The regulatory role of PIF4 and its influence on target genes

To unravel the mechanisms behind plant growth

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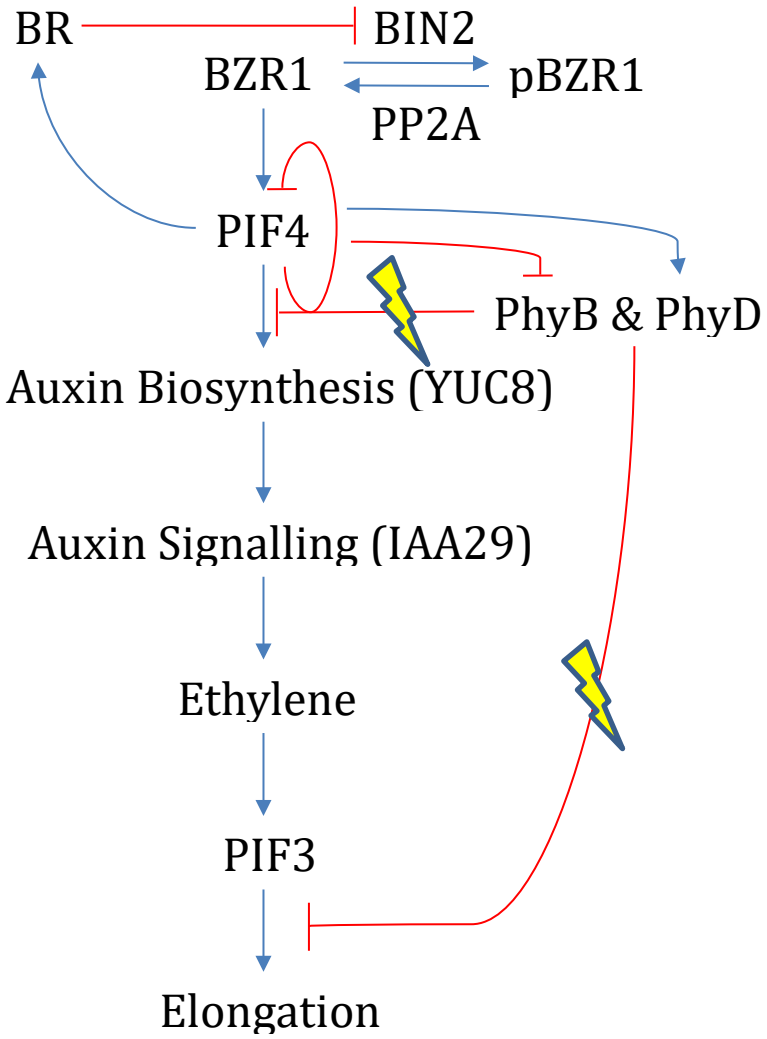

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Abstract

Plant growth is a highly regulated process which is influenced by a lot of internal and external cues. A lot is already known, yet some mechanisms remain elusive. To better understand we looked at one central transcription factor and its influences on several target genes. PIF4 is a transcription factor that binds to a g-box motif in the promoter region of genes, and can upregulate or downregulate with the transcription level, it is found that PIF4 suppresses its own promoter, but upregulates YUC8. It does not change the transcription level of IAA29 or PIL1, whilst all these genes are very important in plant growth regulation, and are (in)directly affected by PIF4. A transient expression assay found that PIF4OE has a negative influence on its own promoter, the same as MED25OE, BZR1OE does not change the promoter activity of PIF4. BZR1 is phosphorylated by BIN2, and later on degraded, making it impossible to affect PIF4. We also performed a split luciferase protein protein interaction assay on PIF4 and BZR1 with MED25, as a mutant screening showed MED25 mutants had enhanced elongation. We found that both BPIF4 and BZR1 had a direct interaction with MED25, and regulate gene expression interdependently. Lastly, we looked at the influence of PIF4 on phytochromes, specifically PhyB and PhyD. We found that PhyB is downregulated and PhyD is upregulated, showing two different ways of PIF4 regulation. To conclude, we can see that PIF4 has a lot of possibilities, and is an important systems integrator.

Preface

At the end of any Master, there comes the Master thesis. This thesis is build-up of everything a student has learned during its years as a student, but this does not signal the end of learning of said individual. This thesis is my thesis on the role of PIF4 in pant growth, and specifically the genre regulatory role. I have worked on in together with Umid Shapulatov and Sander van der Krol as my supervisors.

Both my supervisors were more than willing to answer all my questions, and provide me with expert knowledge on both experimental procedures as well as statistical analysis. My research question was formulated together with my supervisor.

I wish to thank my peers that have helped me in writing my thesis a lot more fun, and especially Job Cohen and Jolien Verweij. It wouldn't have been the same without them.

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Introduction

A plant needs only a few basic items to survive. Just water, CO_2, and sunlight go a long way in helping the plant grow. While one plant can not physically block another plant's access to water or CO_2, it can however, grow over another plant, effectively shading the lower plant. As the lower plant needs sunlight as well, it can respond to the shade in a few ways, depending on the plant species. Shade responses vary widely across plants, and include changes in leaf physiology and anatomy, but can also occur on a molecular level, changing the biochemistry of a plant completely.(SMITH & WHITELAM, 1997)

The first set of responses can be classified as shade-tolerant responses, and are found in plants that can survive with lower light intensities. These plants can adapt to a restricted allocation of resources, resulting in low growth rates, decreased elongation and reduced apical dominance. Another response certain plant species exhibit is a shade avoidance response, or shade avoidance syndrome (SAS). During SAS, a plant tries to find unshaded territory by outgrowing neighbouring plants through enhanced elongation, reduced leaf expansion and decreased branching. The value of a crop plant is mostly determined by the sheer volume it can produce. As farmers want more produce from their land, they sow individual plants closer and closer together. The crops subsequently grow over each other, resulting in shaded leaves. If a plant displays SAS, it will stop developing desirable plant parts, and invest in growth.(Casal, 2012) This is detrimental to the value of the plant, and must be avoided as much as possible.

Plant growth can be achieved in two distinct ways, the plant can make new cells, and it can elongate existing cells. Both cell elongation and cell development help plants reach nutrients and sunlight. Plant growth is regulated through several hormone pathways, making growth very complex. In response to shade, one of the first growth hormones to be affected is auxin. Auxin is used by the plant for cell elongation, and it can be up- or downregulated by several transcription factors and plant hormones.(Roig-Villanova & Martínez-García, 2016) If a plant experiences an increase in auxin, it will start by elongating the hypocotyl. This is the same interaction found in normal plant growth, although the auxin levels during SAS are found to be higher.(Carabelli et al., 2007) By controlling auxin levels, it is possible to control SAS, and thus plant growth.(Keuskamp, Pollmann, Voesenek, Peeters, & Pierik, 2010)

To be able to respond to light, a plant has several phytochromes that are affected by light. These phytochromes, in turn are able to change the biochemistry in a plant by binding to phytochrome-interacting factors (PIFs).(KEBROM, BRUTNELL, & FINLAYSON, 2009) The phytochromes exist in two forms, active and inactive. It is possible to go from one form to the other through the absorption of red and far-red light, where the active form can bind to a PIF. Some PIFs are shown to be able to bind directly to DNA, like PIF3, making it an excellent transcription factor for light-regulated genes, such as CHS involved in the biosynthesis of anthocyanin. (Ni et al., 2013; Shin, Park, & Choi, 2007) Other PIFs can depend on other transcription factors to be able to regulate gene expression, such as PIF4.

PIF4 is mostly known as a systems integrator in plant development, and its influence is widespread in plant gene regulation. It is able to both suppress and stimulate gene transcription by binding to a specific motif in the gene promoter region, called the g-box motif.(Sun, Qi, Li, Zhai, & Li, 2013) This motif is found in a lot of genes, and is fairly common. Currently, the influence of PIF4 on these genes is not a complete overview.

PIF4 has been shown to accumulate in the dark, and when it comes in contact with light-activated phytochrome B (PhyB), it is ubiquitinated and quickly degraded, resulting in SAS.(Lorrain, Allen, Duek, Whitelam, & Fankhauser, 2007) However, overexpression lines of PIF4 show a constitutive SAS. It is not yet known what the exact reason for this outcome is. PIF4 is shown to regulate up-stream genes in the auxin generating pathway. Through this regulation, PIF4 is able to induce SAS, and thus cell elongation, by AUXIN/INDOLE-3-ACETIC ACID (IAA) genes IAA19 and IAA29.(Casal, 2012; Franklin et al., 2011) PIF4 is shown to also bind to its own promoter sequence, forming a feedback loop. Preliminary research shows that PIF4 inhibits its own transcription. PIF4 is able to stimulate the Brassinosteriod pathway, which in turn activates BZR1, a known activator of PIF4. This would mean that PIF4 has a positive feedback on itself. Our main research question then becomes: *"Is PIF4 a positive or negative regulator of PIF4 expression?"*

From literature, it is known that PIF4 also has interactions with other transcription factors, but it is not known which of these interactions all are contributing to cell elongation. One of the interactions that has been shown to influence cell elongation is the BZR1-PIF4 interaction. BZR1 and PIF4 are shown to interact (Oh et al., 2014)and are able to integrate hormonal and environmental cues to regulate up to 2000 genes together, which include cell-elongation-promoting genes.(Oh, Zhu, & Wang, 2012) This shows that BZR1 and PIF1 interdependently regulate cell elongation. BZR1 functions as a regulator in the brassinosteroid pathway, it is currently not known how BZR1 interacts with PIF4, although it is proposed to form a dimer to regulate gene expression. Both over-expression lines and agro-infiltrated lines of BZR1 have shown an increase in plant growth, and lack-of-function mutants show dwarfism, dark-green leaves, and reduced fertility. (Goda, Shimada, Asami, Fujioka, & Yoshida, 2002) When BZR1 is translated, it is formed in its inactive form, phosphorylated. It is only when BZR1 is dephosphorylated that it becomes active. (Q.-F. Li et al., 2017a) The phosphorylation and dephosphorylation are the main on-off switch, and are highly regulated by other transcription factors BIN2 and PP2A. (Gupta, Singh, & Laxmi, 2015; He et al., 2005) BIN2 is also known to phosphorylate PIF4, thus providing more than one switching mechanism. (Q.-F. Li et al., 2017b). The brassinosteroid pathway is able to enhance the presence of BZR1 in the cell by blocking the transcription of BIN2. As BIN2 is blocked, there is no more deactivation of BZR1, and the total amount of active BZR1 increases by PP2A. Figure 1

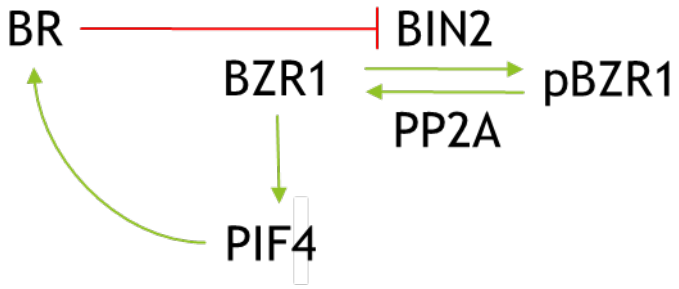


Figure 1 The activation and in activation of BZR1 plays an important role in the effectivity of BZR1. PP2A and BIN2 can dephosphorylate and phosphorylate BZR1 to activate and inactivate it respectively. PIF4 has an up regulatory role in the BR pathway, which in turn downregulates BIN2.

Another transcription factor that can play a role in cell elongation is MED25, it is a sub-unit of the mediator complex in plants that is involved in the transcriptional regulation.(Iñigo, Alvarez, Strasser, Califano, & Cerdán, 2012) The Mediator complex serves as a link between transcription factors and RNA polymerase II. It can acetylate and deacetylate gens by recruiting histone acetylases and histone deacetylases respectively, depending on which transcription factors bind to it. MED25 has been shown to interact with transcription factors that bind to the g-box, and it is important in down-stream processing of PhyB, that regulates flowering time.(Elfving et al., 2011). During mutant screenings, it was found that MED25 mutant plants had an elongated phenotype. This was very peculiar, as there was now not a complete mediator complex to conduct transcription factor cues to the RNA polymerase II Figure 2. A direct interaction between PIF4 and MED25 has not been proven at this point, but it is mentioned in preliminary research. Furthermore, an interaction between BZR1 and MED25 has been hypothesised, but no research has been done in this direction so far. This is one of the objectives of this research. If we were able to couple the function of MED25 to that of PIF4 that would dramatically increase our understanding of the inner workings of the regulatory pathways in plants. Both PIF4 and MED25 seem to have a role in plant growth, as is shown by these mutant screenings and previous literature, and this leads to the research question*: "Do MED25 and PIF4 show interaction on protein level?"* As BZR1 is also involved in the cell elongation process by regulating PIF4, we were also wondering; *"Do MED25 and BZR1 show interaction on protein level?"*

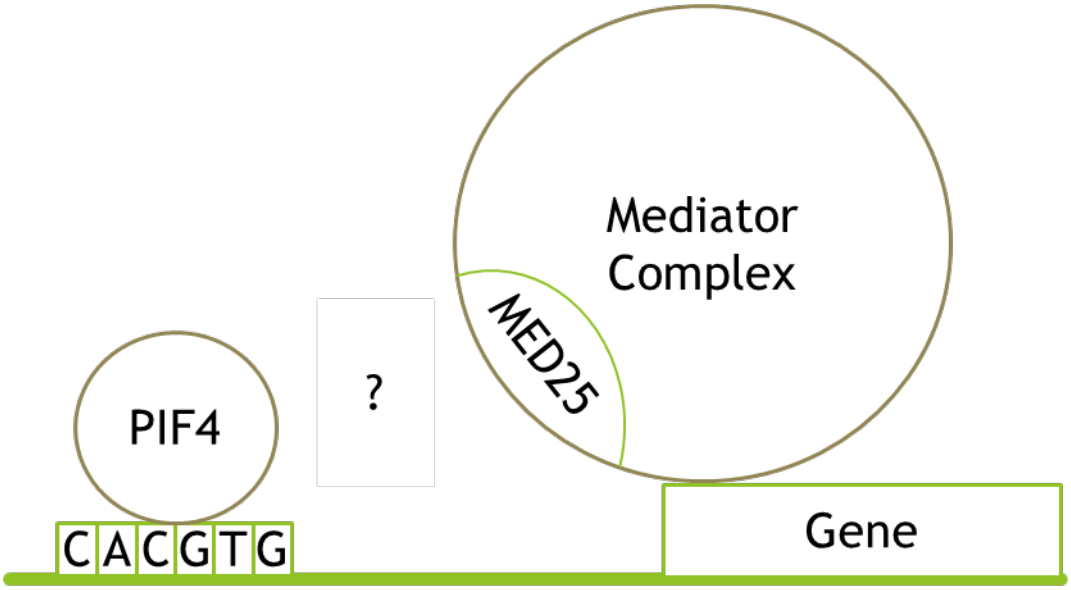


Figure 2 The Mediator complex binds to a gene and forms the link between the gene and transcription factors. Here it is shown to interact with PIF4, but what specific interaction takes place is not yet known.

To be able to establish the mechanism behind all this, we designed a series of experiments. First, we want to see with which other proteins PIF4 has an affiliation. As PIF4 and BZR1 already have been shown to interact(Oh et al., 2012), we now lay our focus on the interaction with MED25. Continuing, we see how certain different regulators affect the expression of PIF4, and what its own feedback mechanism is. We then see how PIF4 influences its target genes, and whether it is possible to influence gene regulation directly, or that it needs a full complex to more efficiently regulate gene expression. Lastly, we look at the influence of PIF4 on PhyB and PhyD, both have a g-box motif in their promoter, meaning that PIF4 would be able to bind to them and regulate transcription levels. Whether PIF4 upregulates or downregulates these genes is still a point open for discussion in literature. (Gangappa, Berriri, & Kumar, 2017; Kim et al., 2017; Kunihiro et al., 2011; Lorrain et al., 2007; Yang et al., 2001). This research should show us the answer to the following research question: *"What is the influence of PIF4 on PhyB and PhyD transcription?"* We focussed only on transient expression assay experiments here, and not on mutant screenings.

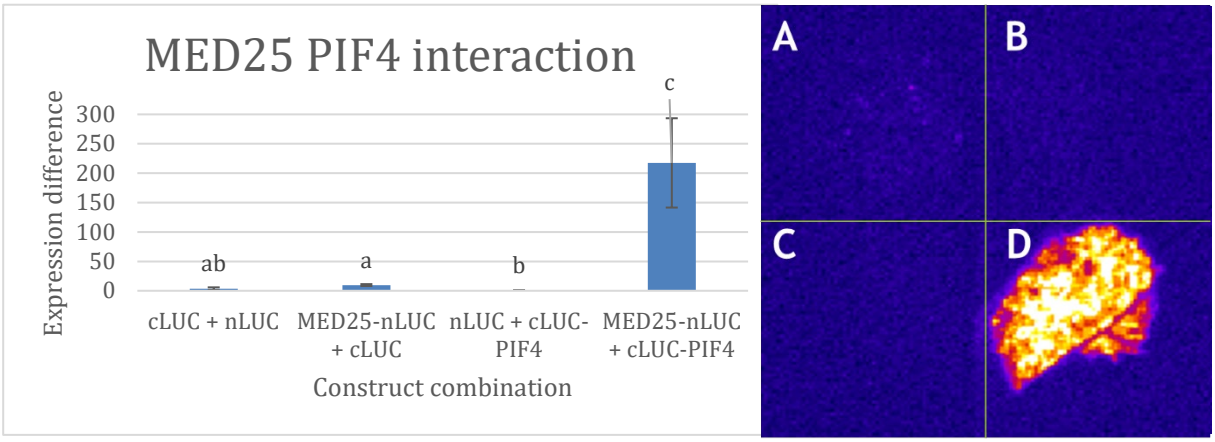
Results

MED25 interaction assay

MED25 is a sub-unit of the mediator complex. In recent research, it has been shown to interact with a lot of other protein and can regulate a lot of genes (Ou et al., 2011). In this experiment, we fused the MED25 protein with the n-terminal end of luciferase, to create MED25-nLuc. The other protein was fused with the c-terminal end of luciferase, to create cLuc-PIF4 and cLuc-BZR1. After co-expression of both combinations, we looked for luciferase activity under the Luminator camera. This split luciferase assay allowed us to see which proteins exactly bind to each other. We included 3 negative controls to check for direct binding between the nLuc and cLuc construct. In both assays, we had no random binding of nLuc and cLuc construct.

MED25 protein interacts with PIF4 protein

After the overexpression of the newly fused proteins MED25-nLuc and cLuc-PIF4 and the control proteins, a clear expression pattern was visible under the Luminator. In Figuur 3, we can see that the last combination, that of MED25-nLuc + cLuc-PIF4 has the highest expression observed. A high increase in luciferase activity is shown, resulting from the reconstitution of luciferase as a consequence from the binding of MED25-nLuc and cLuc-PIF4. The negative controls do not show significant deviation from the background.



Figuur 3 The results from the split luciferase protein assay, showing direct binding of PIF4 to MED25 (n=9). There was no to little spontaneous reconstitution found of the luciferase enzyme. A = nLuc + cLuc; B = MED25-nLuc + cLuc; C = nLuc + cLuc-PIF4; D = MED25-nLuc + cLuc-PIF4. On the left the results are quantified

MED25 protein interacts with the BZR1 protein

Another protein that is very important for plant growth is BZR1. BZR1 stimulates PIF4 expression, and in turn, PIF4 stimulates the BR pathway, which supresses the inactivator of BZR1, BIN2. (Figure 1) This stops the inactivation of formed BZR1 and forms a positive feedback of BZR1 and PIF4 on PIF4.

In the protein-protein interaction assay with BZR1 and MED25, a significant ($p<0.05$) increase in luciferase activity was found. As the three control combinations yielded a small to no increase, we see a clear difference in luciferase activity, and thus a binding of MED25 to PIF4 Figure 4

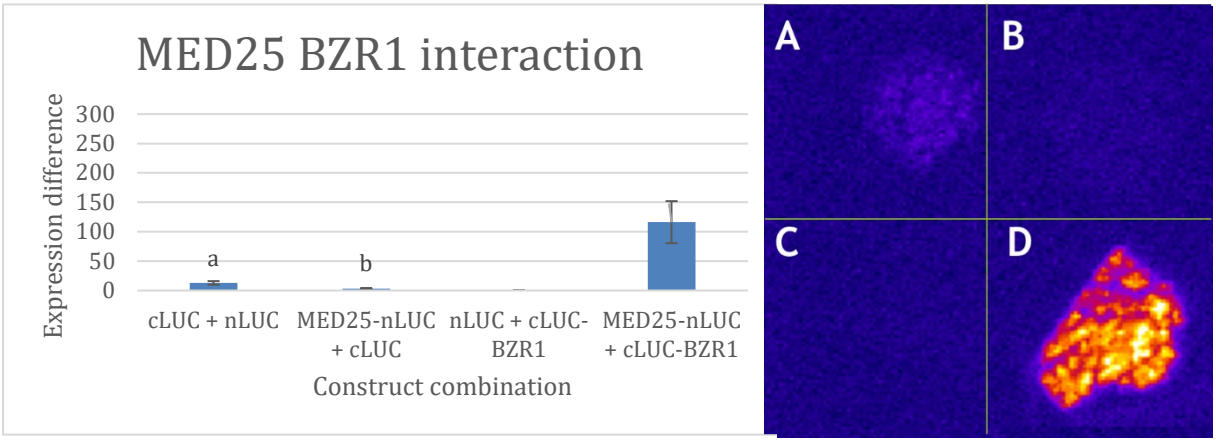


Figure 4 The results from the split luciferase protein assay, showing direct binding of BZR1 to MED25. (n=10). There was no to little spontaneous reconstitution found of the luciferase enzyme. A = nLuc + cLuc; B = MED25-nLuc + cLuc; C = nLuc + cLuc-BZR1; D = MED25-nLuc + cLuc-BZR1. On the left the results are quantified

Other experiments

Protein interaction experiments were repeated using Med25-nLuc and cLuc-(BZR1/HDA9/HMR/PIF3/PIF4). cLuc-PIF4 and cLuc-BZR1 were used as a positive control. All combinations showed no difference compared to the background, and as PIF4-MED25 interaction was previously proven, the results were considered inconclusive and not taken into account for the conclusions. (Results not shown)

Transcriptional regulation of PIF4

PIF4 shows a negative feedback to its own promoter activity

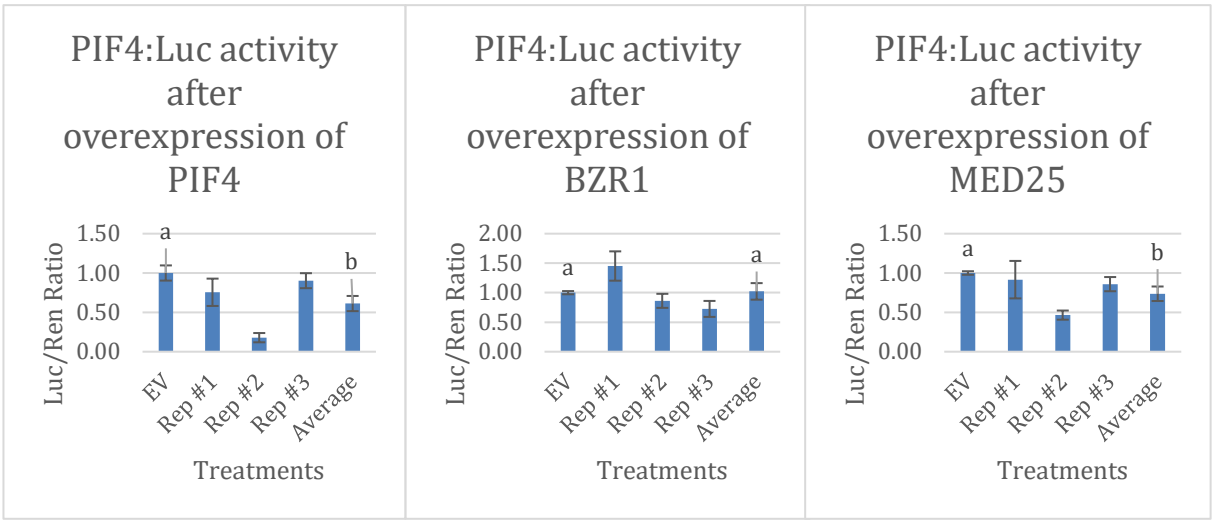
To see the influence of PIF4 protein activity, we analysed in a transient expression assay what the overexpression of PIF4 changed in the expression level of its own promoter. By fusing a PIF4 promotor to the gene encoding for luciferase, we were able to accurately measure these changes. A correction for total protein content and expressivity of the leave was needed, therefore an internal control was co-infiltrated. After correction for these factors, it was found that there was a significant ($p<0.001$) decrease in promoter activity compared to the infiltration with an empty vector control Figuur 5. The total decrease found was 39%.

Does BZR1 stimulate PIF4 expression?

As we looked at growth regulatory genes, another important one was BZR1, short for "Brassinosteroid signalling positive regulator 1". It was hypothesized that an overexpression of this gene would lead to an increase in PIF4 activity. As we compared the control with the overexpression of BZR1, we found that there was no significant change in promoter activity for PIF4 ($p=0.874$), the average change in promoter activity was a 2% increase. In comparison with all other treatments, an overexpression of BZR1 leads to a higher promoter activity ($p<0.1$)

MED25 overexpression regulates a decrease in PIF4 promoter activity

Med25 encodes for a larger complex that regulates RNA transcription. Here, it was found that an overexpression of MED25 lead to a decrease in PIF4 transcription of about 26 %($p<0.05$). This overexpression is significantly lower than BZR1 ($p<0.05$), but not than PIF4 overexpression ($p=0.35$).



Figuur 5 As we compare the results from the different overexpression, we see that PIF4 and MED25 downregulate PIF4 promoter activity, and BZR1 does not influence PIF4 promoter activity.

PIF4 & BZR1 together decrease PIF4 promoter activity

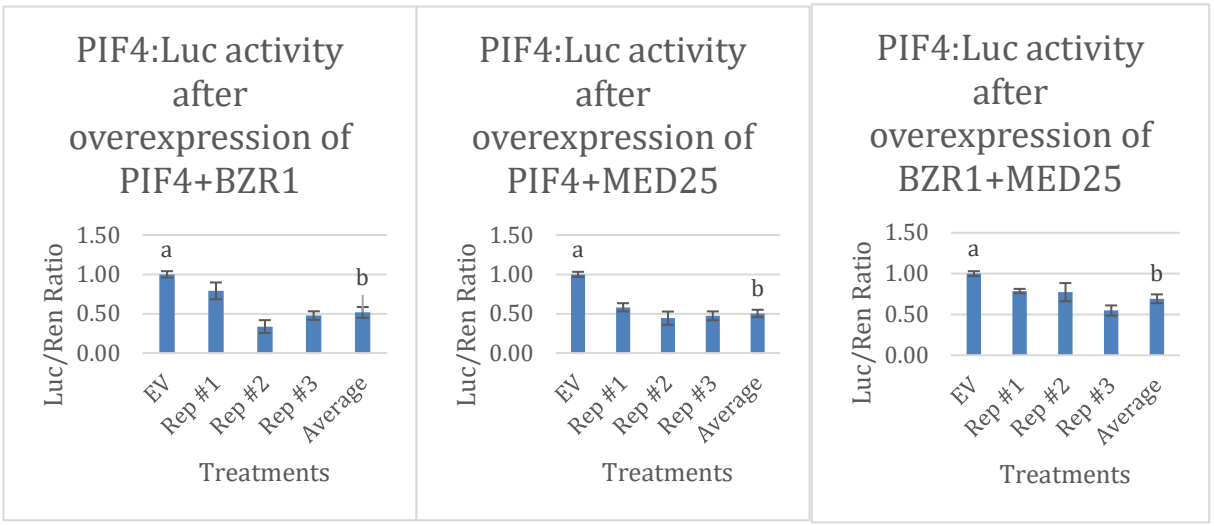
In the co-infiltrations, we overexpressed multiple genes to see the total effect of the two genes. In this assay, we only looked at the total expression, and did not do dosage experiments, to look for possible protein interactions. In the co-infiltration of PIF4 and BZR1, we found a decrease in PIF4 promoter activity of 48%.Figuur 6 This is significantly different from our control ($P<0.05$) and BZR1, but not from any other treatment ($p=0.36$-0.66).

PIF4 & MED25 together decrease PIF4 promoter activity the most

We found that, when we overexpressed both PIF4 and MED25, that we got the highest reduction of PIF4 promoter activity of the whole experiment. A reduction of 50% was measured ($p<0.05$).Figure If we compare the treatment to the other treatments, we see that it is significantly lower than both BZR1, MED25, BZR1+MED25 and the three way co-infiltration of PIF4+BZR1+MED25.

BZR1 & MED25 together decrease PIF4 promoter activity

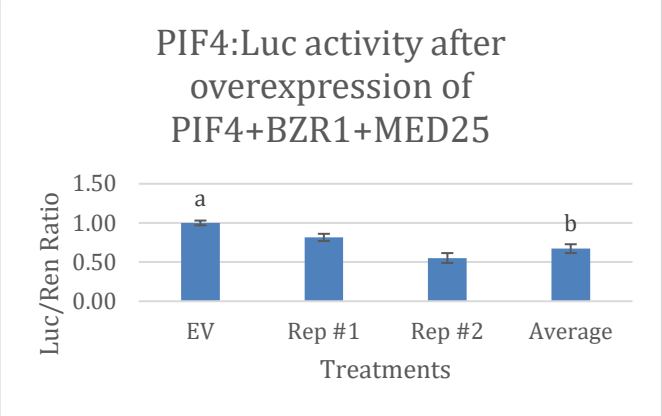
As the last treatment of a dual co-infiltration, we used BZR1 and MED25 overexpression. What we found was a significant decrease of PIF4 promoter activity of 31% Figuur 6($p<0.05$), this decrease was significantly higher than the change in BZR1 alone, and significantly lower than the decrease in PIF4+MED25



Figuur 6 After overexpressing different constructs, we can see their influence on PIF4 promoter activity. Each time, two different constructs were overexpressed. All three combinations affected reporter activity negatively

PIF4 & BZR1 & MED25 together decrease PIF4 promoter activity

Lastly, a three-way co-infiltration was performed using all three constructs, PIF4, BZR1 and MED25. We found a significant reduction in promoter activity of 33% Figuur 7($p<0.05$). In comparison, we see that is significantly different from the overexpression of PIF4+MED25.



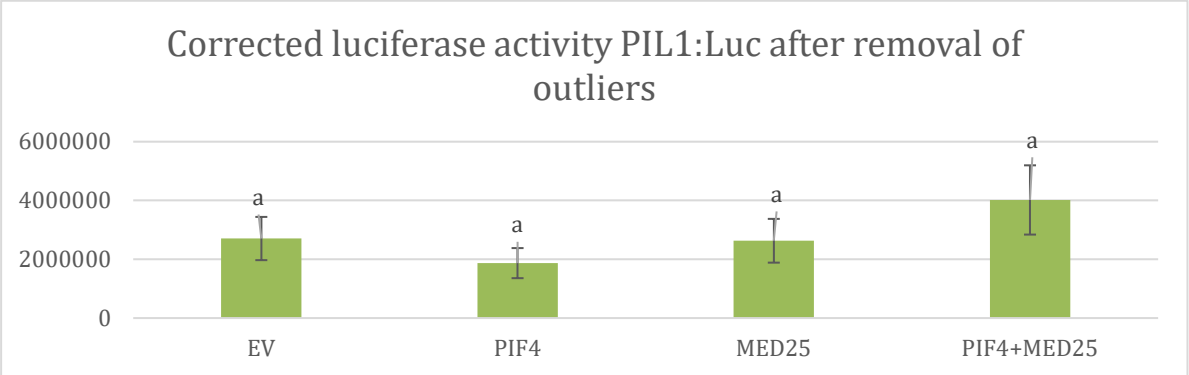
Figuur 7 PIF4 activity after overexpression of PIF4, BZR1 and MED25 is reduced by 33%

Target regulation by PIF4 and MED25

The auxin regulatory pathway is one of the more studied plant pathways there is, and there is good reason for it. As auxin controls one of the most vital parts of plant life, plant growth, a good understanding of this plant hormone can help us improve plants more precise. As we are looking primarily at the regulatory role of PIF4 in plant growth, it was natural to look at some genes that have proven to be very important in plant growth. By comparing single expressions of PIF4 and MED25 and co-infiltrations of both, we can see how we can incorporate light and temperature sensitivity in the SAS pathway. The genes that we are looking at are all known regulators of SAS and plant growth. The main reason we think these genes are affected by PIF4 is that their promoters contain a g-box motif, and thus PIF4 can bind to their promoter.

PIL1 is not affected by either the expression of PIF4, MED25 or the combination of both

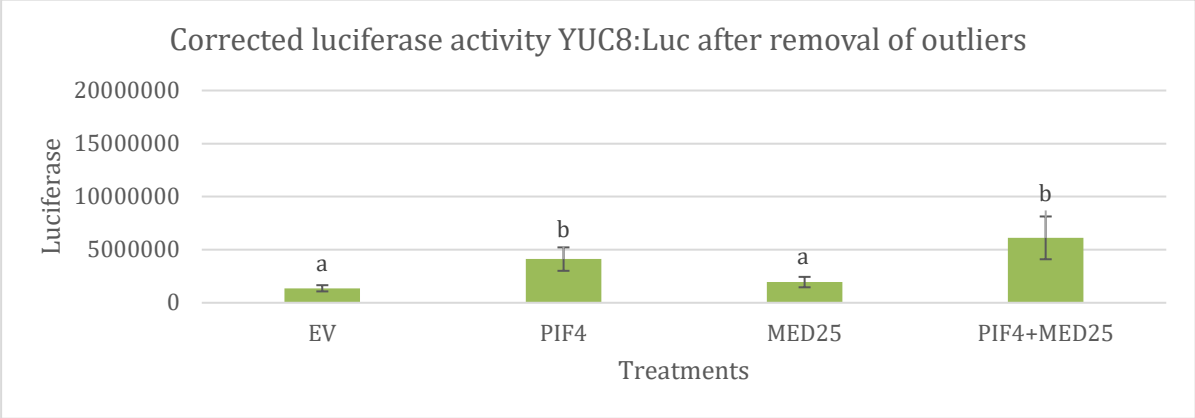
It is known from pull-down experiments that PIL1 and PIF4 have a physical interaction. The role of PIL1 in the whole scheme of SAS is not yet completely known,(L. Li et al., 2014) therefore we did a series of tests where we overexpressed PIF4, MED25 and PIF4+MED25 to see what would happen with the expression of PIL1. By measuring the Luc/Ren ratio, we were able to accurately measure the changes in promoter activity. What we can see from Figuur 8 is that both PIF4OE and MED25OE do not have a significant influence on the promoter activity of PIL1.



Figuur 8 Regulatory role of PIF4 and MED25 on PIL1 by transient expression assay. Outliers were detected using the Univariate method. Both the overexpression of PIf4 and MED25 do not affect reporter and effector activity. (n=10 – 1 outlier removed)

YUC8 is activated by PIF4

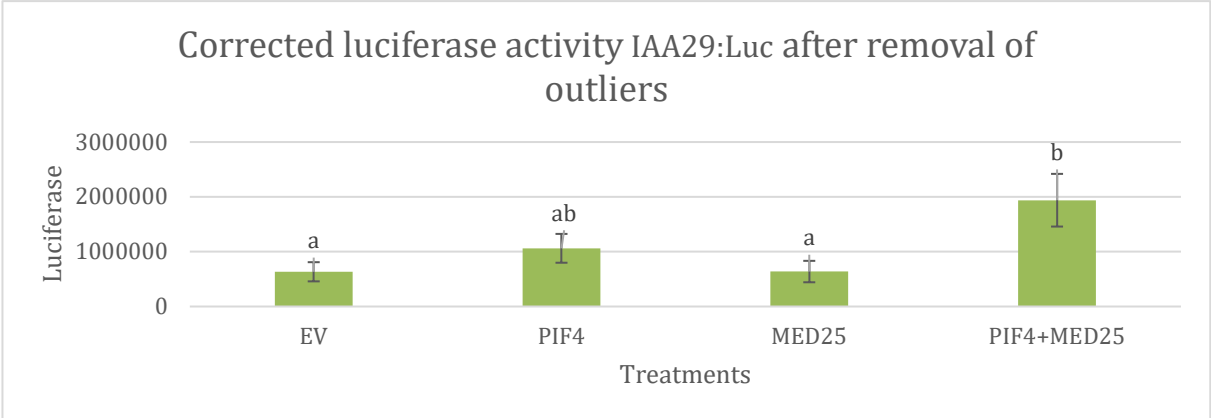
PIF4 is a known activator of the auxin biosynthesis gene YUC8, which integrates temperature into the auxin biosynthesis pathway. Whilst YUC8 mutant lines have shown to suppress the PIF4 overexpression, a direct interaction is not yet proven.(Sun, Qi, Li, Chu, & Li, 2012) In this experiment, we explore the influence of PIF4, MED25 and PIF4+MED25 on the expression of YUC8, and see if the addition of MED25OE makes a significant difference. In Figuur 9, we see that the overexpression of PIF4 upregulates the expression of YUC8 immediately with a threefold increase. An increase in the Luc/Ren ratio is also visible in the MED25 overexpression treatment, although not significant. The biggest increase in promoter activity is seen in the co-infiltration of PIF4OE+MED25OE. This increase is significantly different from the empty vector- and the MED25 treatments, although not from the PIF4 overexpression treatment.



Figuur 9 Regulatory role of PIF4 and MED25 on YUC8 by transient expression assay. Outliers were detected using the Univariate method. MED25 is not affecting reporter or effector activity. PIF4 is stimulating reporter activity. (n=10 – 1 outlier removed)

IAA29 is positively regulated by the combination of PIF4 and MED25

In 2013, it was proven by (Sun et al., 2013) that the direct activation of IAA29, which binds to auxin response factor 7, is enough for PIF4 to negatively regulate auxin signalling. We do see this back in our own results. The overexpression of PIF4 leads to a direct increase in IAA29 promoter activity, and thus to an increase of IAA29 in the plant Figuur 10. MED25OE is not able to change the expression of IAA29, but together with PIF4OE, it is able to double the promoter activity.

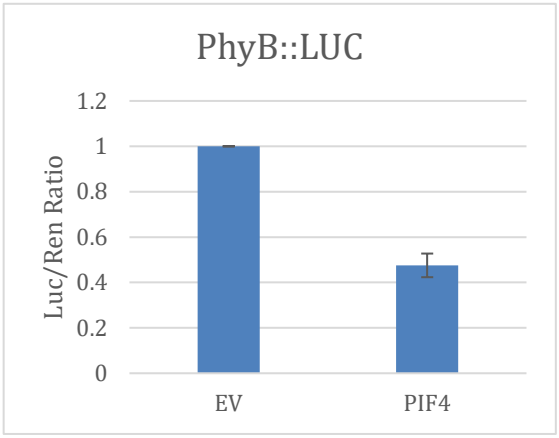


Figuur 10 Regulatory role of PIF4 and MED25 on IAA29 by transient expression assay. Outliers were detected using the Univariate method. MED25 is not affecting reporter activity, but is affecting effector activity. PIF4 together with MED25 is stimulating reporter activity. (n=10 – 1 outlier removed)

PIF4 regulatory role in Phytochromes

PIF4 represses PhyB promoter activity

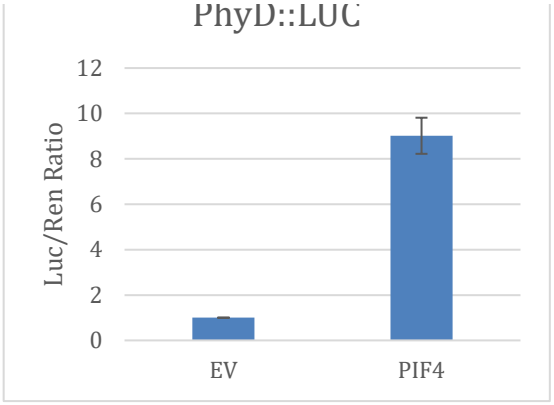
PhyB is one of the most important plant genes in respect to plant growth, and is proven to be interacting with PIF4 (Phytochrome Interacting Factor 4). After overexpression of PIF4, a strong significant decrease is observable in promoter activity of PhyB.



Figuur 11 Influence on promoter activity of PhyB after overexpression of PIF4, PIF4 supresses PhyB transcriptional activity

PIF4 stimulates PhyD promoter activity

PhyB and PhyD share 80% DNA sequence similarity between them, and they are probably originating from the same gene in the past, but do not fulfil the same function anymore. In the past, the respective roles of PhyB and PhyD have changed a few times, sometimes they were thought to be redundant genes/proteins, whereas more recent articles just mention a small overlap in function. (Devlin et al., 1999; Sharrock, Clack, & Goosey, 2003) What is consistent through time, however, is that, like PhyB, PhyD plays an important role in plant growth and SAS regulation. What we see in our experiment, is that an overexpression of PIF4 lead to almost a nine-fold increase in promoter activity for PhyD. To ensure no mistakes were made, we performed a PCR with specific markers. This PCR confirmed that no accidental mishaps occurred. (Results not shown)



Figuur 12 Influence on promoter activity of PhyD after overexpression of PIF4, PIF4 stimulates PhyD transcriptional activity

Discussion

MED25 interaction assay

As we started this experiment, we already had some preliminary data and ideas that MED25 was involved somehow with PIF4 and BZR1, but a clear answer was not yet found. By doing a protein-protein interaction assay, we were able to prove that MED25 formed a direct connection with both BZR1 and PIF4. Shown for both MED25-nLuc+cLuc-PIF4 and MED25-nLuc+cLuc-BZR1 is an efficient reconstitution of the luciferase enzyme. BZR1 does show a lower reconstitution than PIF4, this difference can be explained by the nature of the two proteins. When BZR1 is formed, it is phosphorylated by BIN2, and targeted for degrading. This could lead to a lower amount of active BZR1-cLuc in the cell, and subsequently a lower level of luciferase. PIF4, on the other hand, is not influenced by BIN2, and can immediately bind to MED25. In a yeast-2 hybrid system, no interaction was found between MED25 and either PIF4 or BZR1.(Iñigo et al., 2012; Ou et al., 2011) . This shows that not all protein interactions can be studied in a yeast-2 hybrid system, but some need an in planta approach.

As BZR1, PIF4, and MED25 are all able to bind to each other, we now know that they cooperate to regulate target genes, but we do not know if they are able to bind all together. From preliminary research, we know that PIF4 binds to the GD domain of MED25, but we do not know if this blocks the binding to either BZR1 and PIF4 or MED25 and BZR1. Further experiments are being undertaken, but do not offer more clarity at this moment of writing. It is not known if the interactions are mutually exclusive, or they can happen at the same time.

Transcriptional regulation PIF4

As we compare all the results that were obtained from all the different treatments, we can shed a light on the individual roles that PIF4, BZR1 and MED25 play in the regulatory pathway to either enhance or reduce promoter activity for PIF4. We know that PIF4 plays an important role in plant growth, and knowing what lies beneath the activation of this hormone can help us better understand the growth of plants altogether.

From the first experiment with the single overexpression of PIF4, we can immediately see that PIF4 has a negative feedback on itself. This seems to be an important mechanism, to keep hormone levels, and thus plant growth in check. from the other treatments, we can only strengthen our initial conclusion, as all treatments where PIF4 is overexpressed show a negative relation with PIF4 promoter activity. This is in coherence with earlier findings. As PIF4 regulates plant growth and flowering time (Kumar et al., 2012), we expect a mechanism that keeps the total amount of PIF4 in check, and this appears to be in the role of self-inhibition. This self-suppression of PIF4 is apparently stronger than the stimulation by the increased amount of dBZR1.

The relation of MED25 with PIF4 promoter activity seems also to be a negative one. All treatments where MED25 was overexpressed show a significant decrease in promoter activity. PIF4 compared with MED25 did not show a significant result, but whereas PIF4 shows a difference if we compare the single overexpression with the double overexpression of PIF4+MED25, MED25 does not. This led us to the conclusion that, while both are reducing promoter activity, PIF4 is a stronger reducer than MED25. A link between MED25 and PIF4 can be found in the signalling pathway of photoperiodic regulation, and a direct or indirect interaction must take place to be able to influence its promoter activity, which is discussed later.

At first, it was hypothesized that an overexpression of BZR1 would yield a higher promoter activity for PIF4 (Oh et al., 2012), but this is not what we found. In the single overexpression (Figure 5), we saw an equal promoter activity, and this trend continued for the double infiltrations. There is no different result shown for the infiltrations of either PIF4OE or MED25OE versus PIF4OE+BZR1OE and BZR1OE+MED25OE. There is however one influence detectable of the infiltration of BZR1. If we compare the infiltrations of PIF4OE+MED25OE with PIF4OE+BZR1OE+MED25OE, we do see a higher promoter activity for the three way overexpression infiltration. This can arise from a few different factors; one of them being a statistical error, although not probable. Another explanation can be found in the physiological lay-out of the plant. A direct interaction between BZR1, MED25 and PIF4 is found during other experiments and it is possible that

this complex is needed for BZR1 to fully function and have a significant effect on the plant. As discussed previously, BZR1 needs to be activated by PPA2 to be able to have its proper function in the plant (Q.-F. Li et al., 2017b). By overexpression of BZR1, only inactivated BZR1 is formed, and the plant only has its endogenous BZR1 activated.

In follow-up research, it is recommended to take this into account. We saw minimal influences of BZR throughout the whole experiment. If we are able to effectively activate BZR1, we can better study its function in planta.

Target regulation by PIF4

As mentioned before, there are many processes underlying the phenomenon of plant growth, one of them being SAS. To incur SAS, there are multiple genes that need to cooperate together leading to an appropriate response. The genes that we looked at during our experiment are PIL1, YUC8 and IAA29. YUC8 and IAA29 are integrated in the direct formation and sensitivity of auxin, and are responsible for regulating plant responses more directly than the other 2. PIL1 is proven to be influenced by the environment, thus can conduct certain stimuli from outside the plant to the inside, and is linked with shade avoidance.(Casal, 2012) Both PIF4 and PIL1 have a negative feedback loop on their own promoter activity, and can bind to each other (L. Li et al., 2014). What we see is that PIL1 is not influenced by either PIF4, MED25 or the combination of both. Currently we have no definitive reason for this finding, that is opposite from the hypothesis. The most probable explanation is that exogenous PIF4 and MED25 do not function as the endogenous proteins. Endogenous MED25 is highly affiliated with HDA9, and thus histone modifications, whereas our overexpression only elevated the level of MED25 that was not bound in a histone complex. This also correlated with what we found in the split luciferase interaction assay, but it needs to be researched more in depth.

In low auxin concentration Aux/IAA proteins can bind to Arf proteins, thus stopping the auxin responsive genes, but in high auxin concentrations, these complexes are ubiquitinated and the response genes are activated. (Luo, Zhou, & Zhang, 2018) By activation of these genes, the plant can grow and escape shade environments. This is also what we see in our results. An increase in PIF4 leads to an increase in both IAA29 and YUC8, responsible for auxin sensitivity and productivity, respectively. This increase could lead the plant away from unfavourable conditions, ensuring the plant's survival, showing the role of PIF4 in SAS.

The role of MED25 seems only to be important in the expression of IAA29. Other target genes of PIF4 are not regulated by MED25. As MED25 is strictly not a transcription factor, this was as expected. That MED25 is not important in transcriptional regulation, can be because it is not limiting for the gene transcription. In a cell, there is already enough MED25, and additional MED25 is not needed to cope with the increase in transcription factors.

As is shown in Figure 5 & Figure 6, a large spread in the repetitions of the same experiment. A factor that has to be taken into account when interpreting these results. The reproducibility of the assays is a problem, which means that a large number is needed for proper statistical analysis. This large variation can arise by a number of factors; the first being the difference in temperature between experiments. A higher trend of PIF4 activity seemed to result from higher temperatures (Results not shown), and as experiments were undertaken, the temperature rose to 37° C in the Netherlands. (No temperature measurements were taken in the greenhouse)("Het weer in juli in Wageningen 2018 - De voorspelling van AccuWeather voor Gelderland Nederland (NL)," n.d.). A more temperature-controlled environment could prevent this. Second, the transcriptional level between leaves was off. Some leaves were very active, and some leaves were very inactive. A correction was possible with the Renilla activity, but sometimes lead to large correction factors, which are undesirable.

PIF4 regulatory role in Phytochromes

As we compare the different results from PhyB and PhyD after overexpression of PIF4 (Figure 11& Figure 12), we can see that there is a strong regulatory role for PIF4, being repressing and enhancing, respectively. For now we have mostly been looking at the regulatory role of PIF4 on pant growth, but PIF4 has other functions that link it with PhyB and PhyD, such as plant defence mechanisms. (Gangappa et al., 2017) PIF4 is a positive regulator of plant growth, but a negative regulator of plant defences, and this trade-off lies at the basis of plant survival. PhyB itself is a negative regulator of plant growth, and influences plant growth by changes in the Red/Far-Red ratio of light that falls onto the plant. PhyB is able to degrade PIF4, after light activation, and thus plays a vital part in the stopping of the hypocotyl growth under dark conditions. In this case, PIF4 has a positive feedback on itself, and by reducing PhyB production, it provide the means for the build-up of PIF4 in the cell. The results found back up previous research performed in mutant lines.(Huq & Quail, 2002)

PhyD has previously been proposed to be a degrader of PIF4 (Kunihiro et al., 2011) and, as we have previously established, PIF4 has a negative influence on its own promotor activity. Thus, PIF4 enhancing the activity of PhyD is to be expected, and this is also what we found. The exact role of PhyD is not a well-documented one, and this understanding helps us in further studies. These findings also help us get a grasp on the underlying molecular working of the upstream processing of PIF4.

When we combine the two findings, we see a positive feedback and a negative feedback of PIF4 on its own promotor. Currently, all our findings have been done in natural light conditions. PhyB needs to be activated by light to degrade PIF4. We think that in light conditions, PIF4 has a negative influence on its own promotor, and in dark conditions, PIF4 has a positive feedback on itself, to enable build-up of the protein. More research is to be undertaken to find more evidence for these hypotheses. These findings show there are different roles for PhyB and PhyD to be played in the plant.

Conclusion

The purpose of this series of experiments was to better understand the mechanism of SAS, with a focus on the role of PIF4. This understanding was needed as a basic background for future experiments, and to see how we can manipulate plants to grow in a certain way, or to keep plants small. Both can be useful for crops and ornamentals. As we looked at the influence of PIF4, BZR1 and MED25 on the promoter activity, we saw that both PIF4 and MED25 had a down regulatory role. This negative feedback of PIF4 on itself was confirmed using a PIF4OE line, where an increase of ectopic PIF4 resulted in a decrease in endogenous PIF4 (results not shown). We did not see an influence of BZR1 during this experiment, but this can be explained by the fact that BZR1 needs to be activated. The two- and three-way infiltrations confirmed our hypothesis and were in line with the other results. The role of BZR1 is still unclear, and its activation needs to be taken into account during follow-up research.

In the next experiments, we proved that BZR1, MED25 and PIF4 can form dimers. This excludes a possibility of a mediating or blocking protein in the complex. It also narrows down our potential influencers, and makes research easier. The next step is to see how these dimers, or perhaps complete complex is able to regulate gene expression. We now know that MED25 has a direct interaction with PIF4 and BZR1, and thus forms the link between the mediator complex and these transcription factors.

We know that in the pathway to elongation, a lot of genes are active. IN this study, we focussed on three genes specifically. PIF4 was able to bind to all these three genes, as they have a g-box motif in their promoter. YUC8 was the only gene activated by PIF4. As this gene is the first downstream gene of PIF4, this was as expected, but what we also found was that further downstream, genes were not affected by an overexpression of PIF4, thus PIF4 has a limited range of genes it can affect, even though PIF4 can bind to the promoter, it does not change the transcription level.

As we continued with genes that had this g-box motif, we wanted to know the influence of PIF4 on phytochromes. PhyB and PhyD are both able to interact with PIF4, and both can be transcriptionally

regulated by PIF4. We found that both PhyB and PhyD are influenced by PIF4, but in completely different directions. PhyB is downregulated, while PhyD is upregulated. We do not know why this is, as they are previously thought to be redundant proteins, with an 80% sequence similarity.

Materials and Methods

Plants:

For testing the inner workings of PIF4, *Nicotiana Benthamiana* was used, at 4-6 weeks old. The plants are grown under natural conditions during the whole experiment.

Constructs:

Transient expression and Luminator assay

For the transient assays and the measurements done using the Luminator, the same constructs were used. The constructs; 35S:PIF4, 35S:MED25, 35S:BZR1, PIF4:Luc, PIL1:Luc, IAA29:Luc, YUC8:Luc, PhyB:Luc, PhyD:Luc were made available by Umidjon Shapulatov, working under Sander van der Krol at Wageningen University and Research.

Protein-Protein interaction assay

The constructs used for the protein-protein interaction assay; nLuc, cLuc, MED25-nLuc, cLuc-PIF4, cLuc-BZR1 were made available by Umidjon Shapulatov, working under Sander van der Krol at Wageningen University and Research.

Agro-infiltration:

In preparation for infiltration, bacteria were transfected to complete liquid medium with appropriate antibiotics, and after 24 hours, 50 uL of the cell suspension is transferred to fresh complete liquid medium. After 24 hours the cell suspension is centrifuged at 4000 rpm for 5 minutes. The supernatant is discarded and the cell pellet is resuspended in 20 mL 10mM MES, 10mM MgCl2 and 0.1% Acetosyringone. Plants were not watered for two days prior to infiltration. To test for the separate effects of different transcription factors, several combinations were made of constructs, and tested against the control combination on the same leaf. To make sure every construct is present in the same concentration, the optical density (OD) was measured of each bacterium in buffer, and finally an OD of 0.2 was used for infiltration. P19 was used to stop PTGS from occurring in infiltrated leafs as a response to overexpression of the constructs. (Voinnet, Rivas, Mestre, & Baulcombe, 2003) Every construct was added in equal concentrations, with an exception of empty vector, which was adjusted to ensure an equal gene dosage. Six leaves were infiltrated per construct combination. After infiltration, the infiltrated area was marked with a black marker, and the plant was subsequently provided with water on the soil.

Harvesting

After agro-infiltration, harvesting was done three days post inoculation.

Luminator

Each leaf was harvested from the plant and transferred to the Luminator for the luciferase assay. The leaves were kept in a semi-enclosed box with wet paper to stop them from drying out.

Renilla measurement

Pooling of samples

At first, we tried to pool different biological samples to account for fluctuations between different samples, Different leaf disks were added together in the same tube, and subsequently processed together. This turned out not to give the desired outcomes. The fluctuations between samples were too big, and less samples meant a smaller correction factor. Subsequently, all different biological replicates were treated as independent samples.

Treating independent samples

From each leaf, three leaf disks were taken for technical replicates. These leaf disks were treated independently, and processed as such. Immediately after extracting the leaf disk, each leaf disk was frozen in liquid nitrogen, and stored at -80C° until further processing.

Measurements

Luminator

Luciferase activity was measured using the Luminator made available by Wageningen University and Research with an exposure time of 10 minutes. 30 minutes prior to imaging, the leaves were sprayed with 1 mM Luciferin.

For quantification of the luciferin activity in leaves, ImageJ was used. An equally sized area was used in measurement of the activity per construct combination, and measurement was done on the most active spot per infiltration. The average score of the control combination per treatment, and the background luminescence were used to normalize the results.

Renilla

Each leaf disk is frozen in liquid nitrogen and subsequently pulverised with a stainless steel bead in the shaking machine. 200 mL of passive lysis buffer was added to each sample, vortexed, frozen in liquid nitrogen and put on ice. After 10-15 minutes on ice, samples were vortexed and centrifuged on 14800 rpm for 3 minutes. The supernatant was pipetted on a 96-wells plate for measurement in the Glomax machine.

The subsequent measuring was done according to the specifications of the kit. Through co-infiltration with a vector containing the Renilla gene, it was possible to quantify the amount of protein inside leaf material labelled with luciferase.(Loening, Fenn, Wu, & Gambhir, 2006) By dividing the amount of Luciferase by the amount of Renilla to obtain a normalised value of luciferase activity. This approach was taken, to be able to normalize the activity per leaf. As all samples had 3 technical replicates, the average was taken from these samples. Technical replicates were all similar (Δ>1%). (Results not shown)

Statistical analysis

For detection of outliers, we used Tukey's boxplot method. The outliers were removed for analysis. This method is also known as the univariate method for detection of outliers(*A Review and Comparison of Methods for Detecting Outliers in Univariate Data Sets*, 2006) Significance was tested using the two-sided Students T-test for significance.

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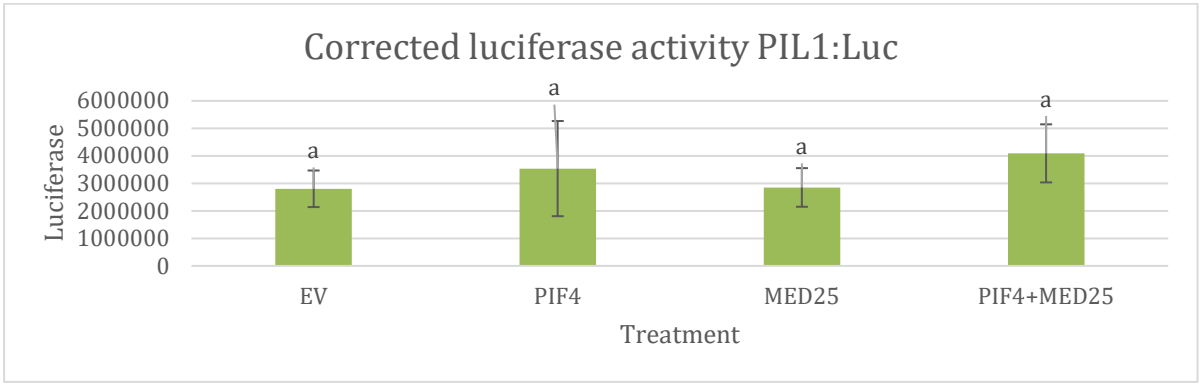
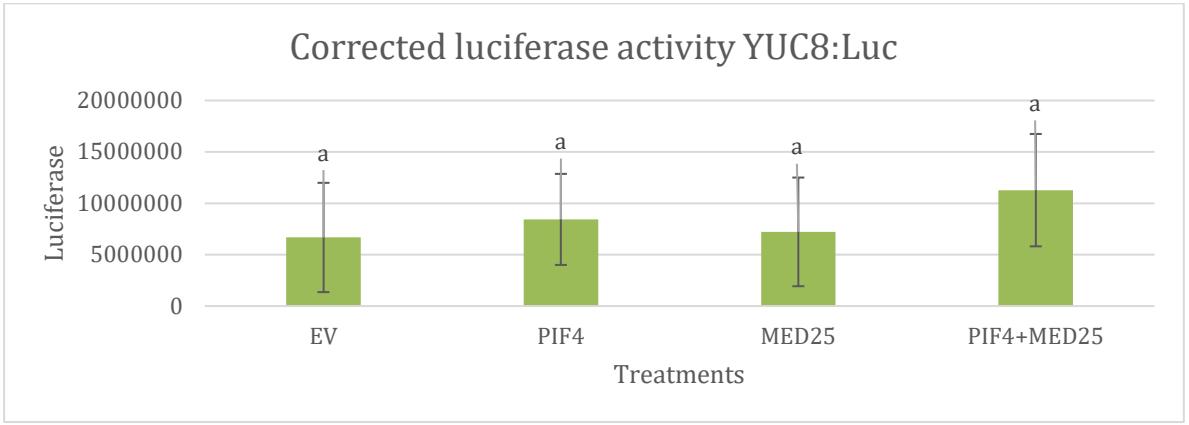
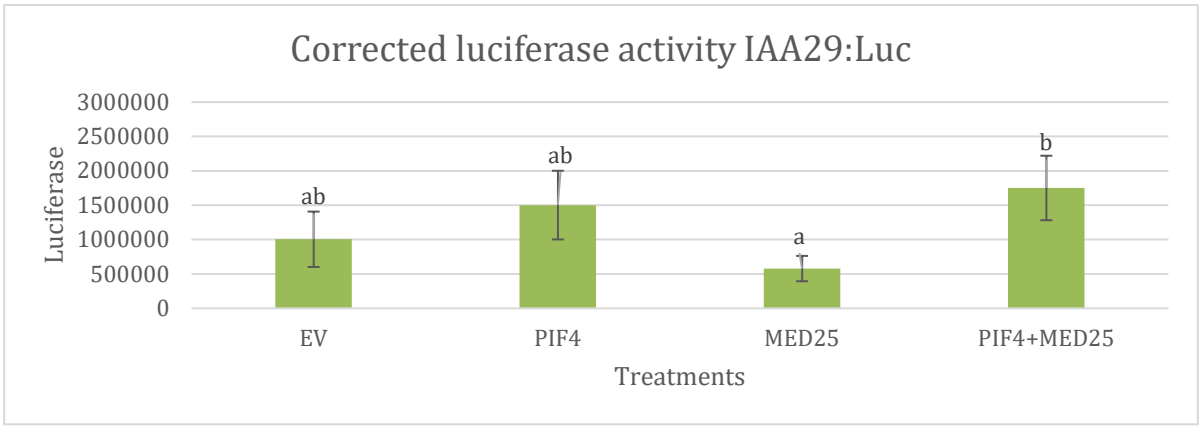
Appendix

PIF4 target genes without removal of outliers:







Planning Luminator:

File/Folder name	Data	Remarks	Location
29May2018_ExpUmid_Laurens	MED25 interaction experiment with BZR1/PIF4/PIF3/HMR/HDA9	Experiment failed, positive control negative	Luminator left
30-31May2018-Laurens	MED25 interaction experiment with BZR1/PIF4/PIF3/HMR/HDA9 Same as previous to see if it reconstituted after time	Experiment failed, positive control negative	Luminator Left
31May2018_Laurens	MED25 interaction experiment with BZR1/PIF4/PIF3/HMR/HDA9 Same as previous to see if it reconstituted after time	Experiment failed, positive control negative	Luminator Left
Interaction assay	Pilot experiment to see binding of MED25 to PIF3&PIF4	No positive control	Luminator right
interaction under temp	Experiment to see influence of light on binding of MED25 and PIF4, data in file attached	Light is negative influencer of reconstitution	Luminator right
Metingen 12-3	EV against PIF4OE, BZR1OE, MED25OE, PIF4OE+BZR1OE, PIF4OE+MED25OE, PIF4OE+BZR1OE+MED25OE, respectively.	Not used for report	Luminator right
Metingen 26-3	EV against PIF4OE, BZR1OE, MED25OE, PIF4OE+BZR1OE, PIF4OE+MED25OE, BZR1OE+MED25OE, PIF4OE+BZR1OE+MED25OE, respectively.	Not used for report	Luminator right