TESTING THE INFLUENCE OF CYP83A1, CYP79F1, AND CYP79F2 IN AUXIN ACCUMULATION AND LATERAL ROOT INITIATION IN ARABIDOPSIS THALIANA

By
ERIKA BROOKE HAWS

A Thesis Submitted to The Honors College
In Partial Fulfillment of the Bachelors degree
With Honors in
Molecular and Cellular Biology
THE UNIVERSITY OF ARIZONA
DECEMBER 2021

Approved by:

Dr. Frans Tax
Department of Molecular and Cellular Biology
Abstract

The role of CYP83A1, CYP79F1, and CYP79F2 in auxin accumulation and lateral root initiation in *Arabidopsis thaliana* was explored. These enzymes are at branch points between auxin synthesis and synthesis of plant defense compounds, found in nearly all plants of the order Brassicales, known as glucosinolates: our hypothesis was to test whether these enzymes functioned with XIP1/CEPR1 to control auxin levels or perform some other unknown function. Two lines of knockout or null mutants for each gene were obtained to cross into a line homozygous for a semi-quantitative auxin reporter (R2D2), a null mutation of an auxin biosynthetic enzyme (NIT1), and a receptor that acts in nitrogen foraging and interacts with NIT1 (CEPR1). The genotypes of the CYP83A1 and CYP79F1 knockout lines were confirmed. Both CYP83A1 knockout lines were successfully crossed into the R2D2 line. One of the CYP83A1 lines, ref2-1, was crossed into all the relevant mutants, selfed for three generations and plants homozygous for ref2-1, xip1-1, and R2D2 were isolated; these plants are heterozygous for nit1-1.

Introduction

Nitrogen is an essential nutrient for development and growth in plants. As nitrogen is not uniformly distributed in soil, plants have developed mechanisms to efficiently acquire it in a heterogeneous nitrogen environment. When different parts of the root system of *Arabidopsis thaliana* are exposed to soil or growth media with high and low nitrogen concentrations, root growth is enhanced specifically in high nitrogen and repressed in low nitrogen (Tabata et al. 2014) (Fig 1.). This phenomenon is called nitrogen foraging behavior and results in differential development of lateral roots and nitrogen-uptake, via transcription of nitrogen transporters in roots growing in more favorable N conditions. Small C-terminally encoded peptides (CEPs) control nitrogen demand signaling and lateral root development. CEPs are secreted in response to local nitrogen conditions then trafficked through the xylem and act as long-distance signals between the root and shoot of the
plant. High nitrogen conditions result in upregulation of *CEP1* and *CEP9*, and downregulation of *CEP3*, *CEP5*, and *CEP13* (Roberts, 2013). A receptor-like kinase called C-terminally Encoded Peptide Receptor 1 (CEPR1), also called Xylem Intermixed with Phloem (XIP1), participates in this long-distance signaling mechanism between the roots and the shoot of *Arabidopsis thaliana* and is the receptor for the small C-terminally encoded peptides. This response is lost in mutants of CEPR1 (Tabata et al., 2014). CEP moves from the roots to the shoot where it is recognized by CEPR1, leading to the production of secondary signals, CEPD1 (CEP downstream 1) and CEPD2 (Ohkubo et al., 2017). Another polypeptide with a high degree of sequence similarity to CEPD1, CEPD-like 2 (CEPDL2), works cooperatively with CEPD1 and CEPD2 to contribute to nitrogen acquisition and mediate root nitrogen uptake. Loss of any of these leads to a disruption of nitrogen homeostasis and reduction in shoot nitrate content. The complete loss of all three proteins severely impairs N homeostasis in plants (Ota, 2020). One group conducted immunoprecipitation experiments on CEPR1 to help understand how this receptor might initiate signaling and to identify potential phosphorylation substrates. This preliminary immunoprecipitation has revealed that CEPR1 interacts with about fifty different proteins, including other kinases, a group of transporters, and a group of metabolic enzymes.

Among the biosynthetic enzymes identified that interact with CEPR1 are several other proteins that could modulate auxin synthesis, including Nitrilase1 (NIT1). NIT1 acts in auxin biosynthesis and hydrolyzes indole-3-acetonitrile (IAN) into indole-3-acetic acid (IAA), better known as auxin (fig. 3). NIT1 is also the most highly expressed of the nitrilase genes in *A. thaliana* (Normanly et al., 1997). Lehmann, T. et al. found that when NIT1 is over-expressed (NIT1ox), plants exhibit increased numbers of lateral roots and shorter primary roots. The NIT1ox plants showed a clear increase in IAA as expected, as NIT1 converts IAN to IAA, but unexpectedly IAN was also increased. A nit1 null mutant showed no significant alterations in auxin content, which may reflect a compensatory effect of other nitrilases in the same family with redundant functions or a compensatory effect via metabolic flux. The role of other nitrilases was supported by data from a RNAi knockdown experiment designed to target Nit1-4. The expression of all NIT- isoenzymes was suppressed and resulted in considerably decreased total
IAA content. The NIT1 protein has several residues that are experimentally confirmed phosphorylation sites under various stress conditions (fig. 2). Phosphorylation may play an important role in regulating its activity; however, the kinases that phosphorylate these sites remain unknown.

Auxin is the oldest studied plant hormone; it is a morphogen and plays a role in almost every stage of plant development. Auxin accumulation occurs in the pericycle just prior to lateral root initiation and lateral roots fail to develop if auxin is inhibited at these sites (Casimiro et al., 2001). The presence and relative accumulation of auxin in a plant tissue can be visualized using the semiquantitative auxin reporter R2D2 (Liao et al., 2015). R2D2 is a transgene composed of three elements. The first is a methotrexate resistance gene with a 35S promoter for selection of plants containing the transgene. Since the 35S promoter is not ideal for expression during many developmental processes, the other elements are expressed from a RPS5A promoter. The RPS5A gene encodes the A. thaliana ribosomal protein S5, one of the proteins from the small ribosomal subunit. The RPS5A promoter is active in the majority of actively dividing cells making it ideal for study of auxin localization in growth and development. The second element is a protein that is degraded in the presence of auxin called auxin-dependent degradation domain II (DII) fused to n3xVenus, a yellow fluorescent protein. Absence of yellow fluorescence indicates auxin accumulation. The third element is an inactivated mutant version of DII, mDII, which is expressed at the same levels, but it is not degraded in the presence of auxin. mDII is fused to ntdTomato a red fluorescent protein. The red fluorescence serves as a baseline for comparison to the yellow fluorescent protein. A decreased relative quantity of yellow to red florescence indicates auxin accumulation, and the levels of florescence are quantifiable at the level of individual cells allowing for precise visualization of auxin accumulation during development. This transgene can be crossed into a mutant Arabidopsis thaliana line to visualize how the loss of a particular gene or environmental treatment impacts auxin localization and concentration.

The ~10.2 kb R2D2 transgene insertion is located on chromosome one. The insertion site was located using TAIL-PCR (Liu & Chen, 2007).

Preliminary data has also implicated the enzymes Cytochrome P450 83A1(CYP83A1), CYP791, and CYP79F2 as interactors of CEPR1. These CYPs catalyze reactions in the biosynthetic pathways of IAA (auxin) and indole and aliphatic glucosinolates. The pathways share an intermediate, indole-3-acetaldoxime.

![Figure 3](image-url)
biosynthesis of aliphatic and indole glucosinolates, organic compounds that play a role in plant defense against herbivory. CYP79F1 and CYP79F2 are tandem repeated genes that share 89% amino acid sequence similarity and have partially redundant functions. Tantikanjana et al. 2004 used a GUS reporter and found these proteins have nonoverlapping gene expression patterns and are developmentally distinct. They found that CYP79F2 expression was developmentally regulated and occurred primarily in the root system, they observed no expression in the shoot. Tantikanjana et al. 2001 found that CYP79F1 expression was restricted to the shoot and was not expressed in roots. They found that expression patterns in vasculature were developmentally regulated. Developmental expression data from Schmid et al. 2005, in the Arabidopsis eFP Browser (fig. 4 and fig. 5) does not show a nonoverlapping gene expression pattern and found that CYP79F1 and CYP79F2 mRNA is expressed in the root procambium, hypocotyl, cotyledons, rosette leaves, and stems. These data imply that CYP79F1 and CYP79F2 mRNA are expressed in the same tissues, but their proteins localize differentially. Additionally, while they exhibit some functional redundancy in substrates, they each have distinct functions in the biosynthesis of glucosinolates. CYP79F1 metabolizes both short- and long-chain methionine derivatives to their corresponding oximes CYP79F2 solely metabolizes long-chain elongated methionine derivatives to oximes (Chen et al., 2003). The hypothesis that these genes have distinct functions in development is strengthened by the phenotypes of knockout mutants. The cyp79F1 mutant displayed severe defects in shoot architecture, and it had a very bushy phenotype, whereas the defects of the cyp79F2 mutant predominantly appear in the root system. The same number of lateral roots were present in the cyp79F2 mutant, but lateral root length was impaired when plants were grown in soil (Tantikanjana et al., 2004). The presence of these root defects only in cyp79F2 mutants and not in cyp79F1 mutants supports that the CYP79F2 gene acts in root growth and CYP79F1 does not. If CYP79F1 and CYP79F2 do in fact have nonoverlapping gene expression patterns, this could explain the phenotypic differences seen in their mutants.

As seen in figure 3 the indole glucosinolate and auxin biosynthetic pathways share an intermediate, indole-3-acetaldoxime (Mano et al., 2012). This link between the pathways and the physical interaction between CEPR1, shown using immunoprecipitation, and enzymes of both of these pathways provide evidence to suggest some kind of feedback between the two. CYP83A1 catalyzes the conversion of aldoximes to thiohydroximates and is expressed in the entire rosette, cotyledons, hypocotyl, siliques, and stem. mRNA expression data is shown in figure 5. Mutants of cyp83A1 have no visible phenotype (Hemm et al., 2003). This may be due to the ability of CYP83B1, another cytochrome p450 enzyme in A. thaliana, to metabolizes aliphatic oximes derived from chain-elongated homologs of methionine, albeit with lower efficiency. CYP83B1 and CYP83A1 both metabolize oximes derived from the aromatic amino acids; phenylalanine, tryptophan, and tyrosine. CYP83A1 has lower affinity for these aromatic oximes than CYP83B1, notably this includes indole-3-acetaldoxime. (Naur et al., 2003). CYP83B1 may be able to compensate to some degree for the loss of CYP83A1.
The goal of this research is to understand how NIT1, CEPR1 and these putative glucosinolate biosynthetic enzymes modulate auxin contributions to the environmental control of lateral root growth using mutants in which the protein products of these genes have been rendered useless or expression completely eliminated. My hypothesis is that a decrease in auxin biosynthesis leads to an increase in glucosinolate biosynthesis because of increased bioavailability of the intermediate indole-3-acetaldoxime and phosphorylation-dependent flux between auxin and glucosinolate pathways. The receptor-like kinase CEPR1 has been shown to interact with NIT1 and may phosphorylate NIT1 at one of its experimentally confirmed phosphorylation sites. This proposed phosphorylation could regulate auxin biosynthesis in response to environmental conditions, particularly nitrogen conditions in soil. In addition, glucosinolate production would be impacted as this pathway is connected with the auxin biosynthesis pathway.

Further strengthening this proposed relationship, a cyp83A1 knockout mutant exhibits auxin overaccumulation in roots (Hemm et al., 2003). This may occur due to disruption of the flux of metabolites between the auxin and glucosinolate biosynthesis pathways. A decrease in indole-3acetaldoxime being used to make glucosinolates may resulting in an increase in substrate for synthesis of indole-3-acetonitrile, the substrate of NIT1 and the precursor to auxin. A cyp79F1 mutant exhibited increased auxin in leaves (Reintanz et al., 2001) and the overgrowth and bushy phenotype seen in cyp79F1 mutants are phenotypes typical for increased auxin. I would expect cyp83A1 and cyp79F2 mutants that also have the R2D2 transgene to display increased auxin accumulation in lateral and primary roots compared to wild-type. In cyp79F1 mutants this phenotype may only be observable if plants are grown in soil (Tantikanjana et al., 2004). If the CYP79F1 and CYP79F2 genes have nonoverlapping expression patterns as reported by Tantikanjana et al. 2004, I would expect cyp79F1 mutants to have auxin accumulation in primary and lateral roots equivalent to the wild-type. The relationship between these biosynthetic pathways and their enzymes has not been fully characterized. Understanding the regulation of auxin synthesis is a critical aspect of plant growth and characterizing the flux between the auxin and glucosinolate biosynthetic pathways and NIT1 regulation will provide valuable insight into this area of research.
Figure 4. Root mRNA expression from Root eFP Browser by N. Provart. Spatiotemporal data from Brady et al. (2007) Science 318:801-808.

(A) CYP3A1  (B) CYP79F1  (C) CYP79F2  (D) NIT1  (E) CEPR1
Figure 5. Developmental mRNA expression from eFP Browser by B. Vinegar drawn by J. Allis N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501 and the Nambara lab for the imbibed and dry seed stages.

(A) CYP3A1 (B) CYP79F1 (C) CYP79F2 (D) NIT1 (E) CEPR1
Methodology

In the experiments described, *Arabidopsis thaliana* ecotype Columbia-0 plants were used. Seeds were planted in a soil and vermiculite mixture at a ratio of 4:1, covered with a plastic dome and kept at 4°C for 3-5 days. They were then moved into a 16-hour light/8-hour dark photoperiod growth chamber at 22°C. To obtain desired genotypes containing multiple individually segregating alleles, reciprocal crosses were performed. Siliques were harvested, dried, and stored at room temperature in 500µL tubes.

Two knockout or null mutant lines for each of three genes of interest, CYP79F1, CYP79F2, and CYP83A1, were obtained from stock centers. The gene diagrams for each are shown in figure 6. The first cyp83a1 line (polymorphism SALK 123405) is a T-DNA insertion into an intron of the gene. The second cyp83a1 line, ref2-1, was a single nucleotide substitution (G to A) leading to a nonsense mutation Trp58STOP. The two cyp79f1 lines, SALK 011806 and SALK 098658 are T-DNA insertions into the first exon of the gene. A pool of potential cyp79f2 lines with a T-DNA insertion in the second intron were obtained (GK-438E07). This set of twelve T4 individual lines was produced from a T3 bulk for which a T2 parental line was confirmed to contain the insert. The individual lines can be wild-type, heterozygous or homozygous for the insertion, and may have additional insertions. This pool was also previously found to contain an additional insertion in a transposable element gene, this was screened for to avoid confounding factors. A second cyp79f2 line was obtained, SALK 129669, which is a T-DNA insertion into the second CYP79F2 exon.

A NIT1 mutant nit1-1, described in Normanly et al., 1997, was obtained. The mutant allele, created using EMS mutagenesis, has a single base substitution leading to a missense mutation (228 Gly→Asp). This mutation also created a Mbol restriction site that can be used for screening as the mutant allele is cut by this restriction enzyme (Normanly et al., 1997). The CEPR1 mutant xip1-1 allele was generated using EMS by the Seattle TILLING Project (Till et al. 2003). The xip1-1 point mutation is located within the kinase domain of the gene and disrupts an HpaII restriction site, the wild-type allele is cut by this restriction enzyme.

DNA was extracted using the DTAB method, then plants were genotyped by PCR. The ref2-1 line was genotyped using the dCAPS method (Neff et al., 1998). A Bsne (BsrI) restriction site was added to the 5’ end of the WT PCR product then digested by a restriction enzyme BseNI (BsrI). PCR temperatures and primers used to genotype each line are described in table 1 and 2. PCR products were visualized using gel electrophoresis on 1- 4% agarose gels run at 120V (90V for ref2-1) for optimal visualization.
Figure 6. Gene Diagrams
(A) CYP3A1 gene structure, ref2-1 mutation site, SALK 123405 T-DNA insertion site (B) CYP79F1 gene structure, SALK 0118066 and SALK 098658 T-DNA insertion sites (C) CYP79F2 gene structure, GK-438E07 and SALK_129669 T-DNA insertion sites (D) NIT1 gene structure and nit1-1 mutation site (E) CEPR1 gene structure and xip1-1 mutation site (F) R2D2 transgene structure.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Polymorphism</th>
<th>Locus</th>
<th>Left Border Primer Sequence</th>
<th>Right Border Primer Sequence</th>
<th>T-DNA Border Primer Sequence</th>
<th>WT PCR Tm (°C)</th>
<th>Mut. PCR Tm (°C)</th>
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<tr>
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<td>GCCTAATAACTCATC</td>
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<td>CS66578 (ref2-1)</td>
<td>AT4G13770</td>
<td>TACACCCAAACGGCT</td>
<td>TCACCTTCCTGCTGACT</td>
<td>Bsne (BsvI) cuts dCAPs modified WT allele</td>
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<td>CS751541-</td>
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Table 1. PCR Primer Sequences and Melting Temperatures for T-DNA Insertion Mutants

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<th>Gene name</th>
<th>Polymorphism</th>
<th>Locus</th>
<th>Left Border Primer</th>
<th>Right Border Primer</th>
<th>Restriction Enzyme</th>
<th>PCR Tm (°C)</th>
<th>Incubation Tm (°C)</th>
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<tr>
<td>CYP83A1</td>
<td>CS66578 (ref2-1)</td>
<td>AT4G13770</td>
<td>TAACCCAAACGGCT</td>
<td>TCACCTTCCTGCTGACT</td>
<td>Bsne (BsvI) cuts dCAPs modified WT allele</td>
<td>60</td>
<td>65</td>
</tr>
<tr>
<td>NIT1</td>
<td>nit1-1</td>
<td>AT3G44310</td>
<td>CTGAGTAAAGCAAGATTTTGCTG</td>
<td>GAGTGT</td>
<td>Mboll cuts mutant allele</td>
<td>56</td>
<td>37</td>
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<tr>
<td>CEPRI1</td>
<td>xip1-1</td>
<td>AT5G49660</td>
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<td>ACGTGCGCATTTTAGCTCT</td>
<td>HpaII cuts wild-type allele</td>
<td>65</td>
<td>37</td>
</tr>
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</table>

Table 2. PCR Primer Sequences, Melting Temperatures, and Restriction Enzymes for Point Mutants

After the genotypes of the mutants were confirmed, they were reciprocally crossed with verified homozygous triple mutant plants that contained the transgene R2D2 (Liao et al., 2015), a semi quantitative auxin reporter, and nit1-1 and cepr1 knockouts or point mutants. Figure 6 describes the crossing schemes. Seeds created through these crosses were collected, planted, and screened using the above methods to determine genotype. Seeds were harvested from plants heterozygous for the R2D2 transgene and the polymorphism. Several generations of plants were grown until plants that were homozygous for the R2D2 transgene and each mutant were isolated.

For future expression and phospho-mimetic studies NIT1 and CYP83A1-GFP plasmids will be created. CYP83A1 and NIT1 RNA was isolation using RNeasy Plant Mini Kit (Qiagen) was performed on wild-type Columbia rosettes, a region of CYP83A1 and NIT1 expression (fig. 5A, 5D). RNA was nano-dropped to ensure purity and quality. cDNA reverse transcription was performed using Super Script II (Invitrogen) on the isolated RNA.
Results

The CYP83A1 line ref2-1 was verified to be homozygous through PCR and successfully crossed with a homozygous R2D2: nit1-1: xip1-1 triple mutant. After three generations plants homozygous for ref2-1, nit1-1 and R2D2 were isolated, xip1-1 is still segregating (see fig. 8 and table 3). The R2D2 reporter signal was verified to be working in the CYP83A1 ref2-1 line using fluorescent microscopy. The SALK CYP83A1 line was verified to be homozygous through PCR and successfully crossed with a homozygous R2D2: nit1-1: xip1-1 triple mutant. Seed germination was delayed in the CYP79F1 line SALK 011806C, and rates of germination were extremely low, less than 1 in 50 seeds was viable potentially due to the age of the seeds. After two generations, germination rates were improved, and the line was verified to be homozygous using PCR. The CYP79F1 line SALK 098658 was determined to be heterozygous and seed germination of this line was also delayed and rates of germination were low, less than 1 in 25 seeds was viable. The primers for CYP79F1 lines have proven to be extremely temperature sensitive, a gradient of melting temperatures was used to find the ideal temperature for these PCRs (table 1). Additionally, the LBB1 border primer of pBIN-pROK2 for SALK lines was the only border primer that yielded results when screening for these mutations. After one generation germination rates were restored, and after two generations a homozygous plant was isolated. Attempts to reciprocally cross both CYP79F1 lines with the R2D2: nit1-1: xip1-1 homozygous triple mutant have been unsuccessful. A pool of fifteen CYP79F2 GK-438E07 individual lines were received. These T4 individual lines were produced from a T3 bulk for which a T2 parental line was confirmed to contain the insertion in CYP79F2. Each individual line could be wild-type, heterozygous or homozygous for the insertion, and could have additional insertions. Two of these fifteen lines have been screened using PCR and did not contain the insertion. The additional insertion within a transposable element gene that was reported in this
line was not found in either of the screened lines. The SALK 129669 CYP79F2 line was ordered but not yet planted and the genotype not yet verified.

![Figure 8. ref2-1: R2D2: nit1-1: xip1-1 F3 generation PCR Results](image)

Plants 8, 10, and 11 were chosen for seed collection based on genotype. Gels from genotyping results of other lines are not shown but are included in table 3.

![Table 3](image)

Table 3. Furthest genotypes isolated, with the goal of quadruple homozygotes and CYP: R2D2 homozygous double mutants for each line.

**Discussion and Future Steps**

Knockout and null mutants were used to study the role of CYP83A1, CYP79F1, and CYP79F2 in auxin accumulation and lateral root initiation in *Arabidopsis thaliana*. With the goal of testing whether these enzymes functioned with XIP1/CEPR1 to control auxin levels or perform some other unknown function. Two lines of knockout or null mutants for each gene were obtained to cross into a line homozygous for a R2D2, NIT1, and CEPR1. The genotypes of the CYP83A1 and CYP79F1 knockout/null lines were confirmed. *ref2-1*, one of the CYP83A1 lines, was crossed into all the relevant
mutants, and selfed until plants homozygous for ref2-1, xip1-1, and R2D2 were isolated; these plants are heterozygous for nit1-1. The other CYP83A1 null line, SALK 123405, was successfully crossed into the R2D2 line. Plants heterozygous for cyp83A1, nit1-1, xip1-1, and R2D2 were isolated.

Both the cyp83A1 lines will be selfed until plants that are homozygous for all four alleles (cyp83a1:R2D2:nit1-1:xip1-1), just the CYP83A1 mutation and the reporter (cyp83A1:R2D2), the CYP83A1 and CEPR1 mutations and the reporter (cyp83A1:R2D2:xip1-1), and the CYP83A1 and NIT1 mutations and the reporter (cyp83A1:R2D2:nit1-1) are isolated. CYP79F1 knockout/null lines will be crossed into R2D2:nit1-1:xip1-1. They will then be selfed until homozygous for all four alleles, homozygous for just the reporter and cyp79f1, and homozygous for the reporter, cyp79f1, and nit1-1 or xip1-1. The genotypes of CYP79F2 knockout lines will be verified with PCR and then cross into R2D2:nit1-1:xip1-1 then selfed until homozygous for all four alleles, homozygous for just the reporter and cyp79f1, and homozygous for the reporter, cyp79f1, and nit1-1 or xip1-1. Since Tantikanjana et al., observed different lateral root impairment in a cyp79F2 mutant only when plants were grown in soil all plants will be grown on agar plates and in soil for phenotyping. Once all the desired genotypes are isolated the root phenotypes will be observed in the various single, double, and triple knockout lines, that also contain the R2D2 transgene, particularly the lateral roots. Fluorescent microscopy on roots will be used to observe relative quantities of yellow to red florescence to determine auxin localization and relative accumulation compared to a control plant that contains the R2D2 transgene but is otherwise wild-type. Root phenotypes, auxin localization and relative accumulation of each combination of knockouts/null mutants will be compared to draw conclusions about the influence of these genes on the biosynthesis and accumulation of auxin. The same number of lateral roots were present in the cyp79F2 mutant, but lateral root length was impaired (Tantikanjana et al., 2004).

For future protein localization and phospho-mimetic studies NIT1– and CYP83A1- GFP plasmids were created. CYP79F1-GFP and CYP79F2-GFP plasmids will be created as well. The NIT1 and CYP-GFP plasmids will be used in protein localization studies. Additionally, the NIT1 cDNA sequence will be modified to alter phosphorylation sites to study the role of phosphorylation on its regulation. Since nit1 null mutants did not show any significant alterations in auxin content, potentially due to compensation by other nitrilases alterations to NIT1 that resulted in loss of function or loss of positive regulation may not show a phenotype. But alterations to phosphorylation that result in a loss of negative regulation or overexpression may show a phenotype similar to NIT1ox plants and exhibit an increase in lateral roots and shortened primary roots. An increase in IAA would also be expected.
References


