAATGAGACTA - 3'

Analysis of Results

The qrt PCR software program calculated a Ct value for each gene. The software calculates the Ct value (the number of cycles it takes for the gene product to start exponentially amplifying) based on baseline and threshold values, which are derived from the assumption that the data exhibits the typical amplification curve. The baseline reference refers to the initial cycles of PCR in which there are undetectable changes in the fluorescence signal, while the threshold is found within the exponential growth phase of the amplification curve just above the baseline. It is the intersection of the threshold line with the amplification curve that determines the Ct value (SYBR Select MasterMix User Guide, 2012).

Once the Ct values were established, the fold difference was calculated using the ΔΔ Ct method. The Δ Ct was calculated by subtracting the Ct values for the treatment from the Ct values for WT on MS (see figure 2). In an attempt to normalize the PCR reaction a Ct value was also determined for an endogenous control template with each cDNA prepared from each treatment. Actin was chosen as this control because its gene expression should be consistent regardless of cytokinin treatment. It was assumed that the Ct value for actin should be identical in all treatments if the same amount of cDNA template was present initially. A Δ Ct was calculated for actin between treatments and was assumed to reflect differences in the amount of cDNA template added to each PCR reaction. In order to correct the PIN Δ Ct values for any difference in cDNA loading they were adjusted by the amount of the Δ Ct for actin. The PIN Δ Ct values normalized for actin by subtracting the actin Δ Ct were then designated the ΔΔ Ct

values of the PIN genes (see figure 2). Finally, the fold difference in gene expression between the four treatments (WT on MS, WT on MS+BA, arr1,12 on MS, arr1,12 on MS+BA) was found by using the formula $2^{\Delta\Delta Ct}$.

	WT on MS #(Ct)	Treatment (1uM BA) #(Ct)	Δ Ct (WT,MS - Treat.)	ΔΔ Ct ($\Delta Ct_{gene} - \Delta Ct_{actin}$)	Fold Difference $2^{\Delta\Delta Ct}$
Actin	19.26	18.99	0.27	0	1.00
Gene of interest	20.72	21.59	-0.87	-1.14	0.45

Figure 2: Example template with the steps used to normalize the data. The first row is showing how each value was calculated, the second row contains the values for actin which were used to normalize the gene of interest, and the second column is the WT on MS which was also used to normalize.

Results

Temperature gradients and primer efficiency tests were performed as mentioned in the methods section to make sure the correct products were being amplified and to ensure the least amount of background noise (primer dimmer and mis-priming) was present.

Genomic DNA (gDNA) was used as the template for the temperature gradient to guarantee that there would be equal amounts of DNA template corresponding to each primer conbination. One result of using gDNA is that introns are included in the synthesis of the PCR product, which can affect the size of the product. For PIN1 and PIN2 this was the case because their primers flank introns which are present in the gDNA template, resulting in larger than expected products and a difference in size between Fig. 3 and Fig. 4. It is also evident that PIN3

is not amplifying as well as the others by the faintness of the band for PIN3. Since the template in this case was genomic DNA this suggests that the amplification problem for PIN3 is with the primer design.

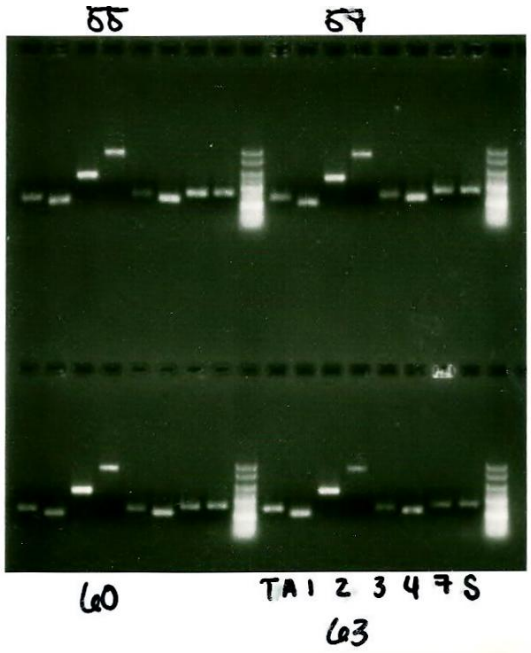

Figure 3: Genomic DNA (gDNA) was used as template in this experiment. Order of the temperature gradient: upper left quadrant at 55º C, upper right quadrant at 57º C, lower left quadrant at 60º C, and lower right quadrant at 63º C. The order of the primers is tubulin, actin, PIN 1, PIN 2, PIN 3, PIN 4, PIN 7, and SHY2. It was determined that the optimum temperature for these primers was 55º C. (The ladder ranges from 500-25 base pairs)

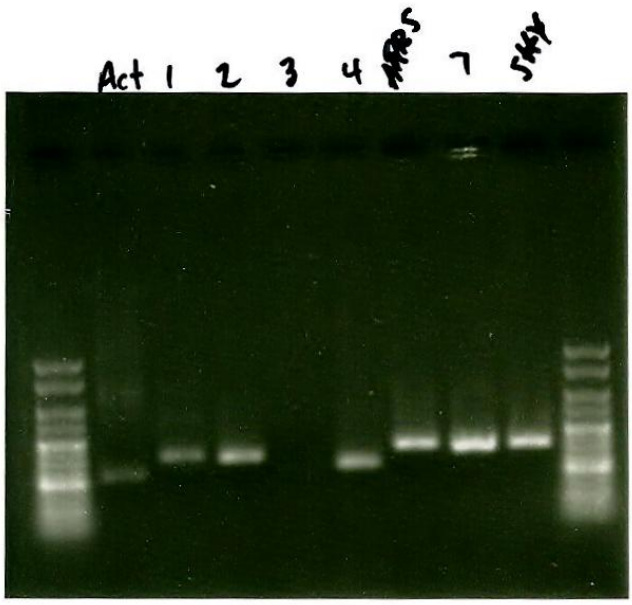

Figure 4: After cDNA was amplified the qrt-PCR the products were run through an agarose gel to check for correct product size. Actin should be 109 bp, PIN1 should be 145 bp, PIN2 should be 138 bp, PIN4 should be 119 bp, ARR 5 should be 159 bp, PIN7 should be 150 bp, and SHY 2 should be 157 bp. (The ladder ranges from 500-25 base pairs).

After attempting to optimize PCR parameters and determining that the correct PCR products were being amplified, we proceeded with the use of the qrt PCR to measure gene expression. The results are a compilation of three trials with error bars to represent the standard deviation between the three trials.

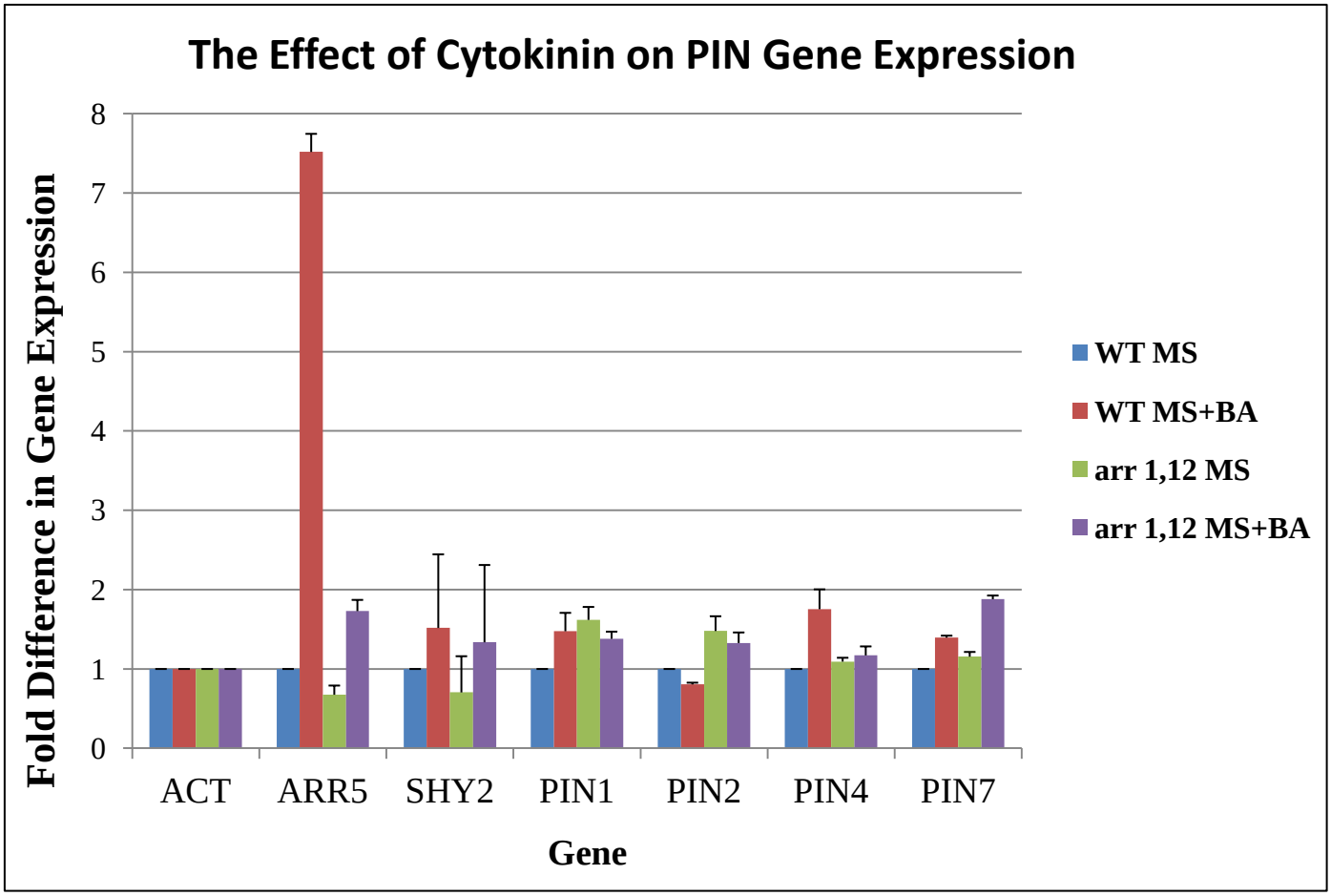

Figure 5: Gene expression values as determined by real-time quantitative PCR and normalized between treatments/genotypes to actin gene expression. Values represent the mean of three separate qRT-PCR trials. RNA was prepared from 1-2 mm root tips of 5-day-old wild-type (WT) and mutant (arr 1,12) seedlings. Mutant arr 1,12 seedlings have reduced cytokinin signaling ability. Seedlings were germinated and grown on MS media or MS combined with 1 uM of the synthetic cytokinin benzyl adenine (BA).

The four treatment groups were, WT on MS, WT on MS with BA (cytokinin) added, arr1, 12 mutant on MS, and the arr1,12 mutant on MS with BA added. The double arr1, 12 mutants is known to be particularly insensitive to cytokinin. Among type-B ARR mutants it has

been shown that the arr1, 12 mutants is the most insensitive double mutant that has been observed for root phenotypes (Mason et al).

Actin, ARR5, and SHY 2 were the controls used during the experiments. Each reaction was normalized to actin due to its fairly consistent gene expression at all times. ARR5 is a type-A response regulator and is known to be highly induced by cytokinin; Mason et al. reported a 5.9 fold induction by cytokinin in the WT. In this study, a large increase, about a 7.5 fold, in gene expression of ARR5 in WT plants grown on BA was seen consistently across all three trials, confirmed by small error bars. This is reassuring because it gives more confidence in other results. SHY2 was less of a reassurance because there were large error bars, meaning the results were fairly inconsistent throughout the three experiments. It has been shown that cytokinin can promote expression of SHY2 (Dello Ioio, 2008). Therefore an increase in SHY2 gene expression should be observed whenever exogenous cytokinin (BA) is added to the growth media and a decrease in SHY2 expression in the arr1, 12 mutants should be seen compared to the WT because it has significantly reduced endogenous cytokinin signaling. These are both evident in the experiments, but to a much lesser extent than with ARR5. Also, the error bars are large meaning there was quite a bit of inconsistency in fold difference for SHY2 between the three experiments, which lowers the confident for these results.

In WT seedlings a moderate increase in PIN1 gene expression, less than two fold, was seen when grown on MS with added cytokinin. An increase in PIN1 expression of similar proportion was seen in the mutant seedlings regardless of cytokinin treatment. In WT seedlings a moderate decrease in PIN2 gene expression with added cytokinin was observed. An increase in PIN2 gene expression in the mutant was also observed as well as an increase in the mutant with added cytokinin, but to a lesser extent. None of the changes were greater than two fold. The

largest increase in gene expression among PIN genes with added cytokinin in the WT occurred in PIN4, but it was still not greater than two fold. A minute increase was seen in PIN4 gene expression in the mutant and in the mutant with added cytokinin. In PIN 7 there was a moderate increase in gene expression with added cytokinin in the WT and a greater increase in gene expression in the mutant with added cytokinin (not greater than two fold). There was no significant change in the mutant without cytokinin. Unfortunately, no results could be obtained for PIN3 because of poor PCR amplification as shown in Fig 3 or no PCR amplification as shown in Fig 4.

Discussion

According to the model described by Dello Ioio et al. proposing a connection between cytokinin signaling and PIN gene expression, there is an inverse relationship between the amount of cytokinin signaling and the amount of transcript for PIN1, PIN3, and PIN7. This was the first model proposing a connection between cytokinin signaling and PIN activity and is still the most widely accepted by the scientific community. Subsequent data reported by others regarding the effect of cytokinin on PIN gene expression have not necessarily supported the earlier data of Dello Ioio et al. At least one more recent study has concluded that cytokinin's major impact on PIN gene expression is post-transcriptional (Zhang el at., 2011).

The current model states that cytokinin signaling is the determinant of root meristem size and therefore of root length by controlling the amount of time meristematic cells remain capable of undergoing division. It has been proposed that greater than normal cytokinin signaling will result in reduced cell division because of an earlier transition to differentiated cells. This would result in a shorter meristem and shorter roots. On the other hand, lower than normal cytokinin

signaling, as in mutants like arr1,12, is thought to allow for a larger meristem and longer roots because postponed differentiation allows for more rounds of cell division by meristematic cells. Dello Ioio proposed that cytokinin regulates the timing of this transition from dividing meristematic cells to differentiated cells by controlling auxin activity. They went on to propose that the way cytokinin regulates auxin activity can be explained by the effect of cytokinin on auxin transport through control of expression of the auxin efflux proteins PIN1, PIN3, and PIN7. They claimed that the level of cytokinin signaling is inversely proportional to the amount of PIN1, PIN3, and PIN7 expression (Dello Ioio el at, 2008).

If the current model developed by Dello Ioio is correct, that increased cytokinin signaling decreases PIN gene expression, a significant decrease in PIN gene expression should be evident when plants are grown on MS with added cytokinin (BA). Likewise, according to this model, an increase in expression of PIN genes should be seen in the mutants deficient in cytokinin signaling like arr1, 12.

In the study described here the overall changes in the level of PIN transcripts were not considered dramatic because there was no increase or decrease greater than 2 fold as shown in Figure 5.

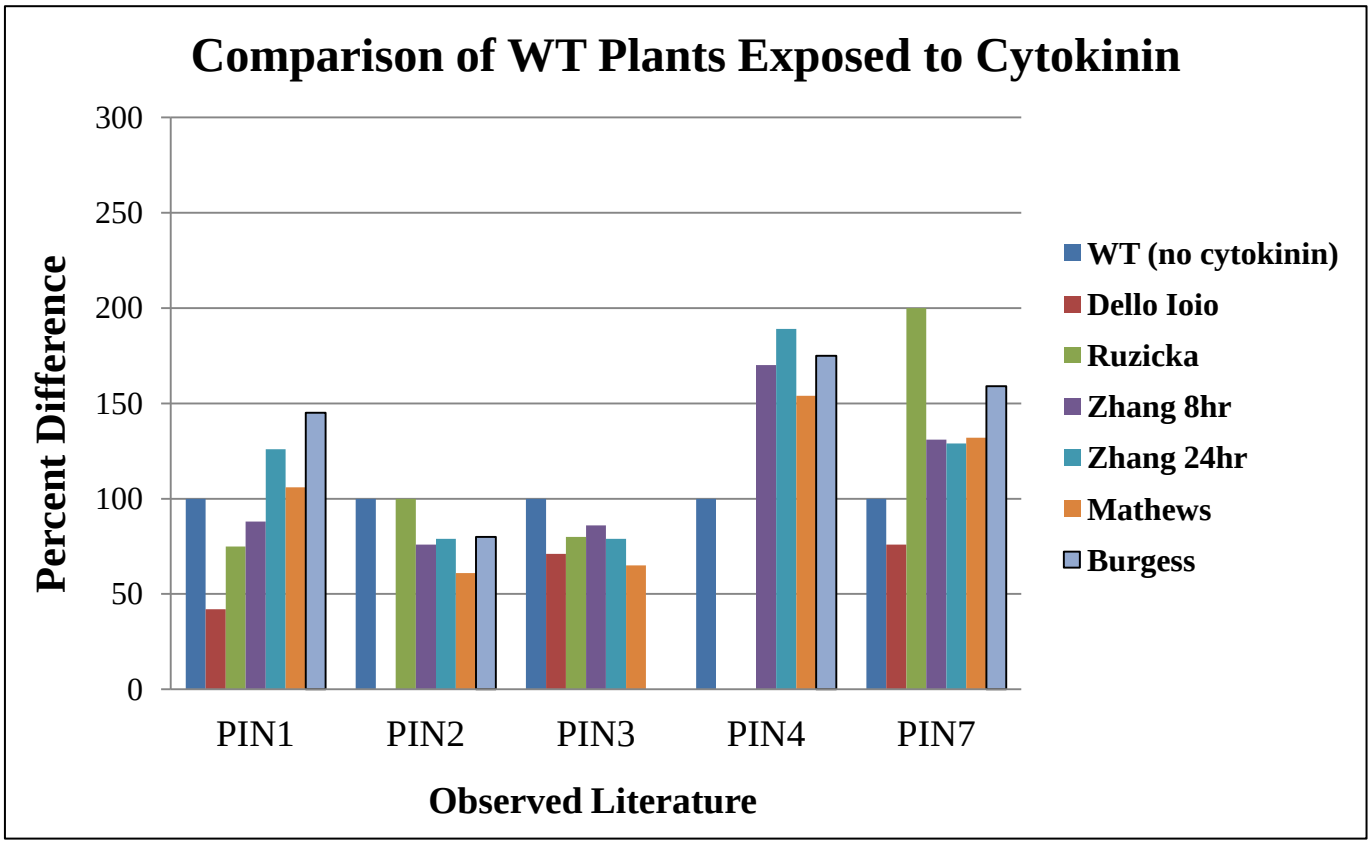

Figure 6: The data collected in this study is outlined in black. Conditions for each study differ: Dellio Ioio grew up seedlings for 4 hours on 5 uM Zt (zeatin a natural cytokinin), Ruzicka grew up seedlings for 6 hours on 5 uM BA (a synthetic cytokinin), Zhang grew up seedlings for 8 hours on 5 uM BA, Zhang performed another study and grew up seedlings for 24 hours on 5 uM BA, Mathews grew up seedlings for 5 days on 1 uM BA, and Burgess grew up seedlings for 5 days on 1 uM BA. 100% is the normal - WT seedlings were grown up on MS without added cytokinin. All studies used qrt PCR for analysis except Mathews who used the nano string approach.

A comparison of the results in this study with results from other related studies is shown in Figure 6.

As is evident by the graph, the results between these five studies are not consistent. The first published report by Dello Ioio is depicted by the red bars. As can be seen, the red bar for

PIN1 is significantly different than any other bar. The result from Dello Ioio shows more than 50% decrease in PIN1 gene expression with added cytokinin, while the results of the study reported here show the complete opposite, about a 50% increase in expression level of PIN1 with added cytokinin. The other studies shown in figure 6 observe little difference in PIN1 gene expression with added cytokinin. As for PIN7, there is a dramatic difference between the Dello Ioio publication (red) and the rest of the experiments. Dello Ioio found a decrease in PIN7 gene expression with added cytokinin of about 25%, whereas an increase in PIN7 gene expression was observed with added cytokinin of about 50%. The other studies hover around a 25% increase with Ruzica observing the greatest increase of about 100%.

This study also observed PIN2 and PIN4 which were not originally studied by Dello Ioio. This study saw a decrease in PIN2 gene expression with added cytokinin of about 25% which is consistent with results found by Mathews (unpublished) and Zhang. Ruzica saw no increase or decrease in PIN2 gene expression with added cytokinin. This study, along with Mathews and Zhang saw an increase in PIN4 gene expression with added cytokinin of about 60%.

Unfortunately, this study was unsuccessful in the attempt to study PIN3, but other studies observed a slight decrease in PIN3 gene expression with added cytokinin.

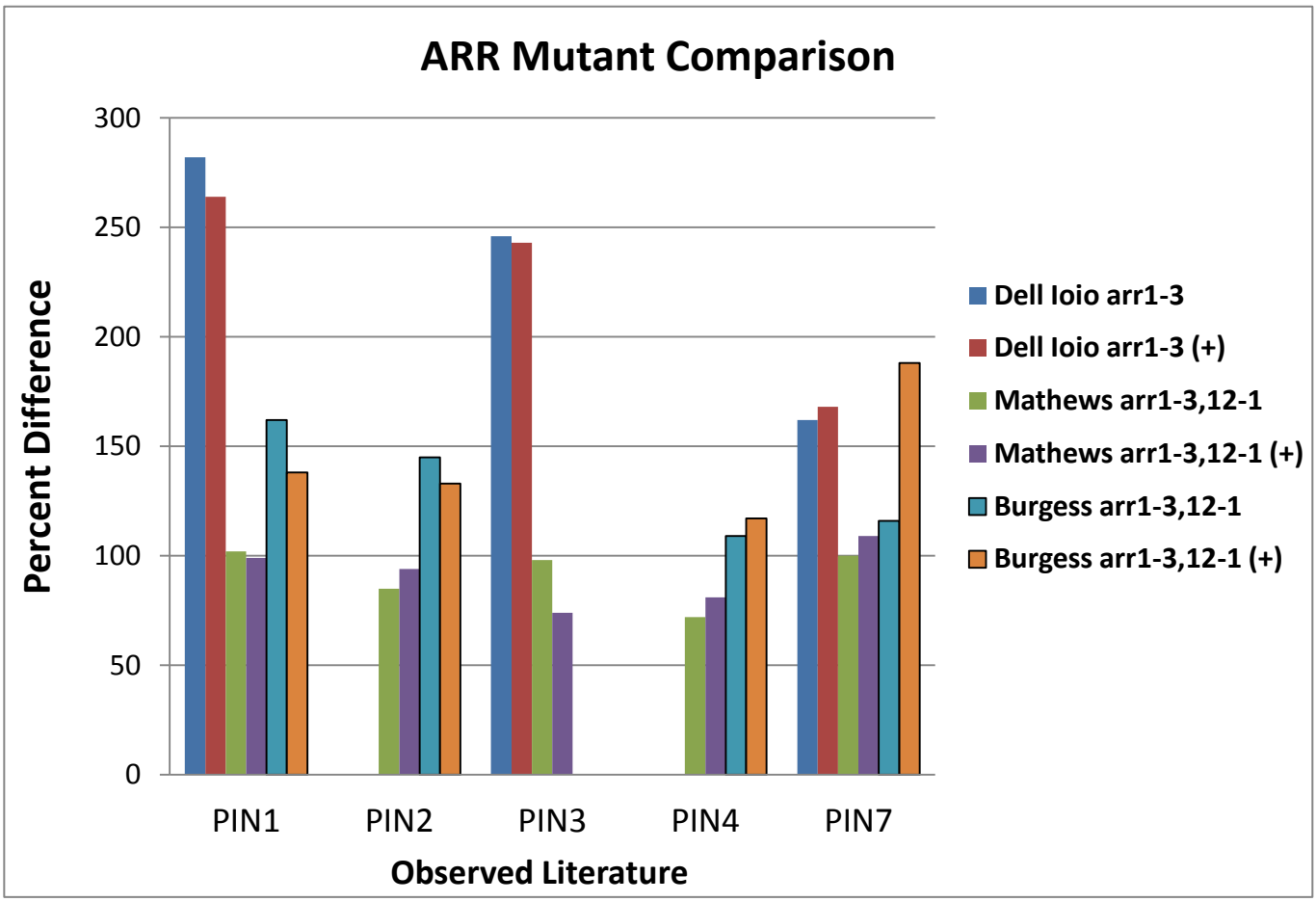

Figure 7: The data collected in this study is outlined in black. Seedlings were grown up on MS media, the plus marks indicate that the seedlings were grown up on MS media with added cytokinin. Conditions for each study differ: Dello Ioio grew up seedlings for 4 hours on 5 uM Zt (zeatin a natural cytokinin), Mathews grew up seedlings for 5 days on 1 uM BA (a synthetic cytokinin), and Burgess grew up seedlings for 5 days on 1 uM BA. Del Ioio and Burgess used the qrt PCR technique to measure gene expression while Mathews used the nano string technique.

If the model proposed by Dello Ioio is correct that there is an inverse relationship between the level of cytokinin signaling and the level of PIN gene expression, then an increase in PIN gene expression should be seen in the arr1,12 mutants because they are deficient in cytokinin signaling. Adding cytokinin to the media that these mutants are grown on should have a limited effect because the mutants are relatively insensitive to cytokinin. So, if a mutant is sufficiently deficient in signaling such that exogenously added cytokinin does not increase the

level of signaling above that in a WT plant without cytokinin then the Dello Ioio model suggests that an increase in PIN gene expression would continue to be observed despite the added cytokinin.

Dello Ioio (figure 7 blue and red bars) observed a very significant, over a 150%, increase in PIN1 gene expression with and without added cytokinin in the arr1-3 mutant. Much less of an increase was observed in this current study with the arr1-3,12-1 double mutant, which is even more deficient in cytokinin signaling than the arr1-3 mutant. Only about only a 50% increase in PIN1 gene expression was observed in the current study. Mathews did not see any change in the expression level of PIN1 using the arr1-3,12-1 mutant. Dello Ioio also saw a significant increase in PIN3 gene expression in the arr1-3 mutant with and without added cytokinin. They saw about a 150% increase in expression while Mathews saw a slight decrease with the arr1-3,12-1 mutant. Unfortunately, no results were obtained for PIN3 in this study. For this study, PIN7 gave the most striking results, with added cytokinin the arr1-3,12-1 mutant increased PIN7 gene expression by about 75%. Whereas without added cytokinin no change in PIN7 gene expression was observed. Dello Ioio reported an increase in PIN7 gene expression in the arr1-3 mutant with and without added cytokinin of slightly over 50%. Mathews, on the other hand, found no change in PIN7 gene expression in the arr1-3,12-1 mutant with or without added cytokinin.

The data collected in this study and data from subsequent studies have not been entirely supportive of the model proposed by Dello Ioio on the interaction between cytokinin and PIN gene expression. Dello Ioio proposed a significant inverse relationship between cytokinin and PIN gene expression. The data in this study found no increase or decrease in gene expression at or greater than two-fold, which calls into question the significance of any difference observed in PIN gene expression related to cytokinin signaling. Many of the other studies according to

figures 6 and 7 found results that were either less dramatic or even opposite to those reported by Dello Ioio. It should be noted that the studies reported here were not identical in their conditions. Dello Ioio used a natural cytokinin, zeatin, while other studies used a synthetic cytokinin, benzyl adenine. Also, the growth periods for seedlings differed between each study (see figures 6/7 captions). This begs the question of whether or not cytokinin does in fact play a significant role in PIN expression at the transcriptional level. Studies using PIN:GFP reporter constructs have seen a difference in PIN gene expression with added cytokinin as viewed on a confocal microscope. Ruzicka saw an increase in PIN3 gene expression with short term cytokinin treatment, but saw a decrease in PIN3 gene expression with longer treatment. Overall with added cytokinin a negative effect in PIN1, PIN2, and PIN3 was observed and a positive effect on PIN7 was observed (Ruzicka, et al.). Dello Ioio also saw a decrease in PIN7 expression using the PIN7:GFP construct and an increase in PIN1gene expression, but saw an decrease in PIN3 which is contradictory. It is possible that cytokinin could influence PIN gene expression more at the prost-transcriptional level. Further exploration is necessary because the results presented in this study serve as further evidence that the original model proposed by Dello Ioio is not completely correct and needs to be edited and reviewed further, since cytokinin induced down-regulation of PIN transcription has not been consistently observed.

Future directions of this study are numerous. Taiz and Zeiger propose in their text "Plant Physiology" that endogenous cytokinin levels in the root have an optimal level. They propose that endogenous cytokinin levels in the root are normally supraoptimal, resulting in some inhibition of root growth. Thus, if cytokinin levels are slightly reduced root growth would increase, but on the other hand if they fall below a critical threshold root growth will decrease

(Taiz & Zeiger, 2006). To explore this proposal, seedlings should be exposed to cytokinin for different periods of time and PIN gene expression should be compared.

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