UNIVERSITY OF CALIFORNIA, SAN DIEGO

Isolating *Arabidopsis* Mutants Affecting Auxin-Mediated Development

A Thesis submitted in partial satisfaction of the requirements
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by

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ABSTRACT OF THE THESIS

Isolating *Arabidopsis* Mutants Affecting Auxin-Mediated Development

by

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Master of Science in Biology

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Professor Yunde Zhao, Chair

Auxin is a plant hormone that plays essential roles in all aspects of growth and development. Plants have multiple pathways to synthesize auxin. The 11 YUCCA genes encode for FMO-like enzymes and catalyze the conversion of tryptamine to N-hydroxyl-tryptamine; they participate in tryptophan-dependent auxin biosynthesis branch. Molecular analysis of auxin mechanisms is difficult because genes involved in auxin

biosynthesis, transport, and signaling oftentimes belong to gene families. Here I conduct EMS forward genetic screening in the partially auxin deficient *yuc1-/-yuc4-/+* background. I have isolated enhancer and suppressor mutants and attempted to identify the corresponding genes. In addition, a weak allele of the auxin transport gene PINOID – pid336, was cloned. Using pid336, I initiate another genetic screening employing T-DNA activation tagging method, in the sensitized *yuc1-/-pid336-/-* background. In total, I have screened more than two thousand lines, isolated several mutants, and have attempted to identify the genes involved. The identification and characterization of genes from my screening help to elucidate the molecular mechanisms of auxin biosynthesis and auxin signaling.

INTRODUCTION

History of auxin

In plants, auxin is a hormone that orchestrates many aspects of growth and development including tropic responses to gravity and light, overall root and shoot structures, vascular differentiation, organ formation and patterning, and senescence. Auxin study is perhaps one of the earliest fields in experimental plant biology. In 1872, Theophil Ciesielski observed that plants roots tend to bend in response to gravity. A few years later, building on this Ciesielski's study, Charles Darwin published *The Power of Movement in Plants,* in which he hypothesized there is a chemical that directs plant tropism towards light and regulates shoot elongation [Woodward and Bartel, 2005]. The chemical was then isolated from plant tissues by diffusion into agar blocks and shown to have growth promoting activity [Went, 1926]. Soon thereafter the chemical was crystallized, and the chemical structure of auxin was identified (see figure 1).

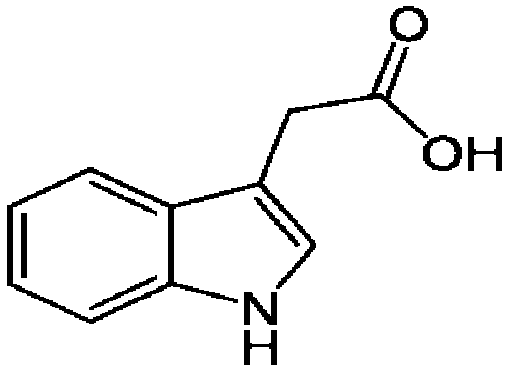

Figure 1: chemical structure of indole-3-acetic acid (IAA), the most common form of auxin in plants. From http://en.wikipedia.org/wiki/File:Indol-3-ylacetic_acid.svg

IAA biosynthetic pathways

In plants, indole-3-acetic acid (IAA) is the most abundant form of auxin, and it was the first molecule to be identified as auxin. Several compounds structurally similar to auxin have been in discovered in bacteria and even humans [Woodward and Bartel, 2005]. In theory, auxin level may be regulated at multiple stages – biosynthesis, de/conjugation, degradation, transport, and signaling responses [Woodward and Bartel, 2005]. The starting point of auxin action is auxin biosynthesis, but the current knowledge regarding auxin biosynthesis is incomplete. It is clear there are many redundant pathways that contribute to auxin production, each to a different extent, and at different developmental stages [Woodward and Bartel, 2005].

Based on genetic and biochemical studies, there is evidence for both tryptophan-dependent pathways and tryptophan-independent routes [Bartel, 1997]. Figure 2 shows four proposed routes for tryptophan dependent auxin biosynthesis and one pathway for tryptophan independent auxin production [Bartel, 1997; Woodward and Bartel, 2005]. The four known pathways for tryptophan-dependent auxin production in plants can be categorized by their intermediates: 1. indole-3-acetaldoxime (IAOx) 2. indole-3-acetamide (IAM) 3. indole-3-pyruvic acid (IPA) and 4. tryptamine (TAM) [Woodward and Bartel, 2005]. Table 1 shows plant genes implicated in *de novo* auxin biosynthesis [Woodward and Bartel, 2005].

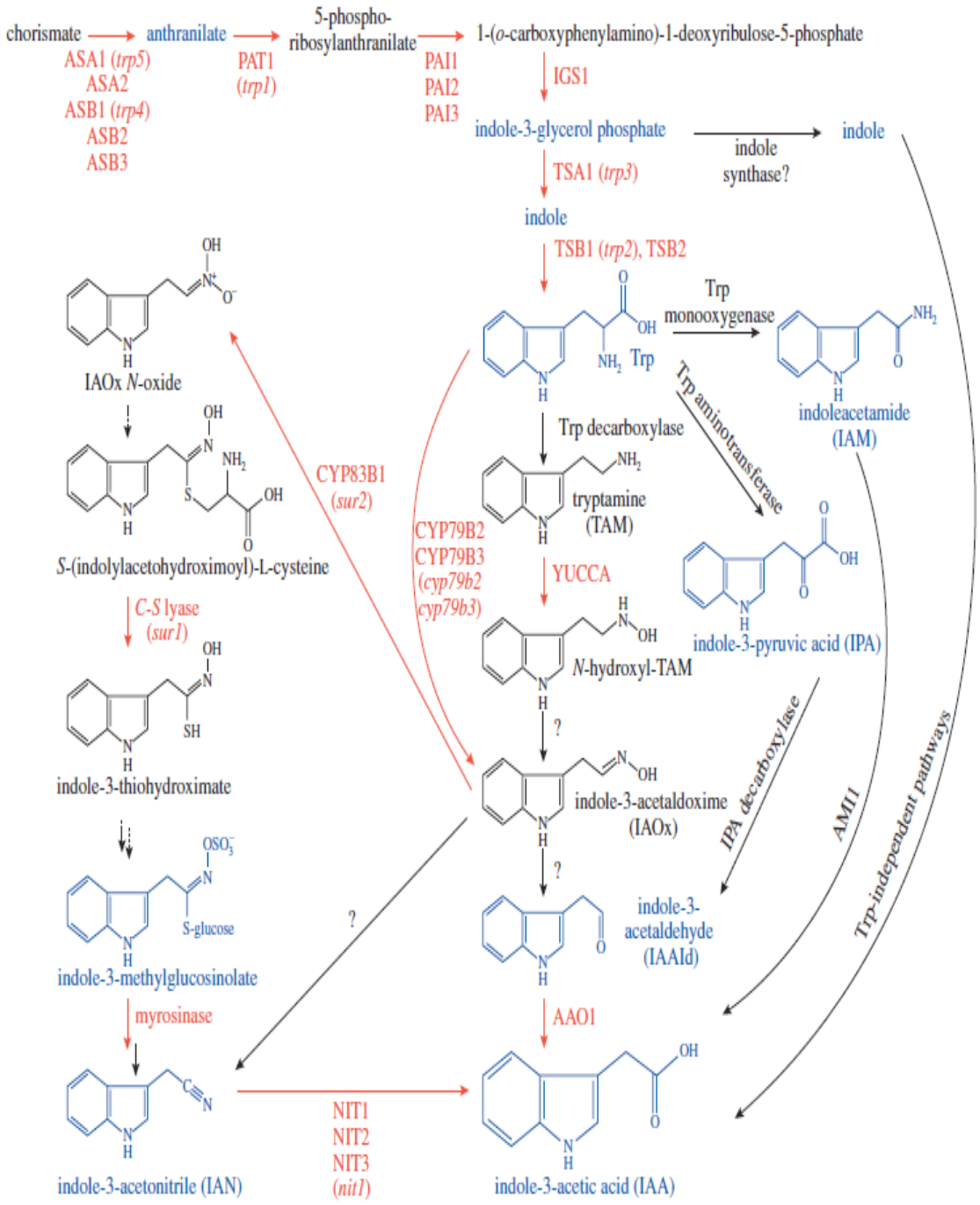

Figure 2: Multiple pathways of IAA biosynthesis in *Arabidopsis*, beginning from tryptophan (Trp) or Trp precursors. Even though plants likely use all of these pathways to make auxin, none of these pathways has been so precisely elucidated to the point where we know every relevant gene, enzyme, and intermediate. In other words, the molecular details of these pathways remain undefined. From Woodward and Bartel, 2005.

Table 1: Plant genes implicated in *de novo* auxin biosynthesis. From Woodward and Bartel, 2005.

Gene	Product*	Putative localization	Loss-of-function (LOF) or overexpression (OE) phenotype	Reference
AAO1	IAAld oxidase	Cytoplasm	OE in *sur1*	Sekimoto *et al.* (1998); Seo *et al.* (1998)
AMI1	IAM hydrolase	Not reported	Not reported	Pollmann *et al.* (2003)
CYP79B2, CYP79B3	P450 monooxygenases	Chloroplast	LOF: *cyp79B2 cyp79B3*: low glucosinolates, IAN, and IAA OE: resistant to Trp analogues; high indolic glucosinolate, IAN, and IAA–X levels	Zhao *et al.* (2002); Hull *et al.* (2000); Mikkelsen *et al.* (2000);
CYP83B1/SUR2	P450 monooxygenase	Cytoplasm	LOF: high IAA, IAAld, and IAA–Asp levels; normal IAN levels; low indolic glucosinolate levels; altered Trp biosynthetic gene expression; defective photomorphogenesis in red light OE: high indolic glucosinolate levels, reduced apical dominance	Delarue *et al.* (1998); Barlier *et al.* (2000); Bak *et al.* (2001); Smolen and Bender (2002); Hoecker *et al.* (2004) Bak *et al.* (2001)
NIT1	Nitrilase	Not reported	LOF: IAN resistant, normal IAA levels	Normanly *et al.* (1997)
NIT2	Nitrilase	Not reported	OE: increased sensitivity to IAN, normal IAA levels	Normanly *et al.* (1997)
ZmNIT2	Nitrilase (maize)	Not reported	Not reported	Park *et al.* (2003)
SUR1/RTY/ALF1/HLS3	*C-S* lyase	Not reported	LOF: high IAA and IAA–X levels, low glucosinolates	Boerjan *et al.* (1995); Celenza *et al.* (1995); King *et al.* (1995); Golparaj *et al.* (1996); Lehman *et al.* (1996); Mikkelsen *et al.* (2004)
TDC	Trp decarboxylase (*C. roseus*)	Cytoplasm	OE: enhanced root curling	De Luca *et al.* (1989); Guillet *et al.* (2000)
TSA1/TRP3	Trp synthase α	Chloroplast	LOF: high IAA–X, IAN, indole-glycerol phosphate, and indolic glucosinolate levels; normal free IAA; low Trp; Trp auxotroph	Normanly *et al.* (1993); Müller and Weiler (2000*a*); Ouyang *et al.* (2000)
TSB1/TRP2	Trp synthase β	Chloroplast	LOF: high IAA–X and IAN levels, normal free IAA, low Trp, Trp auxotroph	Normanly *et al.* (1993); Ouyang *et al.* (2000)
ORP	Trp synthase β (maize)	Chloroplast	LOF: high IAA–X, normal free IAA, Trp auxotroph	Wright *et al.* (1991, 1992)
YUCCA, YUCCA2	FMO-like	Cytoplasm	LOF: no phenotype OE: high IAA levels	Zhao *et al.* (2001)
FLOOZY	FMO-like (petunia)	Not reported	LOF: defective leaf venation and apical dominance OE: high IAA levels	Tobeña-Santamaria *et al.* (2002)

Auxin Overproducers

Several auxin overproducing mutants have been identified (see table 1): *yucca* [Zhao et al., 2001], *superroot1* (*sur1)* [Boerjan et al., 1995], *superroot2* (*sur2)*

[Delarue et al., 1998], and *cpy79b2* [Zhao et al., 2002]. *sur1* and *sur2* are identified as recessive mutants, but neither *sur1* and *sur2* is directly involved in auxin synthesis – mutations in these genes only diverts indole-3-acetaldoxime (IAOx) from glucosinolate synthesis to auxin synthesis [Woodward and Bartel, 2005].

IAOx is the branchpoint between making indole-3-methlglucosinolate and IAA [Woodward and Bartel, 2005]. The CYP79B2/3 genes are P450 monooxygenases in *Arabidopsis*, and *in vitro* they oxidize tryptophan to IAOx [Hull et al., 2000]. Overexpression of CYP79B2 leads to higher levels of indole glucosinolate [Mikkelsen et al., 2000] and IAA / IAN [Zhao et al., 2002]. In loss of function *cyp79b2 /3* plants we see plants with phenotypes indicative of decreased IAA and IAN levels, and with no observable level of indole glucosinolate [Zhao et al., 2002]. From these data, it is evident that IAOx is a precursor that can be used for the synthesis of either auxin or indole glucosinolate.

CYP83B1 is another P450 monooxgenase that makes indole-3-methlglucosinolate by converting IAOx to N-oxide [Bak et al., 2001]. *sur2* is identified through a genetic screen for mutant seedlings with increased auxin phenotypes. It is a loss of function *cyp83b1* allele that accumulates IAAld, the IAA precursor [Barlier et al., 2000] and free IAA [Delarue et al., 1998]. In the *superroot2* mutant, IAOx accumulates and is made into IAAld, which is finally oxidized to IAA [Delarue et al., 1998].

sur1, also known as rooty also overproduces auxin as a result of defective glucosinolate synthesis [King et al., 1995]. The morphological phenotypes of *sur1* are similar to *sur2* and *yucca*. *sur1* accrues free IAA and IAA conjugates [King et al., 1995] while having no detectable levels of indole glucosinolate [Mikkelsen et al., 2004]. Biochemical studies reveal that in the *sur1* mutant, a C-S lyase is defective and cannot cleave S-L-cysteine to indole-2-thiohydroximate, which is the third step making glucosinolate from IAOx [Golparaj et al., 1996].

NIT genes synthesize IAA from IAN

In *Arabidopsis* nitrilases NIT1, NIT2, NIT3 hydrolyze indole-3-acetonitrile (IAN) to IAA [Bartel & Fink, 1994]. After indole-3-methyl glucosinolate is hydrolyzed by myrosinase, IAN is formed [Delker et al., 2008]. It has been observed that IAN levels follow indole glucosinolate levels, suggesting nitrilases function downstream of glucosinolates [Normanly et al., 1993]. In the NIT gene family, NIT1 is the most highly expressed in *Arabidopsis*. NIT1 function in IAA synthesis is an example of genetic redundancy – the *nit1* mutant shows no obvious auxin deficient phenotype, although it is resistant to exogenous application of IAN [Normanly et al., 1997].

Difficulties in dissecting auxin biosynthetic pathways

Because regulation of auxin production is complex, the attribution of a specific regulatory mechanism to a particular physiological response is difficult. Previously,

many studies focused on plants responses to exogenously applied auxin. The important limiting factors of using this approach to analyze mechanisms of auxin pathways include: genetic redundancy in gene families, lack of auxin-deficient mutants, and the existence and contribution of multiple pathways. Lack of auxin deficient mutants suggests that either auxin is essential for plant survival and/or there are redundant mechanisms contributing to auxin production.

Bacterial iaaM

The auxin biosynthesis gene called iaaM found in plant pathogenic bacteria has been well-characterized. iaaM encodes a tryptophan-2-monooxygenase and converts tryptophan to the plant hormone precursor indole-3-acetamide, which in turn is used to produce IAA [Patten and Glick, 1996]. The IAM microbial pathway: tryptophan → IAM → IAA, exists in *Agrobacterium tumifaciens* and *Pseudomonas syringae* [Woodward and Bartel, 2005]. Plants will over-produce auxin when the iaaM gene is expressed under the cauliflower mosaic virus (CaMV 35S) promoter. For example, the seedlings exhibit typical excess auxin phenotypes such as epinastic cotyledons and longer hypocotyls when grown under light [Romano, 1995].

From the well-defined iaaM pathway, we observe that the hallmarks for auxin overproduction are long hypocotyls, epinastic cotyledons, and adventitious roots when grown under light [Zhao et al., 2001]. Therefore, we can use these particular

phenotypes to screen for potential genes involved in auxin biosynthesis when conducting gain of function genetic screens.

YUCCA genes in tryptophan dependent auxin biosynthesis

The discovery of the YUCCA gene family [Zhao et al., 2001] allows a new approach and significant progress towards understanding how the production and regulation of auxin can greatly influence processes in plant development. Using activation tagging and scoring for typical auxin over-producing phenotypes mentioned earlier, the YUCCA genes were discovered to encode for flavin monooxygenase-like enzymes, catalyzing the conversion of tryptamine (TAM, a tryptophan derivative) to N-hydroxyl tryptamine (precursor of IAOx) in the tryptophan-dependent auxin biosynthesis branch [Zhao et al., 2001].

Biochemical analyses reveal YUCCA is a protein containing 414 amino acids, similar to flavin monooxygenase (FMOs) in mammals [Zhao et al., 2001]. It has conserved domains for FAD and NADPH binding. There are elevens genes in the YUCCA family (see figure 3) the other members of the YUC family has approximately 44-64% sequence identity to YUC1 [Zhao et al., 2001]. Other *yuc* mutants have phenotypes resembling *yuc1D*, suggesting genes in the YUCCA family have redundant functions and are functional paralogs.

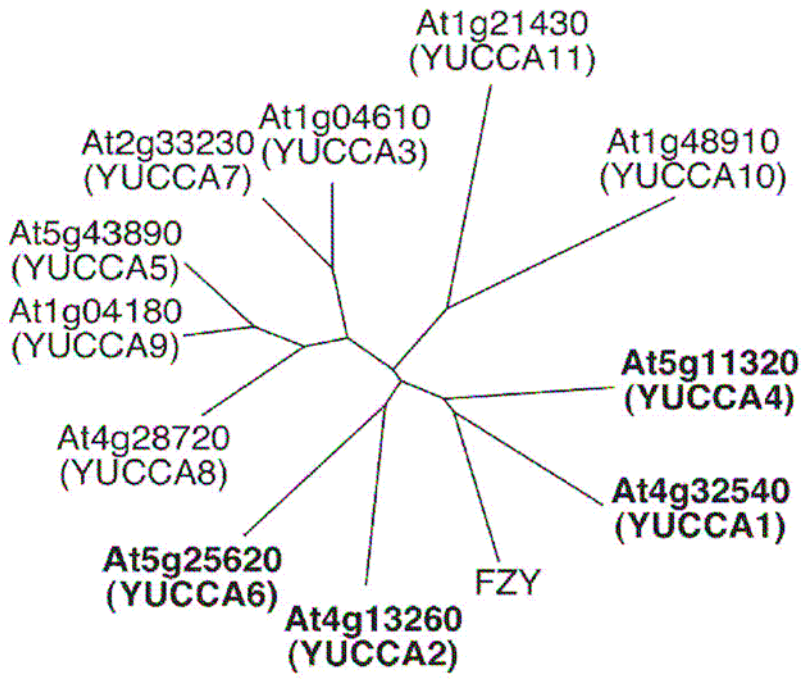

Figure 3: phylogenetic tree of the YUC family of flavin monooxygenases. From Cheng et al., 2006.

Although the previously identified Arabidopsis mutants have increased IAA levels, they either are sterile or innately display heterogenous phenotypes [Zhao et al., 2001]. This greatly restricts their use in investigating auxin pathways. However, the yucca mutant is both a fertile and dominant mutant with increased endogenous auxin. Tryptophan analogues are toxic to wildtype plants, but have no effect on yucca plants, indicating that the accumulating IAA is derived from tryptophan [Zhao et al., 2001].

yucca (*yuc1D*) phenotypes

The *yucca* mutant has clear phenotypes of increased auxin levels throughout all developmental stages: long hypocotyls, epinastic cotyledons, and elongated petioles

when grown under light [Zhao et al., 2001], (see figure 4). These traits are similar to the known auxin overproducing mutants *sur1* and *sur2*, and plants overexpressing the *Agrobacterium* iaaM gene [Zhao et al., 2001]. In addition, the *yucca* plant has shorter but more abundant roots. However when the *yucca* plant is grown in the dark, it has short hypocotyl and no apical hook [Zhao et al., 2001]. In the adult *yucca* plant, it has narrower and epinastic leaves, with longer blades and petioles [Zhao et al., 2001]. Because the natural Yucca plant found in the wild has leaves that curl downward, just like this mutant, it is named the *yucca (yuc1D)* mutant [Zhao et al., 2001].

Analysis of the *yucca* (*yuc1D)* mutant in auxin biosynthesis

Northern blot analysis of total RNA from the two *yucca* alleles identified from activation tagging confirmed higher expression of the YUC gene compared to wildtype [Zhao et al., 2001]. Gas chromatography mass spectrometry, measuring free auxin levels, showed that even a tissue from a weak recapitulated *yucca* line had ~50% more free auxin than wildtype [Zhao et al., 2001]. Normally, wildtype plants can't proliferate on media without auxin, but *yucca* mutant tissues can, indicating that *yucca* makes more auxin than wildtype [Zhao et al., 2001]. Thus, we see this elevated auxin level is physiologically relevant.

If yucca makes too much auxin, then reducing the amount of auxin should mitigate *yucca* phenotypes [Zhao et al., 2001]. The bacterial iaaL gene encodes and enzyme that conjugates free auxin to lysine, reducing the active free IAA pool and

resulting in shorter stature [Romano et al., 1991]. Indeed when the iaaL gene is overexpressed, the *yucca* phenotypes are alleviated, resulting in decreased apical dominance [Zhao et al., 2001]. This further corroborates that auxin overproduction is responsible for *yucca* phenotypes.

A B C D

Figure 4: *yuc1D* phenotypes. (A) – wildtype (left) vs. *yucca* (right) seedlings with longer hypocotyl, grown in white light on MS plate. (B) – wildtype (left) vs. *yucca* (right) with more abundant roots, grown in white light. C – wildtype (left) vs. *yucca* (right) seedling with shorter hypocotyl and no apical hook, grown in dark. (D) wildtype (left) vs. *yucca* (right) adult plant with increased apical dominance. From Zhao et al., 2001.

Conservation of YUCCA genes in the plant kingdom

YUC genes are also present and contribute to development in other plants including rice, petunia (FLOOZY = YUC1), tomato, and corn [Woo et al., 2007; Tobena-Santamaria et al., 2002; Exposito-Rodriguez et al., 2007; Gallavotti et al.,

2008]. Furthermore, when YUCCA is overexpressed in tobacco plants, it induces auxin overproduction phenotypes as well – narrower and epinastic leaves just like in *Arabidopsis* [Zhao et al., 2001]. This indicates that the YUCCA pathway is a common pathway used by most plants to make auxin. On the basis of sequence homology and functional studies of YUC genes in different plants, we see that auxin synthesized by YUCCAs is prevalent and conserved in most plant species [Zhao, book chapter 2010]. YUC-like flavin monooxygenases appear widely distributed throughout the plant kingdom, in contrast to other auxin biosynthesis genes such as CYP79B2/B3, which so far have only been found in *Brassica*, and thus do not appear to be as widely distributed [Zhao, book chapter 2010].

Close YUC1 homologs: YUC2, YUC4, YUC6 are all involved in auxin biosynthesis

Of the eleven genes in the YUCCA family, the three closest YUC1 homologs are YUC2, YUC4, and YUC6 [Cheng et al., 2006]. Overexpression of cDNA from each of these homologs under the control of CaMV35S promoter leads to *yuc1D*-like and iaaM overexpression phenotypes [Cheng et al., 2006]. This indicates that YUC2, YUC4, and YUC6 overexpression induces auxin overproduction and that they are all involved in making auxin. YUC1, YUC2, YUC4, and YUC6 are essential for many processes in plants including: the formation of basal body in embryogenesis, establishment of the embryonic and postembryonic organs, development of vascular tissue and flower [Cheng et al., 2006].

Distinct and overlapping expression patterns of YUC1 and YUC4

RNA *in situ* hybridization, along with YUC promoter-GUS fusion for YUC1 and YUC4 reveal their distinct and overlapping expression patterns [Cheng et al., 2006]. Both YUC1 and YUC4 are expressed in floral primordia, floral and apical meristem, and basal end of young floral buds. In later development, YUC1 expression is limited to specific cell groups in the carpels and stamens, but in fully mature flowers there is no YUC1 expression [Cheng et al., 2006]. For YUC4, during late development, it is expressed in the apical regions of the carpels, and in fully mature flowers it is limited to the gynoecium [Cheng et al., 2006]. In addition, YUC1 and YUC4 have distinct expression in seedlings and young leaves – YUC4 expression is observed in the basal and apical regions of young leaves, while YUC1 expression is limited to base of young leaf primordial [Cheng et al., 2006]. YUC4 is expressed in vascular strands of leaves but YUC1 is not expressed at all in mature leaves [Cheng et al., 2006].

Genetic analysis of YUCCA genes using combinatorial mutants

As mentioned before, it is not uncommon for genes involved in auxin biosynthetic pathways to belong to gene families and therefore function redundantly. Thus, even though overexpression of one YUC gene leads to auxin overproduction and associated excess auxin phenotypes, a single *yuc* loss of function mutant from T-DNA line exhibits no obvious developmental defect [Cheng et al., 2006]. Using reverse genetics to generate higher order loss of function mutants, the analysis of

combined *yuc1, yuc2, yuc4, yuc6* mutants shows their overlapping functions. There are a total of six possible double mutant combinations for these four genes. Of these, *yuc1yuc2, yuc1yuc6, yuc2yuc4*, and *yuc4yuc6,* combined *yuc* mutants in different clades, are similar to wildtype plants in most aspects of growth and development [Cheng et al., 2006]. But developmental defects are apparent for *yuc1yuc4* and *yuc2yuc6* plants, combined *yuc* mutants in the same clade [Cheng et al., 2006]. *yuc1yuc4* seedlings appear normal but has overall decreased apical dominance (see figure 5) and is completely sterile, *yuc2yuc6* plants are also shorter and severely compromised in fertility [Cheng et al., 2006]. Triple mutants and quadruple mutants are even smaller and display more dramatic phenotypes [Cheng et al., 2006]. Table 2 summarizes the defects in all six double mutant combinations of *yuc1, yuc2, yuc4, yuc6* mutants.

YUC genes in floral organogenesis

Normal Arabidopsis flowers have four sepals, four petals, six stamens, and two fused carpels, but there are defects in both *yuc2yuc6* and *yuc1yuc4* mutants (see figure 6). The *yuc1yuc4* plants have more dramatic defects compared to *yuc2yuc6* – *yuc2yuc6* has all four types floral organs with slightly shorter stamens but *yuc1yuc4* are impaired in all whorls of floral organs, with no functional reproductive organs; and although degree of severity varies, all *yuc1yuc4* lack particular organs [Cheng et al., 2006]. For example, some *yuc1yuc4* flowers have no sepal tissues, while others contain most sepal like organs; some also have stamens, but none of the stamens

produce pollen [Cheng et al., 2006]. The triple and quadruple mutants exhibit even stronger floral defects [Cheng et al., 2006]. (see figure 7). From such analysis, it is clear that these four YUC genes are involved in flower development, with YUC1 and YUC4 being more important than YUC2 and YUC6.

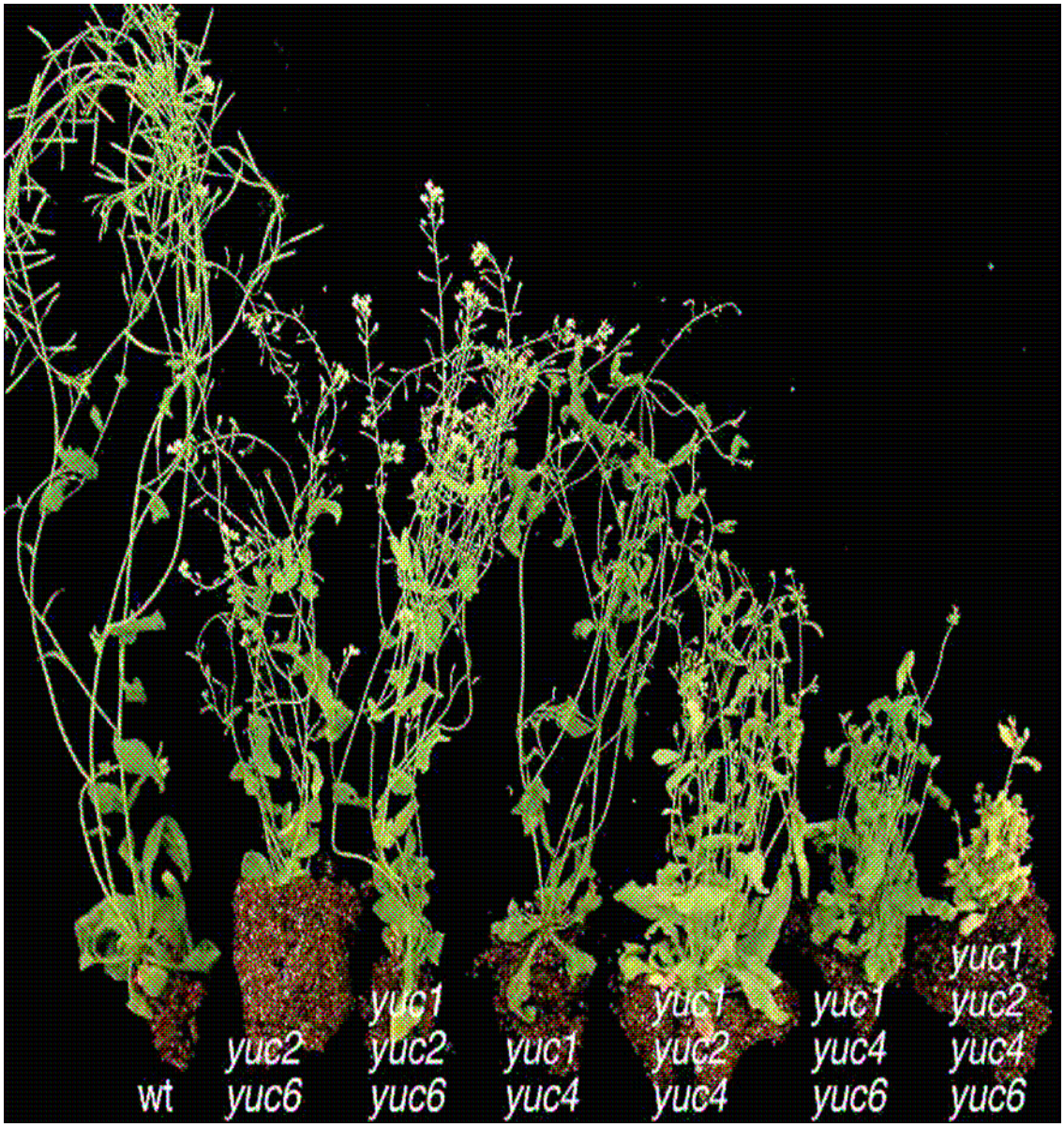

Figure 5: Loss-of-function combinatorial *yuc* mutants with defects in plant stature and apical dominance. From Cheng et al., 2006.

Table 2: Developmental defects in double mutant combinations in four YUC genes – YUC1, YUC2, YUC4, and YUC6. YUC1 and YUC4 belong to one clade and YUC2 and YUC6 belong to another clade, so YUC1 and YUC4 are likely to be evolutionally closer and YUC2 and YUC6 shares more similar functions. Double mutants within the same clade have obvious defects while those in different clade appear normal. Higher order triple and quadruple mutants have additive defects. Data from Cheng et al., 2006.

Mutant combination	Clade	Phenotypes in growth, devp
Yuc1yuc2	Different	Wildtype (wt)
Yuc1yuc4	Same	Defects: shorter stature, curly rosette leaves, dec. apical dominace, 100% sterile
Yuc1yuc6	Different	wt
Yuc2yuc4	Different	wt
Yuc2yuc6	Same	Defects: shorter stature, curly rosette leaves, dec. apical dominance, dec. fertility
Yuc4yuc6	Different	wt
Yuc1yuc2yuc6		More severe than double
Yuc2yuc4yuc6		More severe than double
Yuc1yuc2yuc4yuc6		Most severe phenotypes; smallest

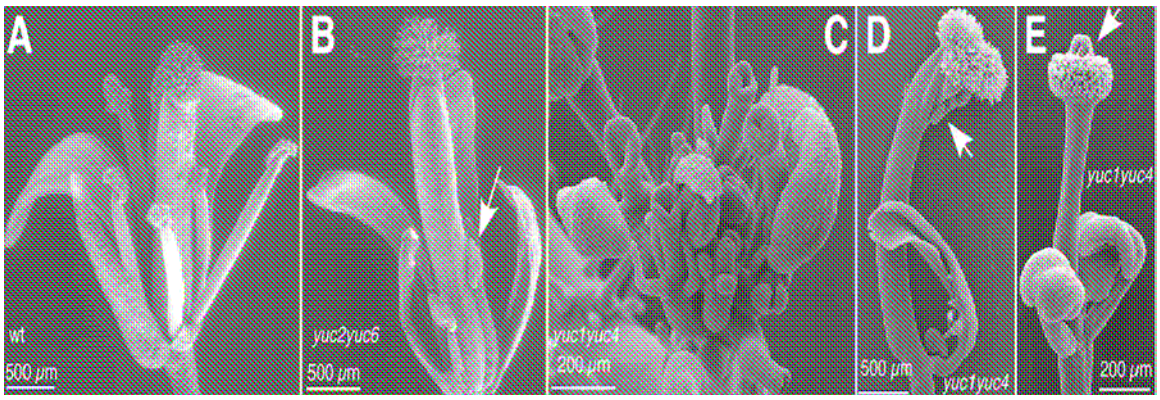

Figure 6: Wildtype vs. flowers of *yuc1,2,4,6* double mutants, some petals and sepals have been removed. From Cheng et al., 2006.

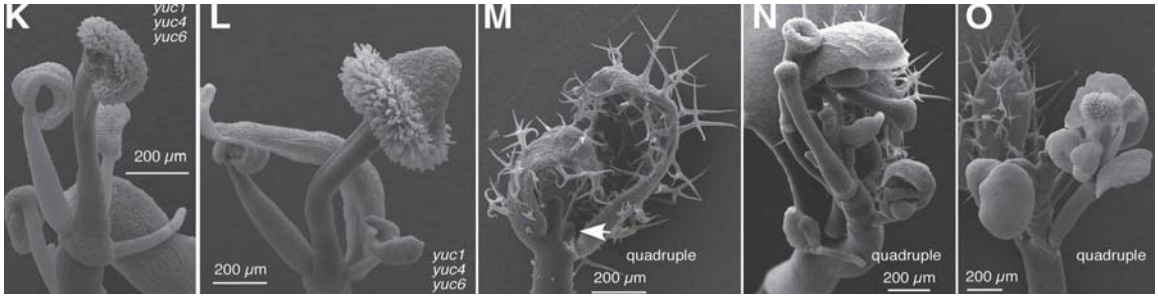

Figure 7: Flowers of *yuc1,2,4,6* triple and quadruple mutants, some petals and sepals have been removed. From Cheng et al., 2006.

YUC genes in vascular development

In normal *Arabidopsis* leaf vascular system, there is a primary vein in the middle with secondary veins emanating from it, connecting to multiple tertiary and higher order veins [Cheng et al., 2006]. In the *yuc2yuc6* mutant there is near normal vascular differentiation but in *yuc1yuc4* leaves there is less quaternary and higher order veins present [Cheng et al., 2006] (see figure 8). Fewer secondary veins and missing tertiary

veins are observed in triple mutants and the quadruple mutant has the least amount of veins and broken connections in the vasculature [Cheng et al., 2006] (see figure 9). The gradient in compromised vein abundance and continuity demonstrates local auxin concentrations established by the YUC genes must reach a specific threshold for proper vascular tissue formation.

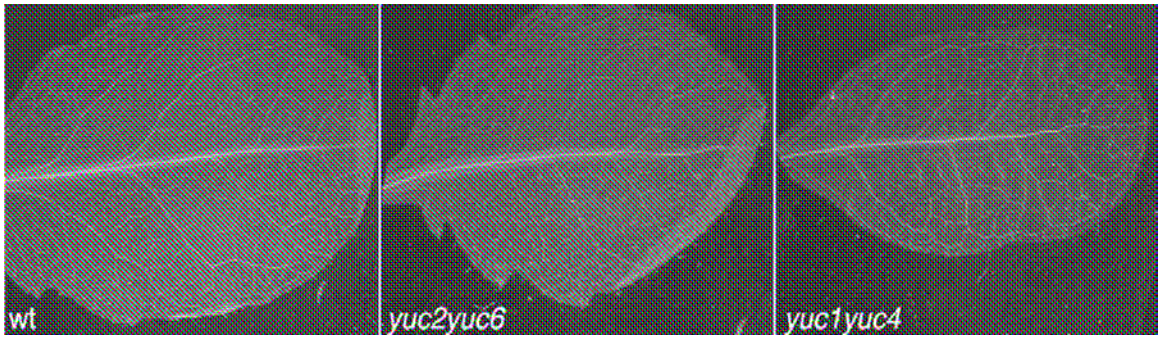

Figure 8: Leaf venation in wildtype vs. *yuc2yuc6* and *yuc1yuc4* double mutants. From Cheng et al., 2006

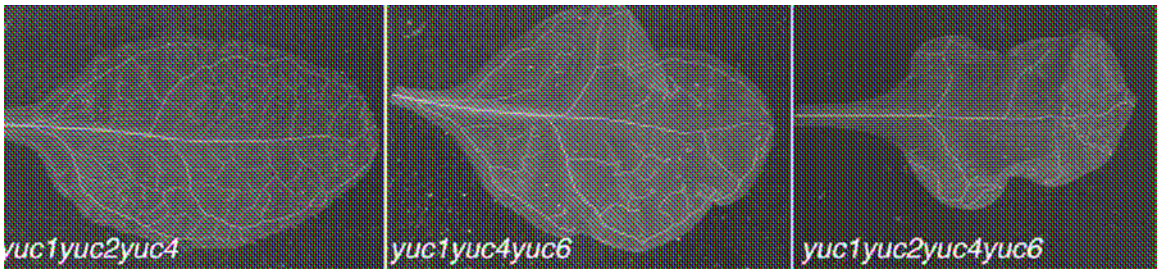

Figure 9: Leaf venation in *yuc* triple and quadruple mutants. From Cheng et al., 2006.

YUC genes in embryogenesis

Not only does YUC1 YUC4 inactivation lead to severe defects in floral and vascular development, further genetic studies show YUC1, YUC4 also have distinct and overlapping expression / functions in embryogenesis. [Cheng et al., 2007].

Because *yuc1yuc4* alone does not exhibit defects in seedlings, it is likely that other YUC genes have overlapping functions with YUC1 YUC4 during embryogenesis [Cheng et al., 2007]. In the phylogenetic tree (see figure 3) YUC10 and its homolog YUC11 are closely related to YUC1,4; RNA in situ hybridization also shows overlapping expression patterns for these four YUC genes [Cheng et al., 2007].

Loss of function mutant *yuc10yuc11* in the *yuc1yuc4* background has dramatic defects in embryogenesis: absence of roots and hypocotyls, only 1 cotyledon, with insufficient amount of vascular tissue (see figure 10) [Cheng et al., 2007]. The deficiencies began early in embryogenesis, during the globular stages – the mutants fail to form hypophysis, which is required for root meristem formation; there is also no elongation of the central cells, which is required for hypocotyl development (see figure 11) [Cheng et al., 2007]. Remarkably, the embryos also did not form primary roots and the basal region is defective [Cheng et al., 2007].

In addition, the *yuc1yuc4yuc10yuc11* seedlings resemble monopteros (mp) mutants [Cheng et al., 2007] – the mp mutants fail to develop a hypocotyl and embryonic roots. MP encodes the auxin response factor 5 – ARF5 [Guilfoyle et al., 1998]. In the presence of auxin, BDL – a transcription factor, is degraded by SCF^{TIR1} complex and MP is allowed to from ARF-ARF dimmers, activating the necessary transcription for development (see ARFs in auxin nuclear signaling) [Kim et al., 1997; Weijers et al., 2005b]. The fact that *yuc1yuc4yuc10yuc11* mutant has phenotypes similar to *mp* mutants

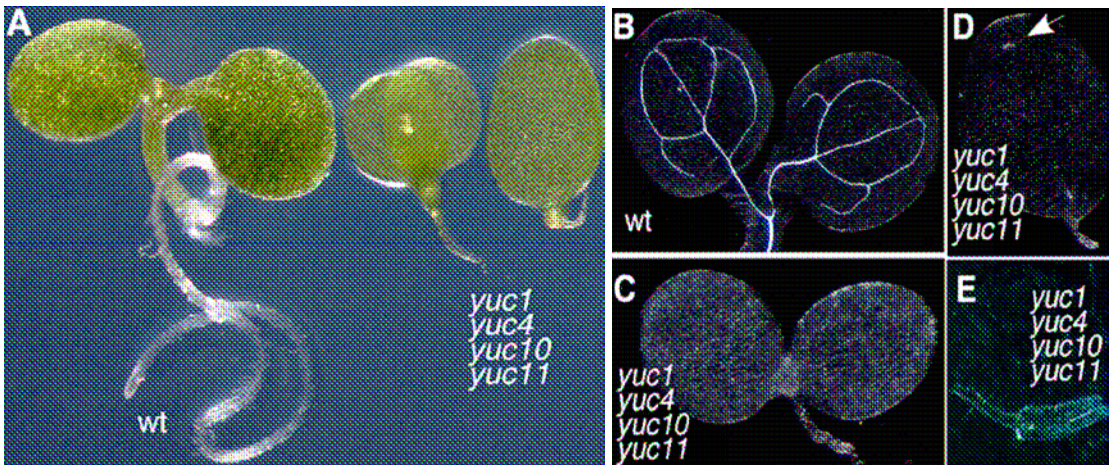

Figure 10: YUCCA genes in embryogenesis. (A) Developmental defects of *yuc1yuc4 yuc10yuc11* at seedling stages. The mutant lacks a hypocotyl and a root. (B) to (E) Vascular defects in *yuc1yuc4yuc10yuc11*. (C) to (E) *yuc1yuc4yuc10yuc11* has short and discontinuous vein. From Cheng et al. 2007.

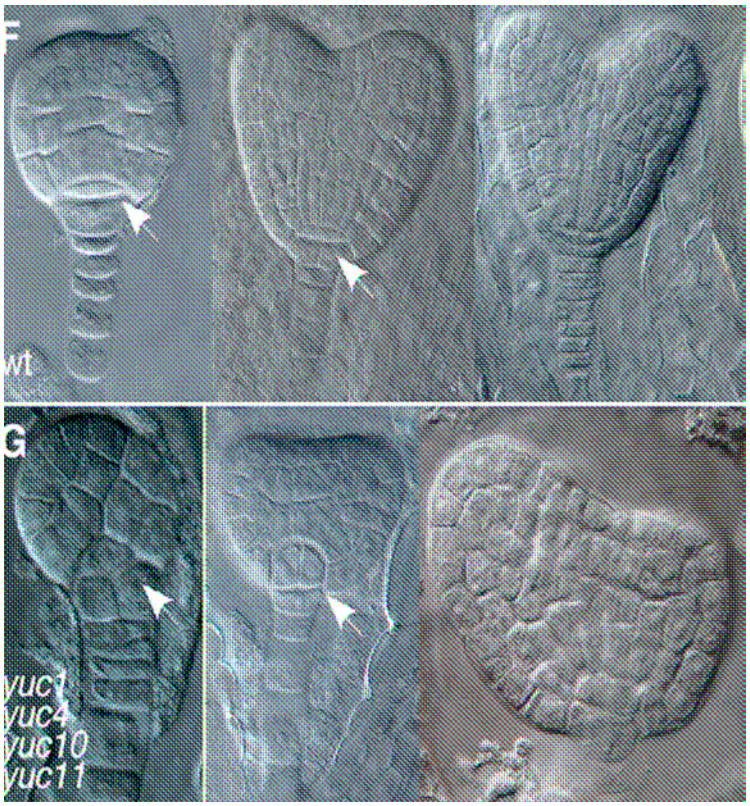

Figure 11: YUC genes in embryogenesis. (F) Stages of wild-type embryos. (G) The corresponding stages of embryos *of yuc1yuc4yuc10yuc11*, it lacks hypophysis. From Cheng et al., 2007.

indicates that auxin made by the YUC genes is a required auxin source for embryogenesis. Combining these results from previous findings it is clear that the YUCCA genes are needed throughout plant development – in flower organogenesis, vascular differentiation, and embryogenesis.

iaaM gene rescues *yuc* defects; YUC genes are indispensible for plant development

Perhaps due to inefficient auxin uptake or accumulation of inappropriate auxin levels, exogenous application of auxin fails to complement any of the *yuc* mutants [Cheng et al., 2006]. Instead, expression of the bacterial auxin biosynthesis gene iaaM with YUC promoters mimics YUC mediated auxin production and does indeed rescue the developmental defects of the yuc mutants, including abnormal flower structures, infertility, decreased apical dominance [Cheng et al., 2006].

Taken together, these studies evince YUCCA genes have unique and overlapping expressions / functions, and that their expressions are temporally and spatially regulated. These experiments also prove that auxin deficiency is responsible for the *yuc* mutant defects, and that YUC genes are important genes in auxin biosynthesis and thus indispensible for plant development. Furthermore, the tryptamine pathway is important for plant development because failure of auxin synthesis from this branch cannot be complemented through other routes of auxin production [Delker et al., 2008].

Project 1: EMS Screening in the *yuc1yuc4* background

Auxin synthesis by the YUC genes is the starting point for auxin action, and it is followed by auxin transport, signaling, and responses. The identification of the YUC genes and the analysis of these partially auxin deficint *yuc* mutants give a new approach the study the molecular mechanisms of auxin regulation. Unlike previous studies where excess exogenous auxin is added, we can use the *yuc* mutants to conduct genetic screening in which the auxin level is decreased. Also, adding exogenous auxin may not influence auxin levels inside the cells, but since the YUC genes have specific spatial and temporal expression, we can see the effects when the localized *in vivo* auxin gradient is disturbed. Moreover, instead of focusing on seedling or root phenotypes like in adding exogenous auxin experiments, we can find genes important throughout all stages of development, because the *yuc* mutants have defects in flowers, vasculature, and leaves as well as embryos. I specifically look for mutants that enhance *yuc1yuc4* phenotypes. For these reasons, EMS forward genetic screening in the sensitized *yuc1yuc4* mutant background makes up for the deficiencies in older screening approaches and subsequent genetic mapping will allow for the identification of new genes involved auxin-mediated development.

TAAs in the IPA auxin biosynthetic pathway

In addition to the characterized YUCCA tryptamine pathway, two independent groups recently identified a small family of tryptophan aminotransferases (TAAs) that

converts L-tryptophan to indole-3-pyruvic acid (IPA), the initial step in the pathway for making auxin [Stepanova et al., 2008; Tao et al., 2008].

Ethylene response gene WEI8 encodes TAA1

Stepanova et al. show genes in the Weak Ethylene Insensitive 8 (WEI8) family are required for tissue-specific responses to ethylene, and that WEI8 encodes TAA1. They isolate the mutants defective in tissue-specific responses to ethylene from genetic screening in *Arabidopsis*, and through mutant analysis of the *wei8* members, they reveal a connection between tissue-specific responses to ethylene and local auxin production. Moreover, the studies evince that WEI8 genes not only regulate ethylene responses, but are also essential for auxin mediated processes including proper formation of the embryo and flower development [Stepanova et al., 2008].

Shade avoidance gene SAV3 also encodes TAA1

At the same time, an independent study done by Tao et al. investigating why plants grow taller to avoid shade, has identified the Shade Avoidance gene (SAV3) gene. SAV3 is necessary for the shade avoidance response in shaded environments of low red / far red light [Tao et al., 2008]. This is especially relevant in modern-day agriculture where high-density planting influences yield [Tao et al., 2008]. *sav3* mutants cannot elongate under reduced red / far red light (shade) [Tao et al., 2008]. Interestingly, SAV3 is the same as WEI8, also encoding for TAA1 in the IPA branch

of the auxin biosynthetic pathway. In wildtype shade-intolerant plants, when the appropriate photoreceptors sense increased shade in their environment (indicated by decreased ratio of red to far-red light), they will trigger responses to avoid shade by increasing in height [Tao et al., 2008]. Short-term exposure to extra shade also induces early flowering [Tao et al., 2008]. Furthermore, shaded conditions trigger increases in auxin production as well as free IAA level [Tao et al., 2008]. TAA1 expression is high in leaves, which causes the auxin source to be transported to sites of cell elongation like hypocotyls [Tao et al., 2008]. In the molecular aspect, shade avoidance triggers fast but reversible changes in the expression of genes involved in auxin transport and auxin response [Carabelli et al., 2007].

In order for plants to survive and to fine tune growth and development, they must be able to properly perceive and translate external cues into appropriate cellular responses. Although the two groups conduct completely different studies, both show that external signals such as ethylene and light can regulate auxin biosynthesis and subsequently change auxin levels to induce specific development responses. WEI8 and SAV3 are identified to be a gene encoding for tryptophan aminotransferase TAA. The TAA genes of the IPA route and the YUCCA family of the indole-3-acetaldoxime (IAOx) pathway together represent the main branches of the tryptophan dependent IAA biosynthesis.

Auxin metabolism

In addition to the multiple pathways that contribute to auxin biosynthesis, elucidating auxin regulated development is further complicated by the complex metabolic networks that conjugate auxin to sugars, amino acids, or proteins. The conjugated forms of auxin mostly appear to be biologically inactive, and they may be stored, transported, or may be degraded and removed [Woodward and Bartel, 2005]. In addition, the auxin IAA can be converted to indole-3-butyric acid (IBA) by IBA synthase; IBA through beta-oxidation, can be made back into IAA and exert auxin effects [Delker et al., 2008] (see figure 12).

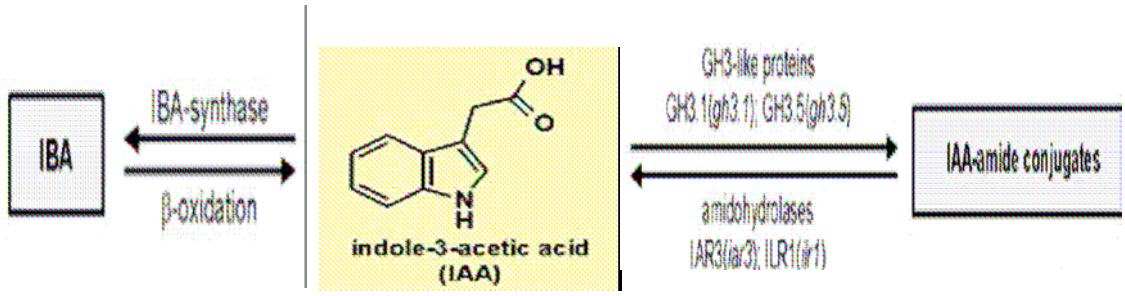

Figure 12: Auxin metabolism – compounds and enzymes in conjugation. Conjugated auxin may be stored or degraded depending on conditions. From Delker et al., 2008.

Auxin conjugation

Some of the molecular components participating in IAA conjugation and hydrolysis have been identified via genetic screens. An example is the GH3 gene family, which functions to adenylate IAA to amino acid conjugates (see figure 12) [Staswick et al., 2002 and 2005]. Functioning in a negative feedback loop, the GH3 genes are induced by auxin, preventing the accruement of excess free IAA [Staswick et al., 2002 and 2005]. After conjugation, IAA may also be released through

hydrolysis by amidohydrolases, ie. IAR3 [LeClere et al., 2002] and ILR1 [Woodward and Bartel, 2005].

Some GH3 enzymes not only act on auxin, they can also catalyze amido-conjugation to salicylic acid (SA), abscicic acid, or jasmonic acid [Staswick et al., 2002], which implies GH3 genes potentially are crosstalk points for different hormone signaling pathways. Recent studies show that the defense mechanism in plants likely involves such crosstalk and integration of various pathways – GH3.5 incorporates signals from both the SA and auxin pathways during defense response [Zhang et al., 2007].

Auxin Transport

Plant growth and development are not only dependent on auxin biosynthesis and metabolism, but also necessitates formation of auxin gradients or maxima. Both auxin biosynthesis and auxin polar transport contribute to the establishment of proper auxin concentration gradient.

Plants use two methods to transport auxin. One of these is fast, long-distance source to sink translocation from young shoot tissues toward sink tissues [Vanneste and Friml, 2009]. This is nonpolar auxin transport that happens by loading auxin into the mature phloem, which is then distributed passively throughout the plant and finally delivered to sink tissues like the root [Vanneste and Friml, 2009]. The second type of

auxin transport is slower and is responsible for moving auxin over shorter distances – mainly between cells [Vanneste and Friml, 2009]. This occurs through transmembrane influx and efflux carrier proteins, and directional or polar transport of auxin is achieved in specific tissues [Vanneste and Friml, 2009].

Auxin transport proteins

Directional auxin transport between cells is mainly created by asymmetric distribution of various transmembrane proteins – these proteins function either as efflux or influx carriers to regulate the release or uptake of auxin in the cell [Vanneste and Friml, 2009].

The protein auxin permease 1 (AUX1) is a well-characterized auxin influx carrier [Bennett et al., 1996]. The *aux1* mutant is identified in a genetic screen for auxin resistant roots [Pickett et al., 1990], it is defective in auxin uptake it is resistant to membrane impermeable auxin that requires active uptake for cellular entry [Pickett et al., 1990]. AUX1 encodes an amino acid permease influx protein [Bennett et al., 1996], and functions as a proton / auxin symporter [Yang et al., 2006]. There are four auxin influx carriers (AUX1 / LAX) in *Arabidopsis* [Swarup et al., 2008]. These influx proteins are involved in a wide variety of developmental process including lateral root and root hair formation, phototropism, flower / leaf arrangement on the stem, and gravitropism [Bennett et al., 1996].

Other proteins involved in both auxin influx and efflux are ABC transporters called multi-drug resistant / P-glycoprotein (MDR / PGP) [Geisler et al., 2005]. The asymmetric distribution of these transport proteins directs the polar flow of auxin and affects multiple developmental processes such as embryogenesis, organ formation, vascular differentiation, and tropisms [Delker et al., 2008]. This polar distribution of transporters is established in part by their constant cycling from the plasma membrane to endosomes [Delker et al., 2008].

PIN family as auxin efflux proteins

Members of the Pin-formed (PIN) family are auxin efflux transporters directing the polar transport of auxin from the site of synthesis to other tissues **[**Křeček et al., 2009]. *Pin-formed (pin1*) *Arabidopsis* mutant is disrupted in auxin transport, the most noticeable characteristic being that it develops pin-like inflorescences and has no flowers [Okadala et al., 1991], (see figure 13). This phenotype is caused by defective auxin transport [Galweiler et al., 1998], and can be rescued by the application of auxin to the pin-stem [Reinhardt et al., 2003].

The PIN family contains 8 transporter membrane proteins directing the polar efflux of auxin [Petra´sˇek et al., 2006]. Like many other genes participating in auxin pathways, the PIN proteins have distinct, yet overlapping functions, and the most homologous family members complement each other in loss-of-function mutants [Blilou et al., 2005]. In the *pin1 Arabidposis* mutant for example, PIN4 expression

expands to locations where PIN1 normally would exist [Blilou et al., 2005]. The precise biochemical functions of PINs have not been elucidated, but so far genetic studies show that PINs generate specific auxin distribution patterns through their own polar localization, and that they participate in a wide variety of plant developmental processes [Teale et al., 2006].

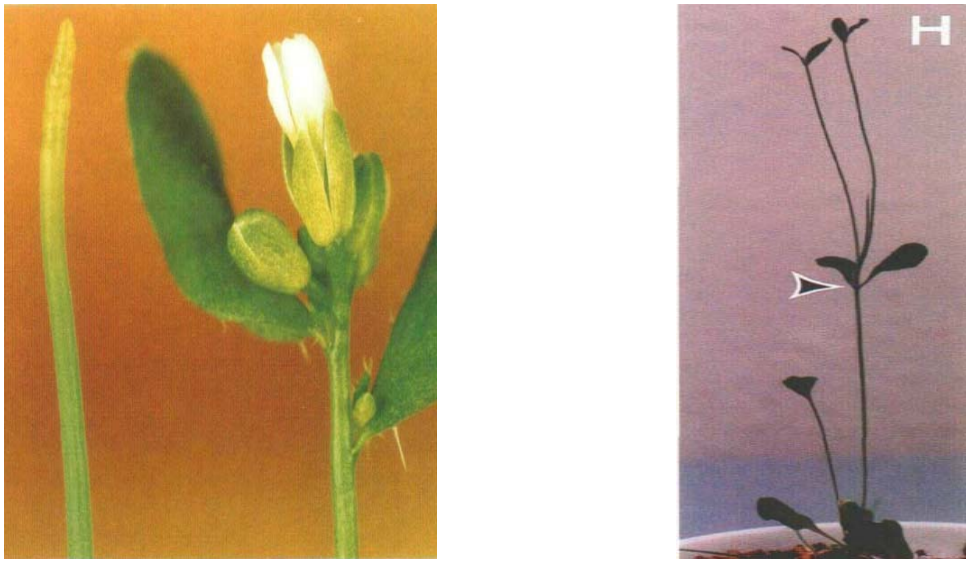

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Figure 13: Phenotype of the *pin1* mutant. In (A) *pin1* (left) has no flowers but only pin-like inflorescence vs. wildtype flower (right). (B) adult *pin1* plant. From Okalada et al., 1991.

Factors Affecting PIN Distribution

PIN protein localization is subject to change in response to both intrinsic and environmental cues, and this directly influences plant development. The exact

mechanisms responsible for localization of the transporters are not completely clear. However, studies suggest that auxin, PINOID (PID), protein phosphatase 2A (PP2A), endocytotic recycling of PIN proteins [Geldner et al., 2003], and the expression of other auxin carrier proteins, (PGPs, MDR1) are all responsible for their localization [Geisler et al., 2006].

Factors Affecting PIN Distribution: PGPs

P-glycoprotein (PGP) is a transporter protein working together with PIN to facilitate auxin cellular efflux – PGP19 stablizes PIN1 on the plasma membrane [Blakeslee et al., 2007]. In the shoot apex, PGP1 and PGP19 colocalize with PIN1, as well as with PIN1 and PIN2 in root tissues [Blakeslee et al., 2007]. Co-IP and yeast two hybrid further corroborate specific PGP-PIN interactions [Blakeslee et al., 2007]. On the other hand, no PGP and AUX1 influx carrier interaction is observed [Blakeslee et al., 2007]. Phenotypic analysis of independent *pin* and *pgp* loss of function mutants reveal their separate roles in auxin efflux, but *pin,pgp* double mutants display more severe and additive pin inflorescence phenotypes [Blakeslee et al., 2007]. These studies show auxin efflux activity is coordinated by both PINs and PGPs, and although both are distinct transport mechanisms, they function interactively and synergistically in plant tissues.

Factors Affecting PIN Distribution: Auxin

In addition, several lines of evidence suggesting that auxin itself participates in PIN protein distribution. First, auxin can affect transcription and degradation of PIN1 [Vieten et al., 2005]. Second, auxin response factors (ARFs) and transcription repressors AUX/IAA proteins are needed to directionally target the PIN proteins [Vieten et al., 2005]. Moreover, changes in auxin concentration and distribution either through loss of single PIN proteins, or treatment with NPA (auxin transport inhibitor) engender ectopic expression of other PIN proteins [Vieten et al., 2005].

Factors Affecting PIN Distribution: PINOID

Another important contributor to PIN localization and polar auxin transport is the serine / threonine kinase protein PINOID. PINOID belongs to a family of 23 protein kinases, which all have differential expression patterns [Friml et al., 2004]. Overexpression of the PID gene causes a basal to apical shift in PIN localization, resulting in loss of auxin gradients leading to strong defects in embryo and seedling roots [Friml et al., 2004]. For example in 35S::PID seedlings, the hypocotyls and roots become agravitropic, and the primary root system collapses [Friml et al., 2004]. These effects are due to a decrease of auxin levels, as indicated by reduced auxin-responsive DR5::GUS expression and IAA levels [Friml et al., 2004]. Conversely, loss of function *pid* creates apical to basal shift of PIN protein, resulting loss of auxin in the inflorescence apex, and leading to defective organ formation represented by extra flower petals and formation of a pin-like inflorescence (see figure 14), [Friml et al., 2004; Benjamins et al., 2001]. Thus, PID regulates PIN polarity and polar auxin

transport, influencing several developmental processes. Interestingly, PINOID's own level is also influenced by auxin, which implies that auxin synthesized locally is able to control its own accumulation or removal through feedback control of PID transcription and PIN polarity [Chandler, 2009].

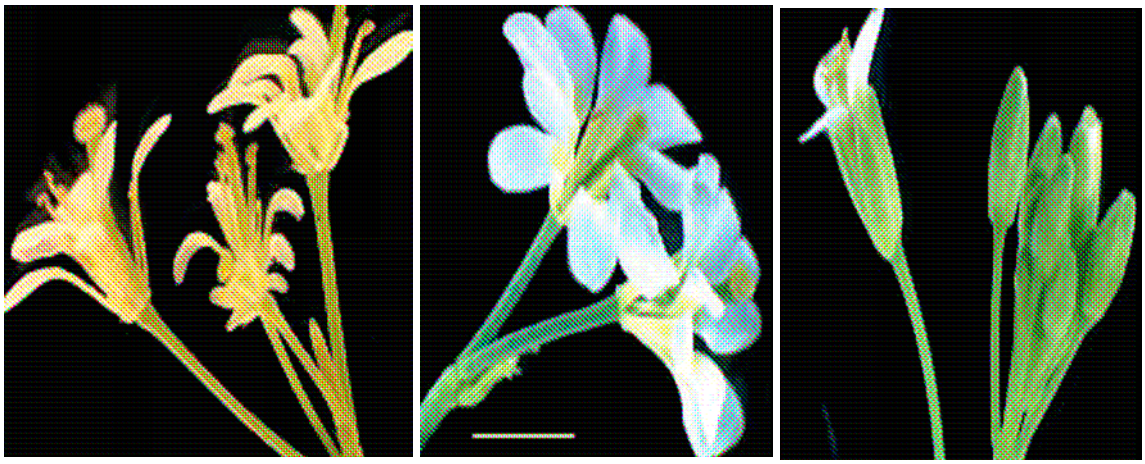

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Figure 14: PINOID in flower development. (A), (B) *pid* mutants have pin inflorescence and few aberrant flowers with extra petals. Flowers generally have few or no sepals and stamens, more petals, a trumpet shaped pistil and produce no or only few seeds. (C) wildtype flower. From Benjamins et al. 2001.

PID and PP2A counterbalance PIN localization

The mechanism by which PINOID (PID) and protein phosphatase 2A (PP2A) determine localization of PIN proteins is via de/phosphorylation, which in turn dictates the direction of auxin flow [Michniewicz et al., 2007]. Genetic and phosphorylation studies demonstrate that PID and PP2A localize together with PINs, governing the phosphorylation status in the central hydrophilic loop of PINs [Michniewicz et al., 2007]. PID and PP2A work in an antagonistic manner – loss of

function *pid* causes PIN movement to the basal side, while *pid* gain of function and *pp2a* loss of function engender apical distribution of PINs [Michniewicz et al., 2007]. Therefore, phosphorylated PINs are recruited to apical side while dephosphorylated PINs are targeted to the basal side; this determines the direction of auxin transport and mediates plant development.

Auxin biosynthesis and polar auxin transport (PAT) together control development

Many scientists studying auxin have held the prevailing notion that polar auxin transport is of utmost importance for plant development, and that it is the main contributing factor in establishing auxin maxima / gradients. Thus, they have viewed auxin biosynthesis as somewhat insignificant. For example, Grieneisen et al. uses computer modeling and wrongly predicts that polar auxin transport alone is responsible for generating auxin maxima and maintaining stable auxin gradient in the root tip. However now we have unequivocal evidence that *both* local auxin biosynthesis and auxin transport play essential roles in establishing local auxin concentration, mediating a wide variety of plant developmental processes including: flower organogenesis, vascular tissue formation, leaf development, embryogenesis, and lateral root growth, as described below.

Auxin biosynthesis and PAT in flower organogenesis

Inactivation of YUC genes sometimes gives rise to similar flower phenotypes seen in auxin transport mutants (ie. *pin1, pinoid, ettin*) [Chandler, 2009]. There is great variation in *pin1, pinoid* and *yuc* flowers, with defects in any of the four whorls, and the plants may develop pin-like inflorescences [Chandler, 2009]. Because higher order *yuc* mutants have various floral defects similar to auxin transport mutants, we see that floral organogenesis is mediated by both polar auxin transport and auxin biosynthesis.

Auxin biosynthesis and PAT in vascular tissue development

Mutations in auxin transport and signaling mutants – *pin1 and mp,* and NPA treatment lead to defects in vascular formation [Mattsson et al., 1999]. Interestingly, combined *yuc* mutants – *yuc1yuc4* and *yuc2yuc6*, also have impaired vascular development like the transport and signaling mutants [Cheng et al., 2006]. In addition, higher order *yuc* mutants show increasingly severe vascular defects, demonstrating dose-dependent effects, and confirming the canalization hypothesis [Sachs, 1981], which states that a specific auxin level must be reached for vascular formation. It has been observed that when *yuc* mutants are treated with NPA, the auxin transport inhibitor, plants are incapable of growing new leaf, and this phenotype is not seen NPA treatment or yuc mutants alone [Cheng et al., 2007].

Auxin biosynthesis and PAT in leaf development

Since there are multiple genes and gene families with overlapping functions involved in auxin transport and auxin biosynthesis, single loss of function *pin1* mutants or double loss of function *yuc1yuc4* mutants are only partly disrupted in auxin efflux and biosynthesis [Cheng et al., 2007]. For example, in the *pin1* mutant alone or *yuc1yuc4* double mutant, there are defects in floral organogenesis and vascular tissue, but no obvious abnormalities in leaves and lateral organs [Cheng et al., 2007]. However, the combined *yuc1yuc4pin1* triple mutant could not form true leaves, a trait not seen in either *yuc1yuc4* or *pin1* [Cheng et al., 2007]. This indicates that PIN1 and YUC genes synergistically control leaf development.

Combining PIN weak alleles *pin1-5* with *yuc1yuc4* further corroborates YUC and PIN synergistic interactions in leaf growth. The *pin1-5* weak alleles plants are quite similar to wildtype – fertile with no pin-like inflorescences, and only a bit shorter in stature. However placing *pin1-5* in either *yuc1* or *yuc4* background the plants have stronger phenotypes such as pin-like inflorescences and much less apical dominace [Cheng et al., 2007]. These studies show that auxin production from YUCs along with polar auxin transport from PINs have strong genetic interactions and work together in leaf development.

Auxin biosynthesis and PAT in embryo development

In cotyledon initiation, PIN-mediated auxin transport is thought to be the main contributor in generating the auxin maxima at the flanks of the globular embryo

[Benkova et al., 2003]. However, analysis on multiple *yuc* mutants show severe impairments in the embryo and cotyledon, and this clearly indicates that PIN-dependent transport isn't the only regulator and that instead, embryonic patterning is also mediated by local auxin biosynthesis from YUC genes [Cheng et al., 2007]. Recently, Leafy Cotyledon 2 (LEC2) has been discovered to positively regulate auxin-dependent transcription by binding directly to the YUC4 promoter and inducing auixn responses [Stone et al., 2008]. LEC2 is a B3 domain transcription factor functioning throughout all stages of embryonic development [Stone et al., 2001]. Auxin biosynthesis via YUC genes and LEC2, together with PIN mediated auxin transport both contribute to proper embryogenesis.

Lateral Root Growth

In incipient lateral roots and root tips, PIN-dependent polar auxin transport generates and maintains the auxin maxima [Benkova et al., 2003]. The CYP79B2/3 genes convert tryptophan to IAOx and are expressed in the root meristem and precise locations of root outgrowth [Ljung et al., 2005]. Auxin is much lower in *cyp79b2/3* double mutants [Ljung et al., 2005]. These observations show that local auxin biosynthesis along with PAT, contribute to the auxin sources for lateral roots formation.

Project 2: activation tagging T-DNA screening in *yuc1pid336* background

Because it is apparent that both auxin biosynthesis and auxin polar transport are important in coordinating plant development, it is interesting to analyze mutants that combine inactivated YUC genes and inactivated transport / signaling genes.

As mentioned earlier, PID regulates PIN polarity and polar auxin transport and influences development. In loss of function *pid* there is apical to basal shift of PIN proteins, resulting in loss of auxin in the inflorescence apex and leading to defective organ formation represented by formation of pin-like inflorescences and aberrant flowers [Friml et al., 2004].

Therefore, my project uses T-DNA activation tagging to screen for mutants in the *yuc1pid336* background. The *pinoid336 (pid336)* weak allele contains a single base pair mutation at position 336 of the PID gene; it shows no phenotypic defects alone. Techniques such as TAIL-PCR is used to locate the site of T-DNA insertion within the *Arabidopsis* genome. The goals are to identify and characterize novel factors in auxin mediated processes, and to further examine the genetic interactions between components of auxin biosynthesis and polar transport.

Activation T-DNA tagging and its advantages

To do this, the genetic screening approach I use is activation T-DNA tagging. Classical loss of function screen approach has major limitations: 1. it cannot discover genes with redundant actions (ie. the YUCCA gene family) 2. it cannot identify genes

that are complemented by alternative biosynthetic / regulatory pathways (ie. several proposed routes for auxin biosynthesis). In either of these two cases, loss of function in 1 gene shows no obvious phenotype. Moreover, genes that have essential roles in early embryogenesis (inactivation leads to embryonic lethality) and those that function during multiple developmental stages, also cannot be uncovered by traditional loss of function screening. Activation T-DNA tagging is a way to overcome such problems. By using activation T-DNA tagging strategy, we have enhanced expression of such genes through gain of function analysis, which will facilitate the assessment of gene functions in auxin pathways.

The Ti plasmid is carried by *Agrobacterium tumefaciens* and is able to insert a piece of its own DNA called the transfer DNA (T-DNA) into a plant's genome [Chilton et al., 1977]. In nature, the *Agrobacterium* has a set of genes within its T-DNA that converts plant tissues into a suitable environment for itself [Clough et al., 1998]. In the lab we exploit this mechanism for genetic transformation of *Arabidopsis*. For example, taking advantage of the T-DNA insertion into the plant genome, we can generate insertional mutants with disrupted gene functions; however, it is implausible to generate insertions in targeted genes since the mechanism of T-DNA insertion is random and uncontrollable. There are four consecutive copies of cauliflower mosaic virus 35S (CaMV 35S) enhancers, which increase the expression of genes in its vicinity, leading to genetic gain of function [Odell et al., 1985]. Many important genes involved in plant development processes have been discovered using this approach, ie. the YUCCA genes.

I focus on screening of mutants in the T2 generation, which may be either gain of function or loss of function mutants - if T-DNA insertion is within the exon coding region, it is a loss of function mutant; whereas if T-DNA insertion happens to be inserted in the promoter region, it is a gain of function mutant (see materials and methods). This approach involves a T-DNA vector, providing a target for PCR primer and facilitating analysis of gene functions.

Auxin nuclear signaling

Auxin concentration established by both auxin biosynthesis and polar auxin transport dictate auxin-dependent developmental processes, but in the end, plant cells must be able to correctly interpret these auxin concentrations to decide the developmental output. Although auxin induces a variety of cellular responses, the transcriptional regulation mediated by two types of regulators – Aux/IAAs and ARFs, is the essence of auxin signaling.

Aux/IAAs and Auxin Response Factors (ARFs) in auxin signaling

Studying genes upregulated upon auxin treatment, a group of negative regulators of auxin signaling called Aux/IAAs are identified, and there are 29 members in Arabidopsis [Abel and Theologis 1996]. There are four conserved domains in AUX/IAAs, all of which are required for transcriptional repressor function [Tiwari et

al., 2004; Szemenyei et al., 2008]. The first domain has an ERF-associated amphiphilic repressor motif that is necessary to recruit the transcriptional corepressor TOPLESS [Tiwari et al., 2004; Szemenyei et al., 2008]. The second domain is needed for auxin-stimulated Aux/IAA degradation [Gray et al., 2001], and mutations in this domain lead to auxin insensitivity [Rouse et al., 1998]. Homo and heterodimerization interactions with another group of transcriptional regulators – the auxin response factors (ARFs), involves the last two domains of Aux/IAAs [Kim et al., 1997].

The ARFs are transcription factors govern auxin-dependent transcriptional activities. There are 23 members in the ARF family, which either serve to activate or repress transcription, contingent upon the central domain's non-conserved amino acid sequence [Guilfoyle and Hagen, 2007]. The ARFs bind to a consensus sequence (named auxin response elements) located in the promoter regions of genes inducible by auxin [Ulmasov et al., 1997].

In low auxin conditions, the Aux/IAA transcriptional repressors are stable and dimerize with auxin response factors (ARFs) via domains III and IV. At the same time the corepressor TOPLESS is recruited to the Aux/IAA-ARF complex, inactivating transcription [Szemenyei et al., 2008]. When auxin levels are higher, Aux/IAA is ubiquinated by SCF^{TIR1} E3 ligase, followed by proteolysis by the 26S proteasome. Aux/IAA degradation stops TOPLESS repression and frees the activating ARFs to induce the transcription of auxin-dependent genes [Szemenyei et al., 2008].

Auxin perception / response: TIR1

In order to solve the question of how auxin is perceived, studies aimed to search for auxin receptor(s). Genetic screens led to the identification of the F-Box TIR1, which modulates the Skp1-cullin-F box protein (SCF) E3 ubiquitin ligase [Leyser et al., 1993]. This finding suggests that the protein stability status is important in auxin signaling. Because Aux/IAAs have been known to have a fast turn-over rate, it is logical to think that ubiquitin-dependent degradation is involved. In an auxin dependent manner, SCF^{TIR1} recruits Aux/IAAs, and via domain II of Aux/IAAs there is direct interaction with TIR1. Subsequently the Aux/IAAs are ubiquinated and targeted for degradation [Gray et al., 2001]. At relevant concentrations (20-80nM), auxin binds to TIR1 in its hydrophobic pocket, without causing a conformational change [Dharmasiri et al., 2005]. This is a necessary step that stabilizes the Aux/IAAs interaction with TIR1 [Dharmasiri et al., 2005a]. These observations are strong evidence that TIR1 is the auxin receptor. Additionally, more studies reveal three other TIR1 related, auxin binding F box proteins (AFBs) also directly interact with Aux/IAAs in an auxin dependent way, and it is possible they function redundantly with TIR1 [Dharmasiri et al., 2005b]. In the genomes of all land plants, there are homologs for every component of this pathway, and this proves the significance and indispensability of the SCF^{TIR1} mechanism [Rensing et al., 2008].

MATERIALS AND METHODS

Project 1: EMS Screening in the *yuc1yuc4* background

Starting plant material

All *Arabidopsis thaliana* lines used are from the Columbia background. *yuc1-/-yuc4-/+* EMS mutants are from Dr. Yunde Zhao's lab.

Plating, transplanting, and scoring

~50 seeds per *yuc1-/-yuc4-/+* EMS line are put out for initial screening. Seeds are washed with 100% and 75% ethanol, dried for ~30 minutes and plated out on agarose MS media. Seedlings are then placed in complete darkness, at ~4 degrees Celsius for two days, and are moved to the incubator with constant light, at ~ 20 degrees Celsius for ~1 week before they are transplanted into soil. Approximately 24-32 seedlings are transplanted for each line, 700 lines are screened. Mutants with phenotypes that either enhance or suppress yuc1yuc4 are isolated.

Crosses

Selected *yuc1-/-yuc4-/+* mutants are usually sterile. To recover them, sister plants from the same mutant line are crossed to homozygous wildtype *Landsberg-*

Erecta plants. F1 and F2 generations are planted in attempt to recover the mutant phenotype again for future mapping.

Plant DNA extraction and mutant genotyping

DNA of interesting mutants lines are extracted according to DNA prep protocol from Dr. Yunde Zhao's lab. Genotypes are determined by PCR with YUC1, YUC4 gene-specific primers from Dr. Yunde Zhao's lab.

Project 2: activation tagging T-DNA screening in *yuc1pid336* background

Starting plant material

All *Arabidopsis thaliana* lines used are from the *Columbia* background. *yuc1pid336* activation tagged T-DNA mutants are T2 plants, generated from *yuc1pid336* T1 plants, screened by Genji Qin (post-doc) of Dr. Yunde Zhao's lab.

T-DNA Activation Tagging

The activation tagging vector pSKI105 (see figure 15, 16) is constructed by Igor Kardailsky in the Weigel lab, its complete sequence in GenBank is AF187951. http://www.ncbi.nlm.nih.gov/sites/entrez?cmd=Retrieve&db=nucleotide&dopt=GenBank&list_uids=6537289

The constitutively active cauliflower mosaic virus 35S (CaMV 35S) enhancers present in the vector are enhancer elements originally developed by Rick Walden at the Max Planck Institue in Cologne [Weigel et al., 2000]. There are a total of four copies of CaMV 35S enhancers and they correspond to -417 to -86 relative to transcription start [Weigel et al., 2000]. These enhancers engender transcriptional activation of nearby genes when inserted in the promoter region, or cause gene inactivation if inserted in the exon coding regions [Weigel et al., 2000]. The affected genes are associated with a T-DNA insertion and this method is known as activation tagging [Weigel et al., 2000].

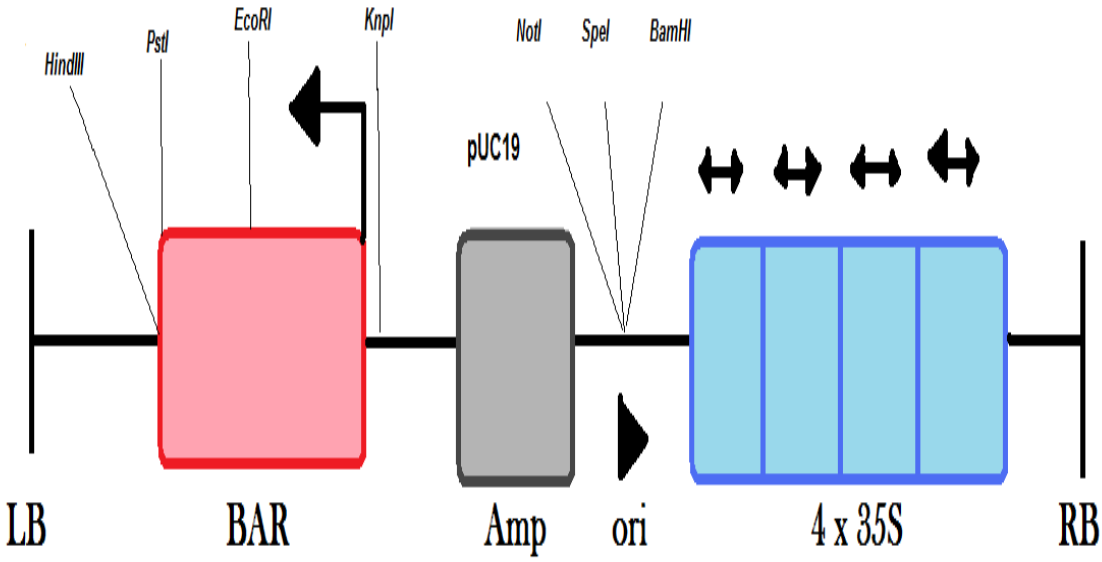


Figure 15: Molecular architecture of activation tagging T-DNA vector pSKI015, according to Weigel et al., 2000. BAR: herbicide glufosinate resistance gene facilitates selection of transgenic plants in soil. The construct has ampicilin gene (Amp) and origin of replication (ori). The restriction enzyme sites on both sides can be used to rescue the flanking genomic sequence next to the left and right borders.

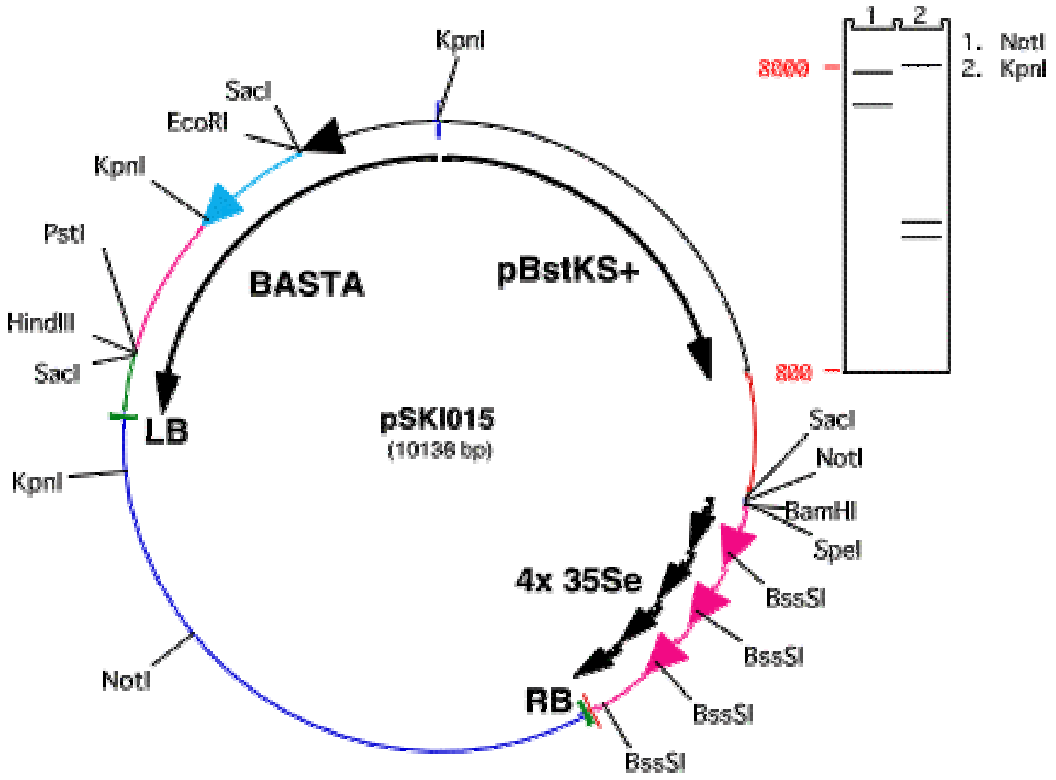


Figure 16: The activation tagging vector pSKI105 from Weigel lab, Salk Institute

Important Features of Activation Tagging

The vector also contains the Bar gene that confers Basta resistance for selection in soil. The majority of the overexpressed genes are immediately adjacent to the CaMV 35S enhancers, and usually it causes enhanced endogenous expression pattern rather than constitutive ectopic expression [Weigel et al., 2000]. The activation tagging vector pSKI105 confers resistance to the herbicide Basta, also known Finale^{TM}; it is commercially available containing 5.78% glufosinate-ammonium as 1000x stock. Selected mutant lines are sprayed 2-3 times per week with an aqueous 1x dilution [Weigel et al., 2000].

Plating and transplanting

~50 seeds per *yuc1pid336* T-DNA line are put out for initial screening. Seeds are washed with 100% and 75% ethanol, dried for ~30 minutes and plated out on agarose MS media. Seedlings are then placed in complete darkness, at ~4 degrees Celsius for two days, and are moved to the incubator with constant light, at ~ 20 degrees Celsius for ~1 week before they are transplanted into soil. Approximately 15-20 seedlings are transplanted for each line.

Scoring for mutants

yuc1pid336 by itself is phenotypically similar to wildtype *Columbia* plants. Seedlings are examined under light microscope and abnormal seedlings are noted and selected for transplant. Adult plants with phenotypes that show defects in seedlings, flowers, siliques, and leaves are isolated.

Plant DNA Extraction

Plant DNA prep for genotyping and TAIL-PCR is done according to DNA prep protocol from Dr. Yunde Zhao's lab and Plant DNeasy protocol from Qiagen, respectively.

Mutant Genotyping

DNA of interesting mutants lines are extracted according to DNA prep protocol used in Dr. Yunde Zhao's lab. Genotypes are determined by PCR with YUC1, PID336, and BAR gene-specific primers from Dr. Yunde Zhao's lab.

Thermal Asymmetric InterLaced PCR (TAIL-PCR)

Thermal asymmetric interlaced PCR is an efficient way to amplify unknown sequences adjacent to T-DNA insertion sites in *Arabidopsis*. The many advantages of TAIL-PCR include: simplicity, high specificity and efficiency, speed, direct sequencing, and high sensitivity [Liu & Whittier, 1995].

TAIL-PCR primers

I use 8 shorter arbitrary degenerate (AD) primers along with 3 longer, nested T-DNA specific primers (SP1-3) that have differing annealing temperatures to carry out three cycles of PCR. The specific primers are complementary to T-DNA vector sequence and has high melting temperature - Tm~58-63 Celsius. The AD primers have lower melting temperature - Tm~47-48 Celsius [Liu & Whittier, 1995]. The primers are from TAIL-PCR material stock in Dr. Yunde Zhao's lab.

Three TAIL-PCR reactions

The relative amplification efficiencies of specific and nonspecific products can be thermally controlled throughout the three rounds of TAIL-PCR - primary, secondary, and tertiary (see figures 17-21). Each reaction round has varying stringency cycles: the high-stringency cycle (thermal asymmetric) has Tm~63 Celsius; reduced-stringency cycle (thermal symmetric) has Tm~44 Celsius; and low stringency cycle has Tm~30 Celsius [Liu & Whittier, 1995].

Specific products are not always present in the primary reactions because of their relatively low concentration. However, the specific products become visible after the subsequent secondary reaction since most non-specific product bands from the primary reaction disappear after the secondary round. Thus, high specificity of TAIL-PCR allows for the direct sequencing of the products [Liu and Whittier, 1995].

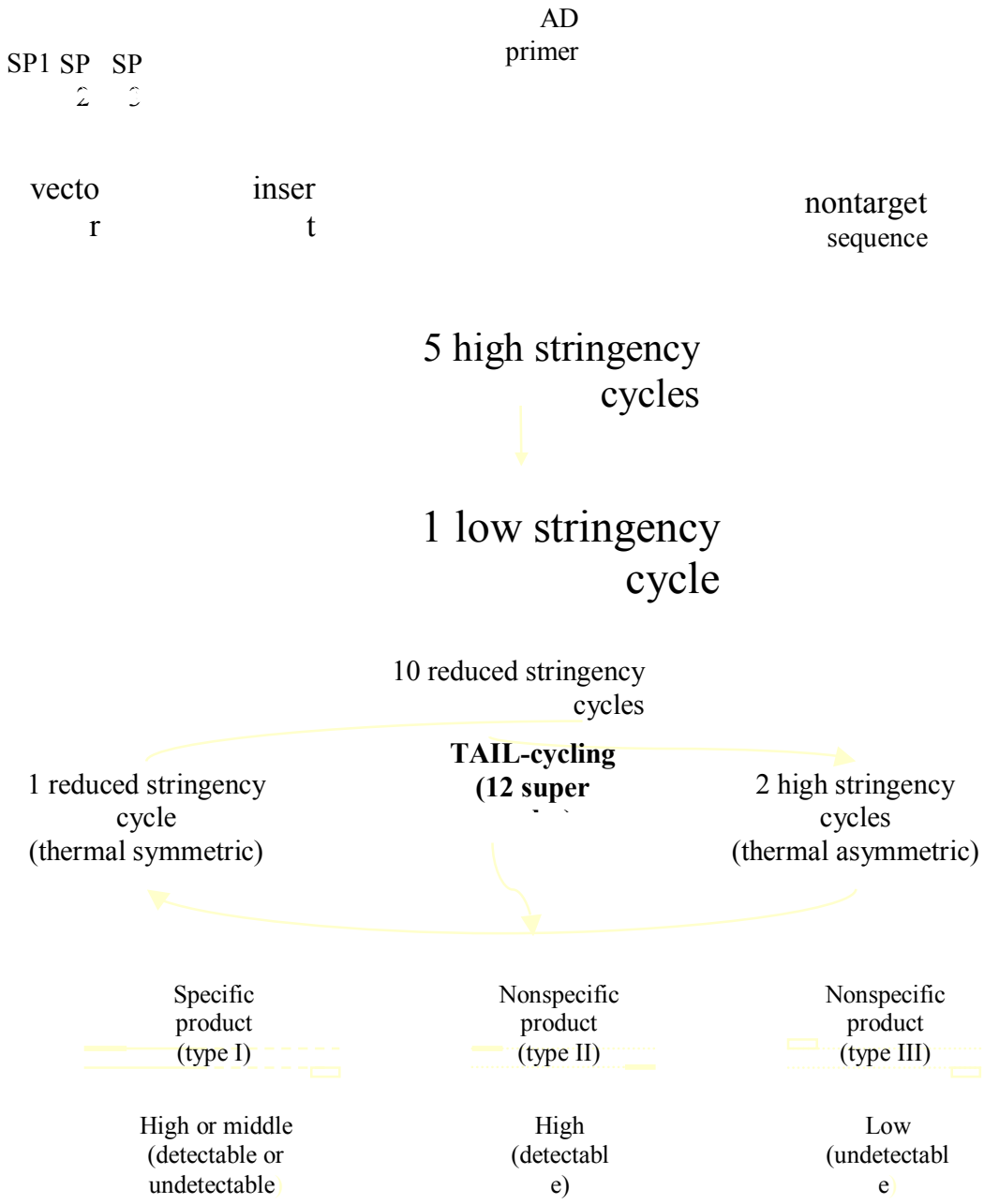


Figure 17: TAIL-PCR primary cycle amplification, using SP1 / AD primers. The following figures 17-21 are according to Liu and Whittier, 1995.

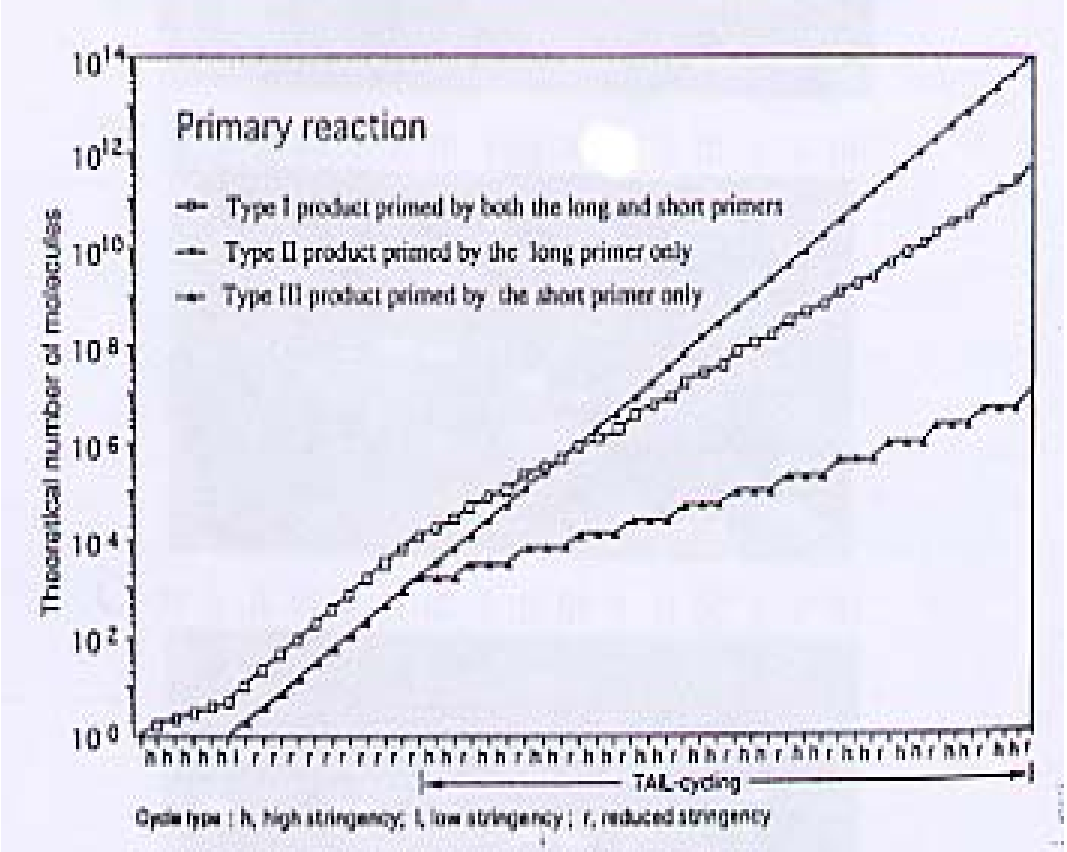


Figure 18: Products after primary TAIL-PCR reaction

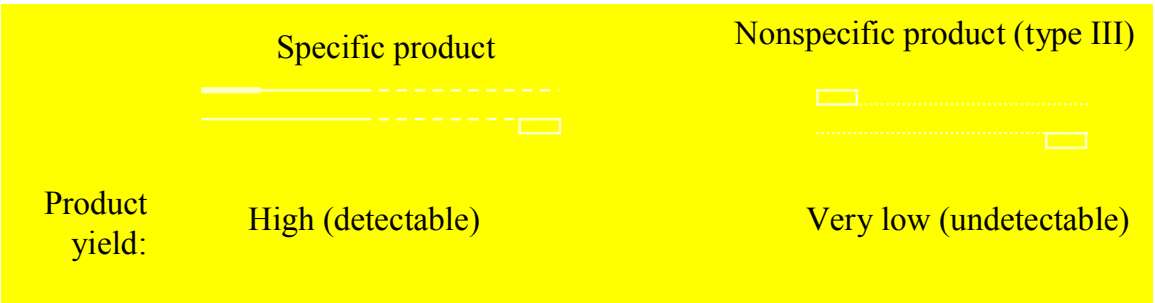


Figure 19: TAIL-PCR secondary cycle amplification, using SP2 / AD primers and diluted primary PCR products

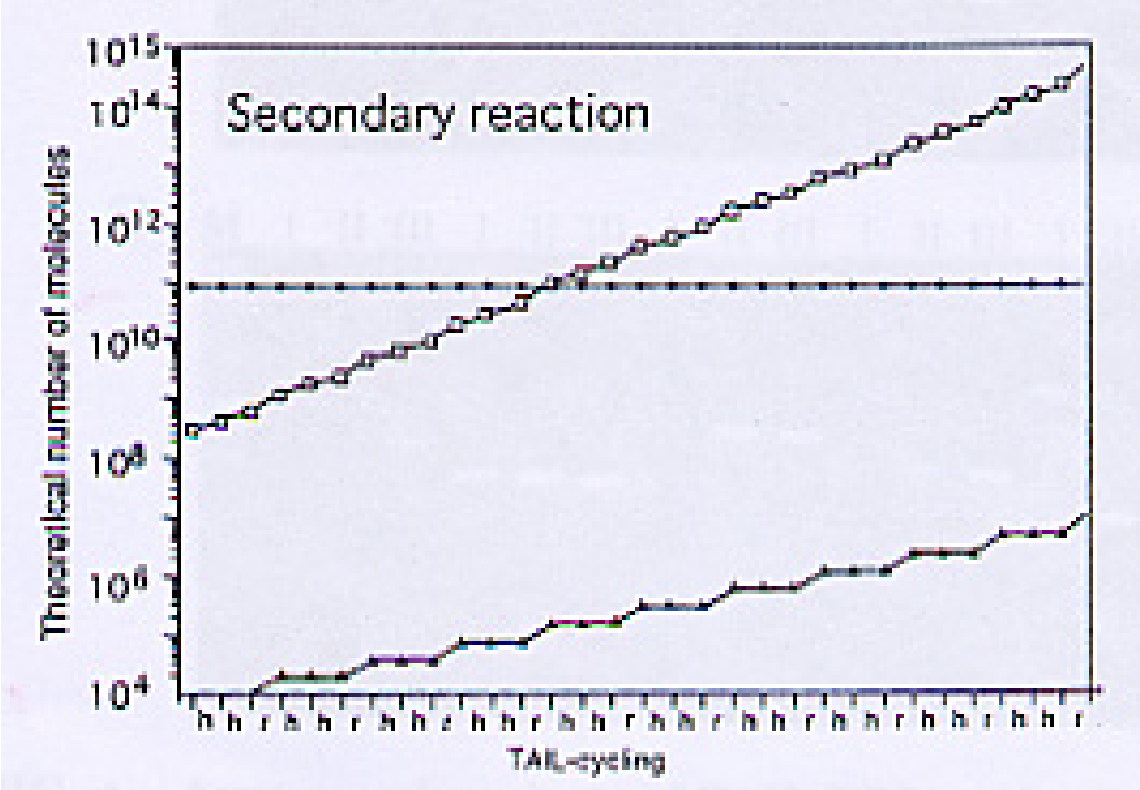


Figure 20: Products after secondary TAIL-PCR reaction

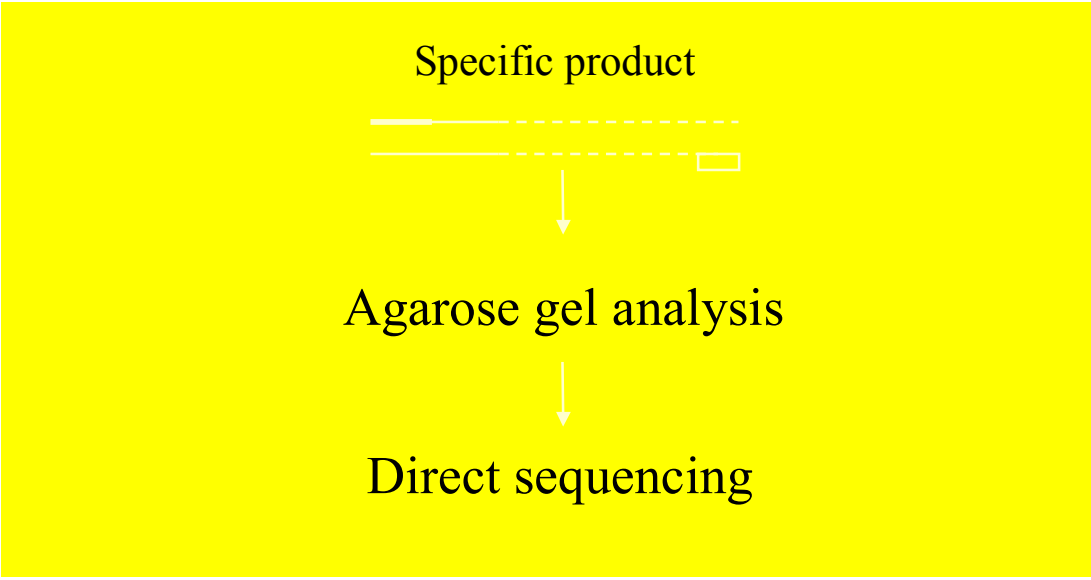


Figure 21: TAIL-PCR tertiary cycle amplification, using SP3 / AD primers and diluted secondary PCR products. The specific products are sent for direct sequencing.

Specific TAIL-PCR cycling conditions

Table 3: Primary TAIL-PCR reaction cycle

Lid = 100 Celsius	Temperature in Celsius	Time in minutes
1	92	3
2	95	1
3	94	0.5
4	65	1
5	72	2.5
6	Go to step 3	Repeat 4x
7	94	0.5
8	25	3
9	72 + 0.0 R=0.3/s G=0.0	2.5 + 0:00 + 0.0/s
10	94	0.25
11	65	1
12	72	2.5
13	94	0.25
14	65	1
15	72	2.5
16	94	0.25
17	44	1
18	72	2.5
19	Go to step 10	Repeat 14x
20	72	5
END		

Table 4: Secondary TAIL-PCR reaction cycle

Lid = 105 Celsius	Temperature in Celsius	Time in minutes
1	94	1
2	94	0.25
3	65	1
4	72	2.5
5	94	0.25
6	65	1
7	72	2.5
8	94	0.25
9	45	1
10	72	2.6
11	Go to step 2	Repeat 14x
12	72	5
END		

Table 5: Tertiary TAIL-PCR reaction cycle

Lid = 105 Celsius	Temperature in Celsius	Time in minutes
1	94	2
2	94	0.5
3	56	0.5
4	72	0.5
5	Go to step 2	Repeat 40x
6	72	2

Gel band extraction, purification; product sequencing

Gel products are visualized under UV light and specific bands of the tertiary cycle are cut out and purified for direct sequencing. Gel purification is done according to Qiagen's protocol. Specific TAIL-PCR products are sent for sequencing, using the specific T-DNA left border primer from Dr. Yunde Zhao's lab.

Adapter Ligation PCR

Adapter Ligation PCR contains three basic steps – 1. restriction enzyme-mediated ligation of adapter to genomic DNA 2. PCR of the T-DNA, genomic DNA junction using primers specific to the adapter and T-DNA 3. sequencing of T-DNA, genomic junction to map the reference genome [O'Malley et al., 2007]. My experimental design mainly follows the protocol form O'Malley et al. For details of my design see Results section.

RESULTS

Project 1: EMS Screening in the *yuc1yuc4* background to dissect auxin biosynthesis

YUC genes producing auxin mark the beginning of auxin action, auxin transport, perception / signaling, and responses follow. The identification of the YUC genes and the characterization of partially auxin deficient *yuc1yuc4* mutants serve as the starting material for my project; it also provides a new approach to study the molecular mechanisms of auxin biosynthesis and signaling.

Normal Arabidopsis flowers have four sepals, four petals, six stamens, and two fused carpels, but there are defects in *yuc1yuc4* mutants The *yuc1yuc4* plants have dramatic defects in all four whorls of floral organs, with no functional reproductive organs; and although degree of severity varies, all *yuc1yuc4* lack particular organs [Cheng et al., 2006]. For example, some *yuc1yuc4* flowers have no sepal tissues, while others contain most sepal like organs; some also have stamens, but none of the stamens produce pollen [Cheng et al., 2006]. On the other hand, *yuc1yuc4* mutants do not exhibit defects in embryogenesis [Cheng et al., 2006].

In forward EMS screening, I look for mutants that display more severe phenotypes than *yuc1yuc*4 alone – ie. defects in embryo and seedling structures, formation of pin-like inflorescences with complete abolishment of any type of floral organs. In addition, I also look mutants that can suppress the *yuc1yuc4* phenotype – ie.

formation of near normal or extra floral structures and fertile plants. By scoring for and isolating *yuc1 yuc4* enhancers / suppressors through forward EMS screening we can identify and characterize genes important in auxin-mediated plant development and fill in the gaps of our current understanding of auxin mechanisms.

Isolated EMS *yuc1yuc4* mutant seedlings

yuc1yuc4 double mutants do not have any obvious defects in embryogenesis and the formation of leaves; it is similar to the wildtype seedling shown in figure 22 below. Figure 23 and 24 shows *yuc1yuc4 so652* and *yuc1yuc4 so607* mutants with abnormal seedling development. (~50 seeds are plated for each EMS *yuc1yuc4* screening line). Both *yuc1yuc4 so652* and *yuc1yuc4 so607* lines later have 2-4 adult plants (out of 24 in 1 pot) with pin-like inflorescences (see table 6).

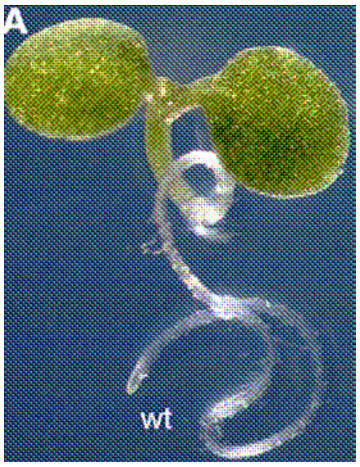


Figure 22: wildtype seedling. From Cheng et al., 2006.

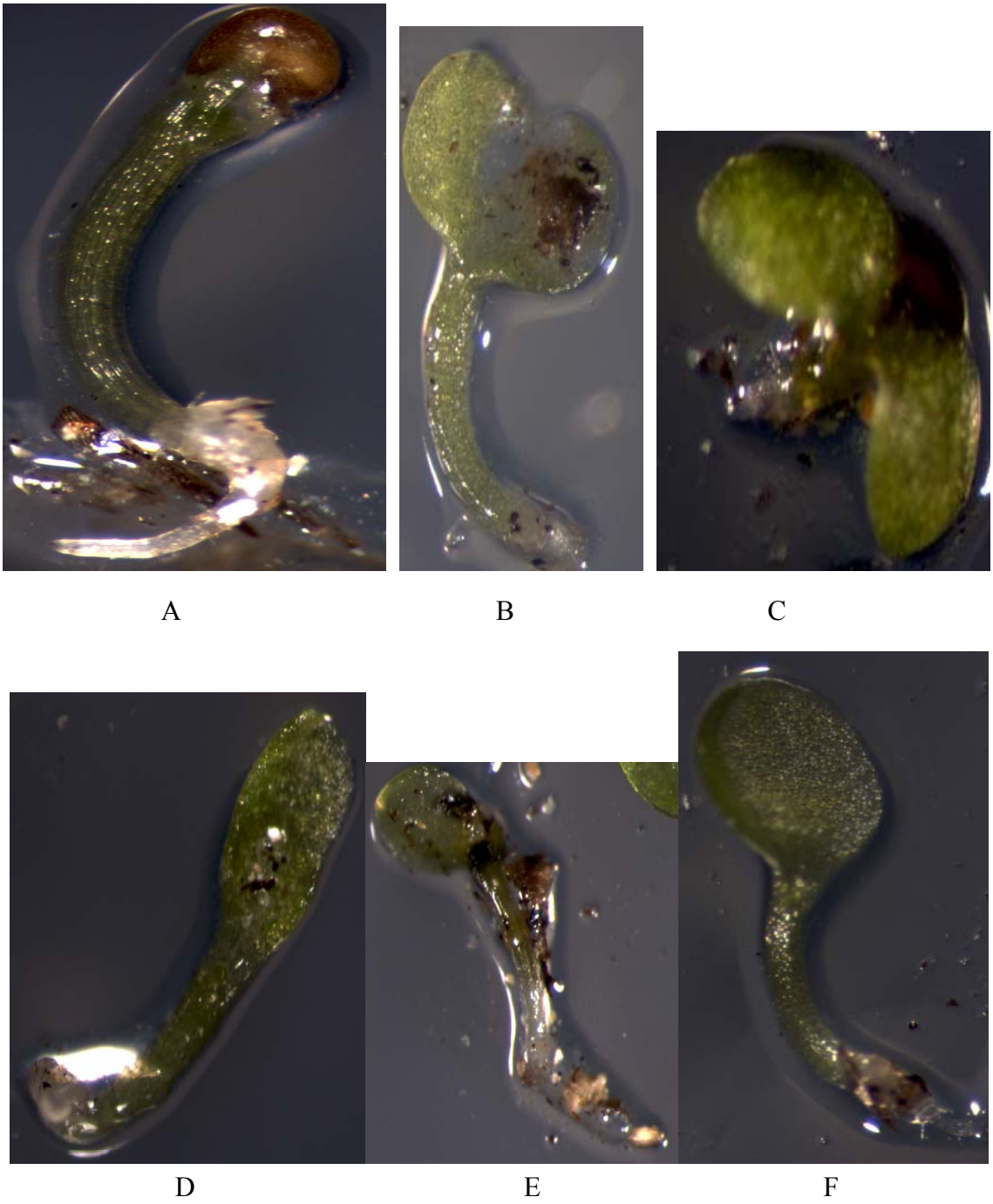
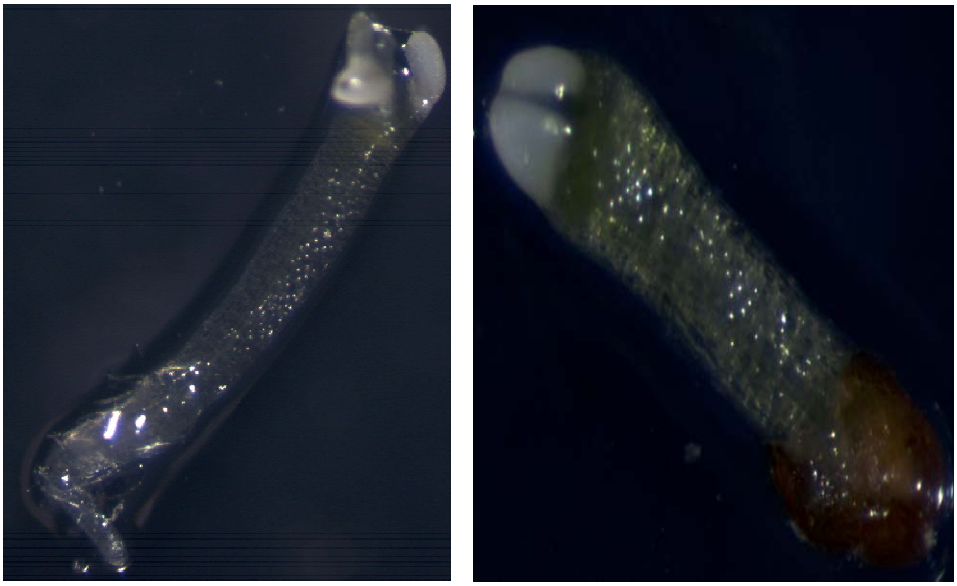


Figure 23: (A-F) EMS *yuc1yuc4* so652 mutant seedlings.

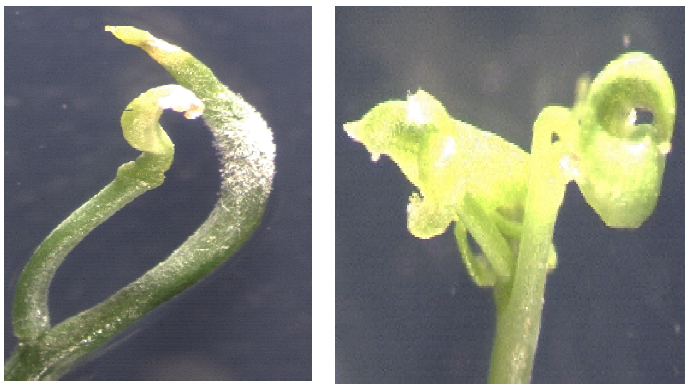


A B

Figure 24: (A-B) EMS *yuc1yuc4* so607 mutant seedlings.

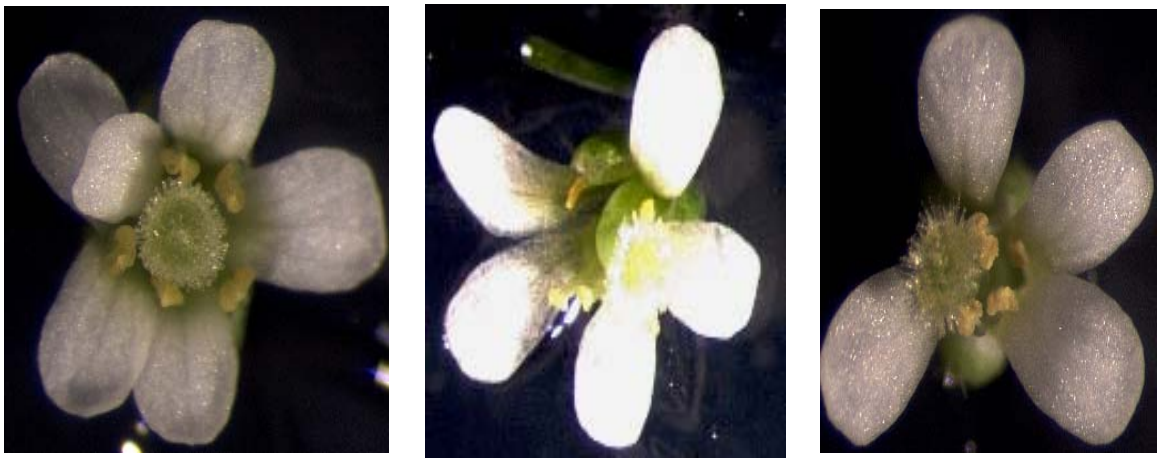
EMS *yuc1yuc4* mutant flowers

yuc1yuc4 double mutants exhibit defects in all four whorls and are entirely sterile. A strong enhancer of *yuc1yuc4* mutant is found – 583. It develops aberrant flowers as well as pin-like inflorescences with complete abolishment of all floral organs (see figure 25). In the 583 line, three plants out of 24 (1 pot) show enhanced *yuc1yuc4* flower phenotype. A potential suppressor of yuc1yuc4 mutant is also isolated – 585. Four plants out of 24 (1 pot) have suppressed *yuc1yuc4* phenotype. This mutant has near normal flower structures with extra petals and is fertile (see figure 26).



A B

Figure 25: (A-B) EMS *yuc1yuc4*-583 mutant flowers.



A B C

Figure 26: (A-C) EMS *yuc1yuc4* supressor – 585 mutant flowers with normal floral organs, extra petals, and is fertile.

yuc1yuc4 EMS enhancer / suppressor lines (M1) isolated

In total 750 lines were plated, transplanted, and scored; each line contained 24-28 plants. Table 6 lists lines with enhanced / suppressed *yu1yuc4* phenotypes.

Next generation M2 of isolated _yuc1yuc4_ EMS enhancer / suppressor lines

For all M1 lines with either enhancer or suppressor phenotypes, seeds are harvested from their sister plants (~20 sister plants for each line); and 15-18 seeds from each sister plant were transplanted as M2 generation. No original enhancer or suppressor phenotypes are recovered in the M2 generation except in these four lines: 612, 607, 615, 625. However the enhancing phenotypes – pin-like inflorescences are weak compared to the original ones seen in M1 generation, making mapping difficult.

Table 6: yuc1yuc4 EMS lines with enhanced / suppressed *yu1yuc4* phenotypes. (A) lines with enhanced phenotypes, with pin-like inflorescences, 2-4 mutants out of 24 plants total. (B) lines with suppressor phenotype in flowers, 2-4 mutants out of 24 plants total.

A	B
715	669
631	585
632	651
625	
615	
607	
645	
652	
656	
696	
583	
612	

~20 crosses to *Landsberg-erecta* using 612, 607, 615, 625 M1 sister plants. All F1 and F2 seeds were transplanted, but mutant phenotypes are not recovered in F2; thus I was unable to perform mapping to identify the gene.

Project 2: activation tagging T-DNA screening in *yuc1pid336* background

Since both auxin biosynthesis and auxin polar transport are important in coordinating plant development, I analyze mutants that combine inactivated YUC gene *(yuc1-/-)* and inactivated transport / signaling gene *(pid336-/-)*. I use T-DNA activation tagging to screen for mutants in the *yuc1pid336* background.

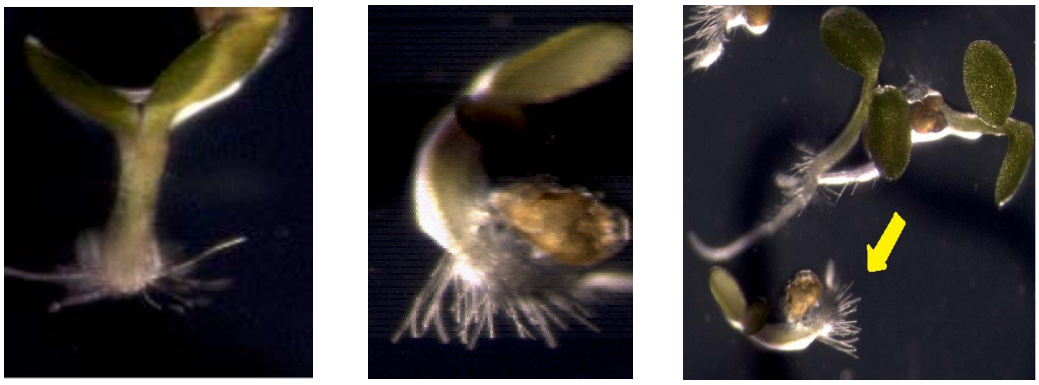
Hallmarks of defective auxin biosynthesis and transport include aberrant seedlings, abnormal flower structures and/or the formation of pin-like inflorescences in place of flowers. The *pid336* weak allele contains a single base pair mutation at position 336 of the PID gene; it shows no phenotypic defects alone. The single *yuc1* loss of function mutant also does not exhibit obvious defect alone. In T-DNA activation tagging screening, I specifically look for mutants that display severe defects throughout development – in embryogenesis (see figures 27-43), floral organogenesis, or have pin-like inflorescences (see figures 44-53).

I have screened a total of 1300 *yuc1pid336* T2 lines, 15-20 plants for each T2 line. I collected the seeds of isolated mutant T2 lines and transplanted T3 mutants, to recapitulate the phenotypes and examine segregation ratios. To identify the genes associated with mutant phenotypes I collected high purity DNA and performed TAIL-PCR for many mutants. By scoring for and isolating these mutants my hope is to identify and characterize genes important in auxin-mediated plant development and

improve of our current knowledge of auxin biosynthetic, transport, and signaling mechanisms.

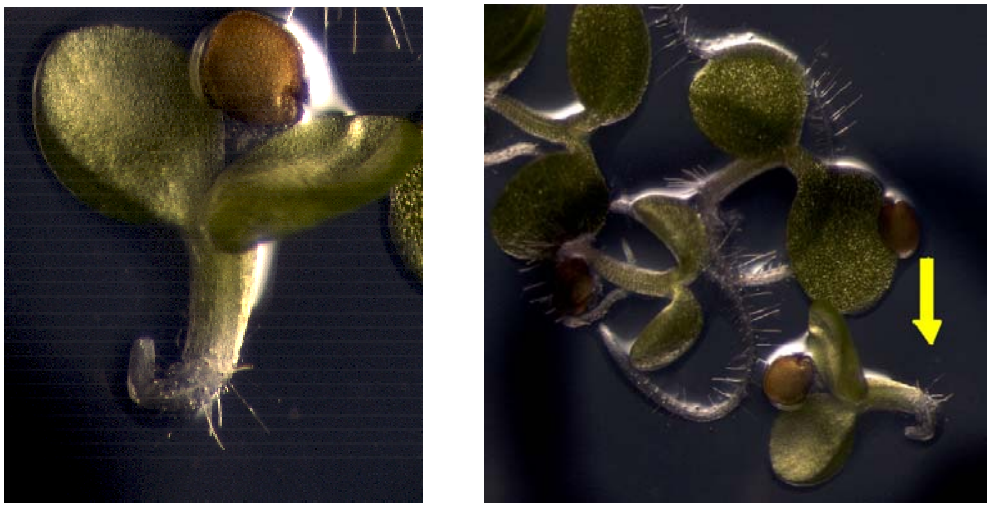
Isolated T-DNA *yuc1pid336* T2 mutant seedlings

yuc1pid336 double mutants do not have any obvious defects in embryogenesis, except that sometimes seedlings will have three cotyledons, as seen in *pid*. The figures below (27-43) show *yuc1pid336* T2 mutant lines with abnormal seedling development. ~40 seeds are plated for each T2 *yuc1pid336* screening line, 15~20 seedlings are transplanted for each line. Seedlings have been growing on MS media, in light for at least seven days before photos are taken.



A B C

Figure 27: *yuc1pid336* T2-303. (A) 303-1 (B)303-2 (C) 303-2 seedling in contrast with seedlings from the same line. This mutant is defective in root development.



A B

Figure 28: *yuc1pid336* T2-252. (A) 252-1 (B)252-1 seedling in contrast with seedlings from the same line. This mutant has defects in root development.

A B

Figure 29: *yuc1pid336* T2-111. (A) 111-1 (B)111-2 This mutant is defective in cotyledon development.

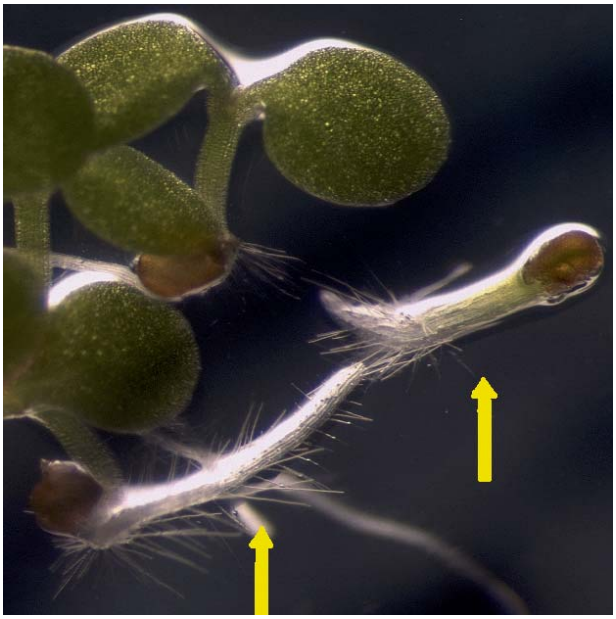
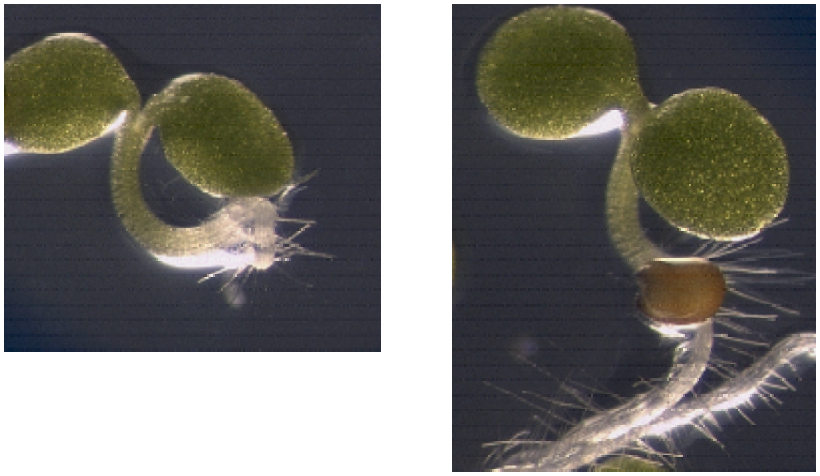


Figure 30: *yuc1pid336* T2-110. 2 seedlings in contrast with seedlings from the same line. This mutant is defective in cotyledon development.

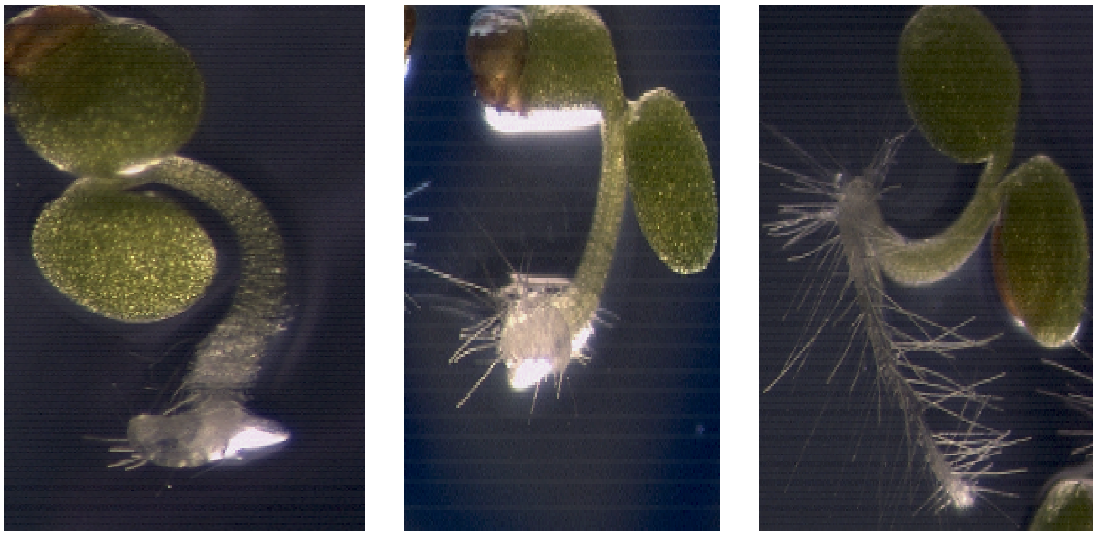
A B C

Figure 31: *yuc1pid336* T2-106. (A) 106-1 (B) 106-2 (C) normal. This mutant is defective in root development. All pictures are taken on the same day.



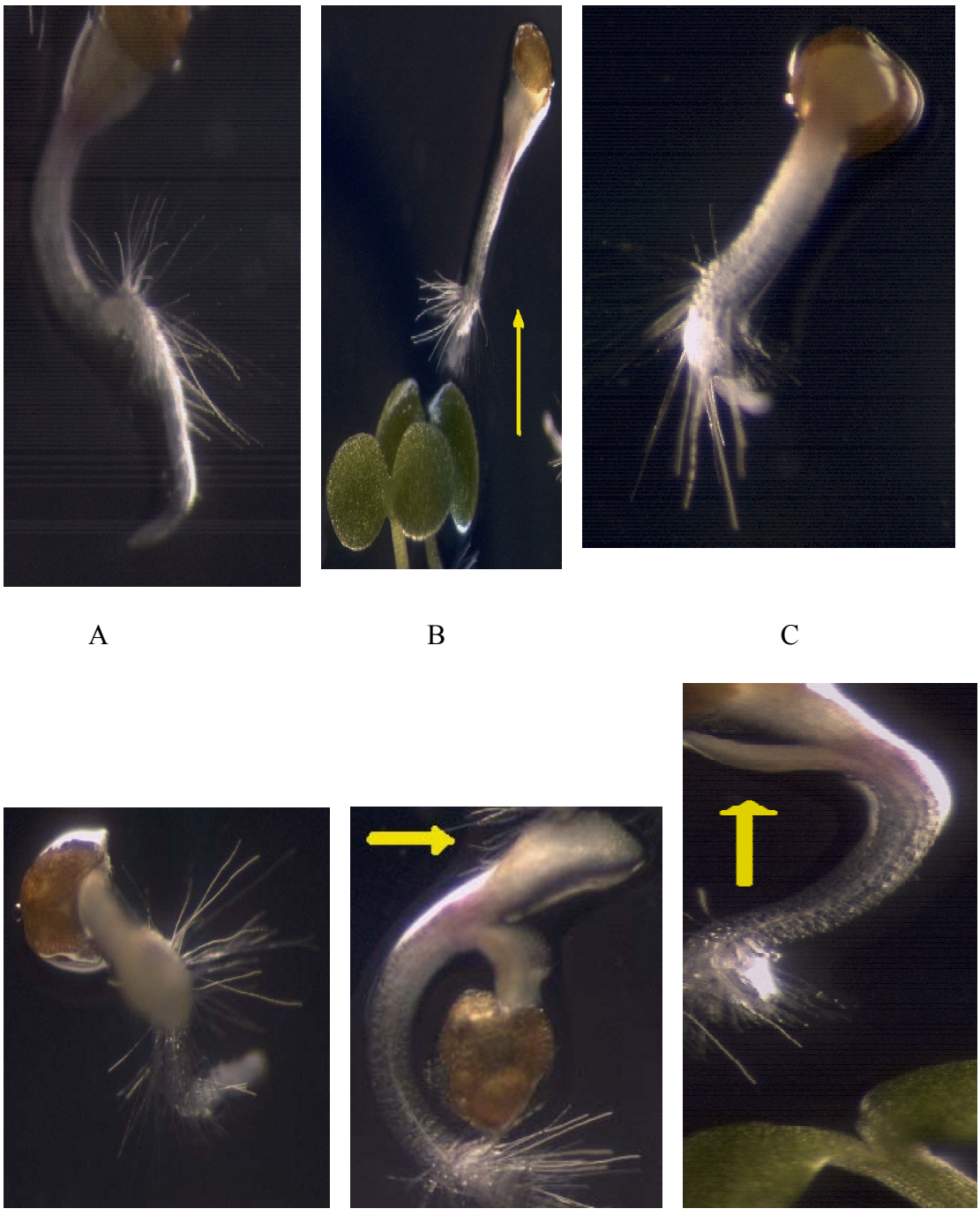
A B

Figure 32: *yuc1pid336* T2-101. (A) 101-1 (B) normal. This mutant is defective in root development.



A B C

Figure 33: *yuc1pid336* T2-98. (A) 98-1 (B) 98-2 (C) 98 normal seedling. This mutant is defective in root development.



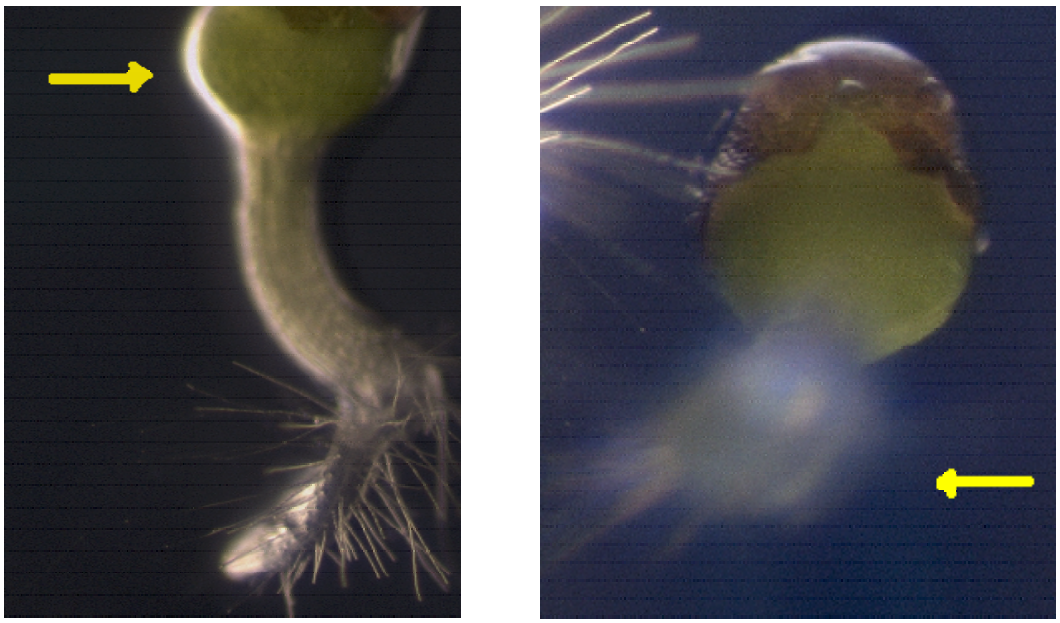
A B C

D E F

Figure 34: *yuc1pid336* T2-96. (A-F) 96-1, 96-2, 96-3, 96-4, 96-5, 96-6 seedlings. This mutant is defective in root and cotyledon development.

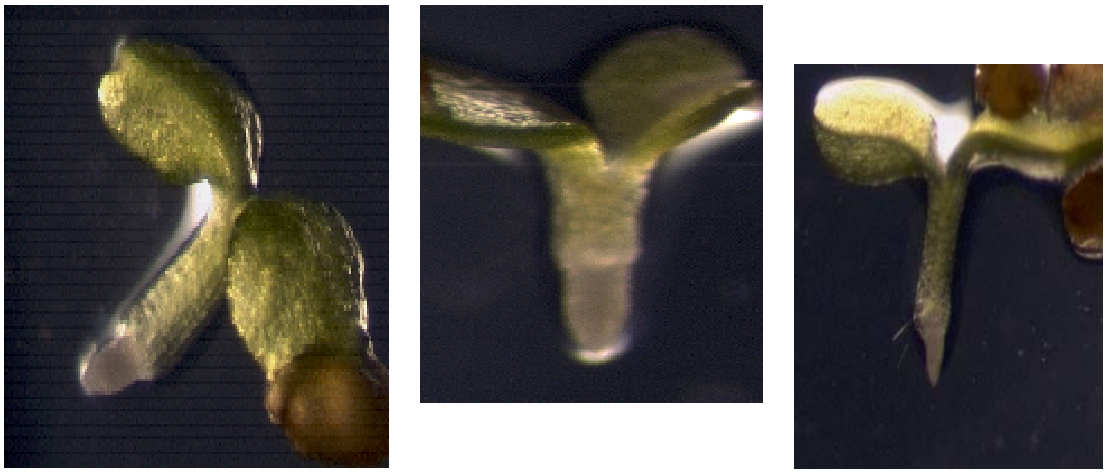
Figure 35: *yuc1pid336* T2-76. This mutant is defective in root development.

Figure 36: *yuc1pid336* T2-73. This mutant is defective in root development.



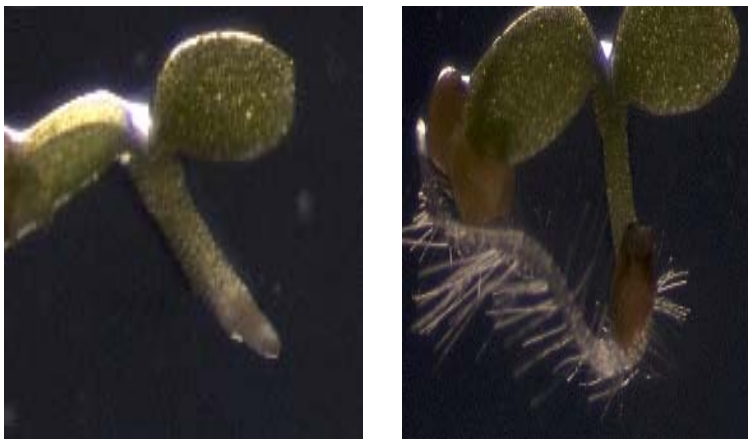
A B

Figure 37: *yuc1pid336* T2-70. (A) 70-1, fused cotyledon (B) 70-2, no root. This mutant is defective in root and cotyledon development.



A B C

Figure 38: *yuc1pid336* T2-258, 259. (A) 258-1, short root (B) 259-1, short root. (C) 259-2, short root. These mutants are defective in root development.



A B

Figure 39: *yuc1pid336* T2-260. (A) 260-1, short root (B) 260, normal. This mutant is defective in root development.

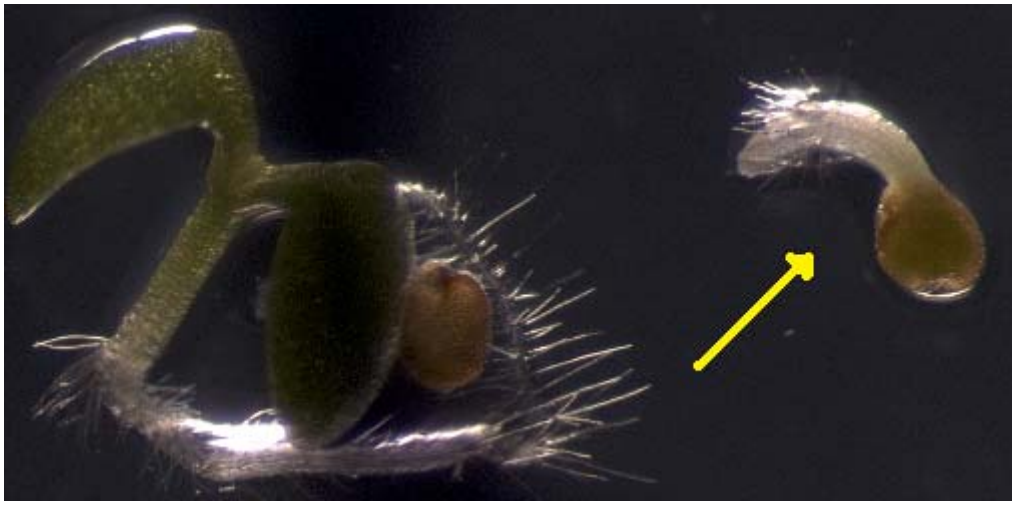
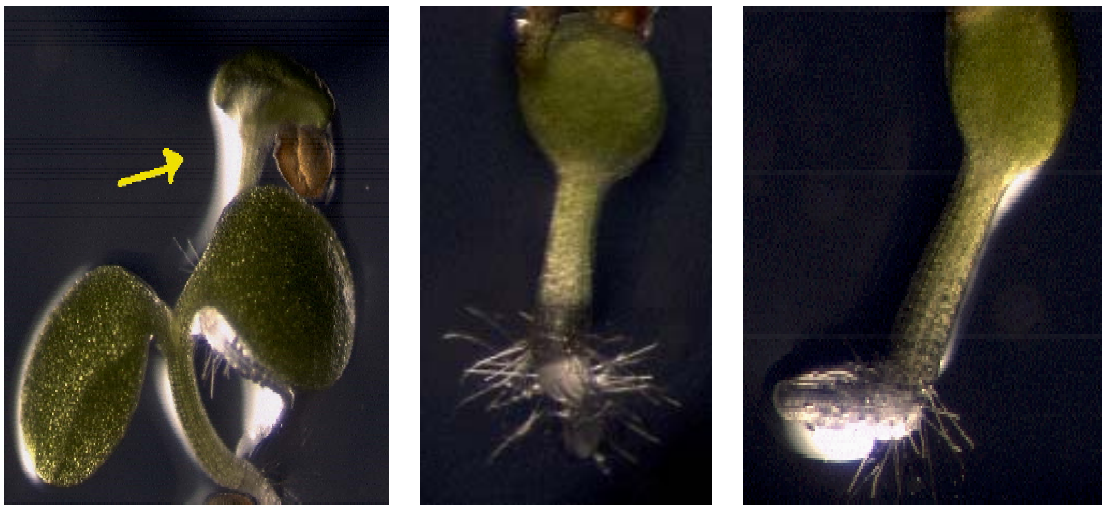
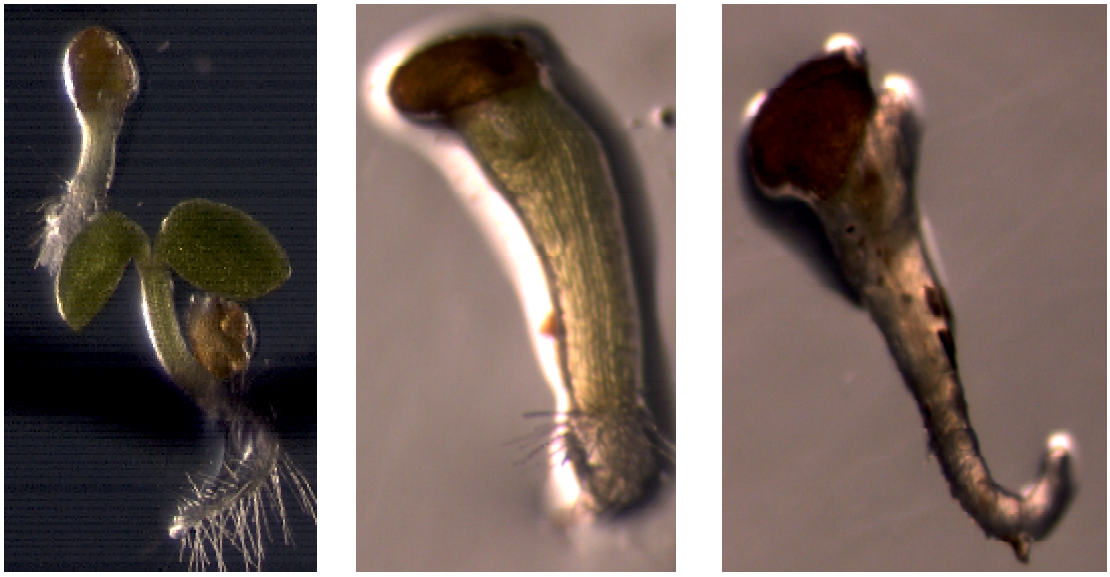


Figure 40: *yuc1pid336* T2-265. This mutant is defective in root and cotyledon development.



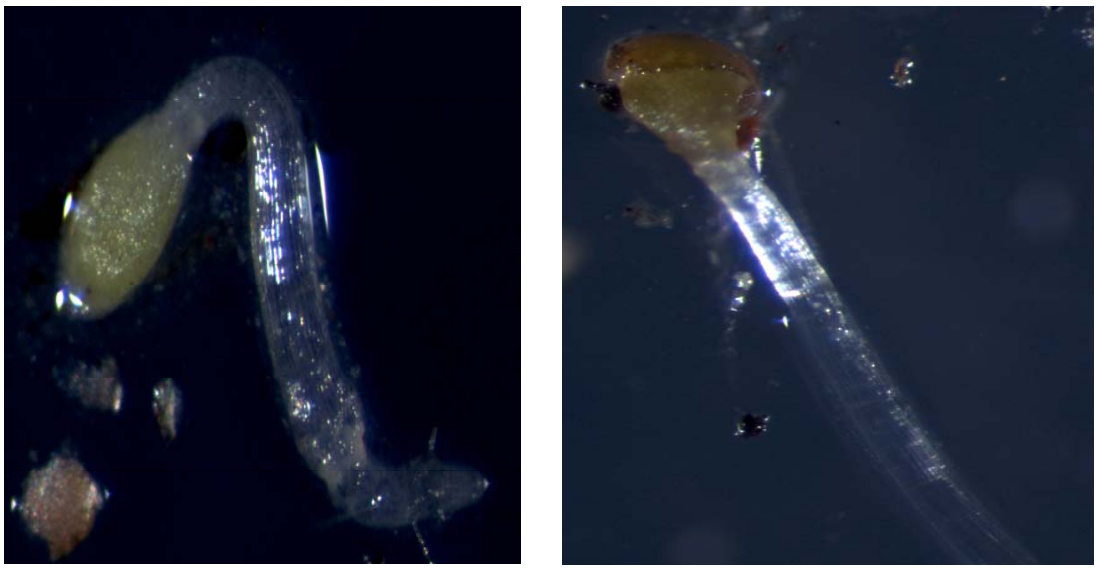
A B C

Figure 41: *yuc1pid336* T2-299. (A) 299-1, fused cotyledon (B) 299-2, defective root (C) 299-3, defective root.



A B C

Figure 42: *yuc1pid336* T2-310. (A) 310-1, no cotyledon (B) 310-2 (C) 310-3



A B

Figure 43: *yuc1pid336* T2-1038. (A) 1038-1, 1 cotyledon (B) 1038-2, 1 cotyledon

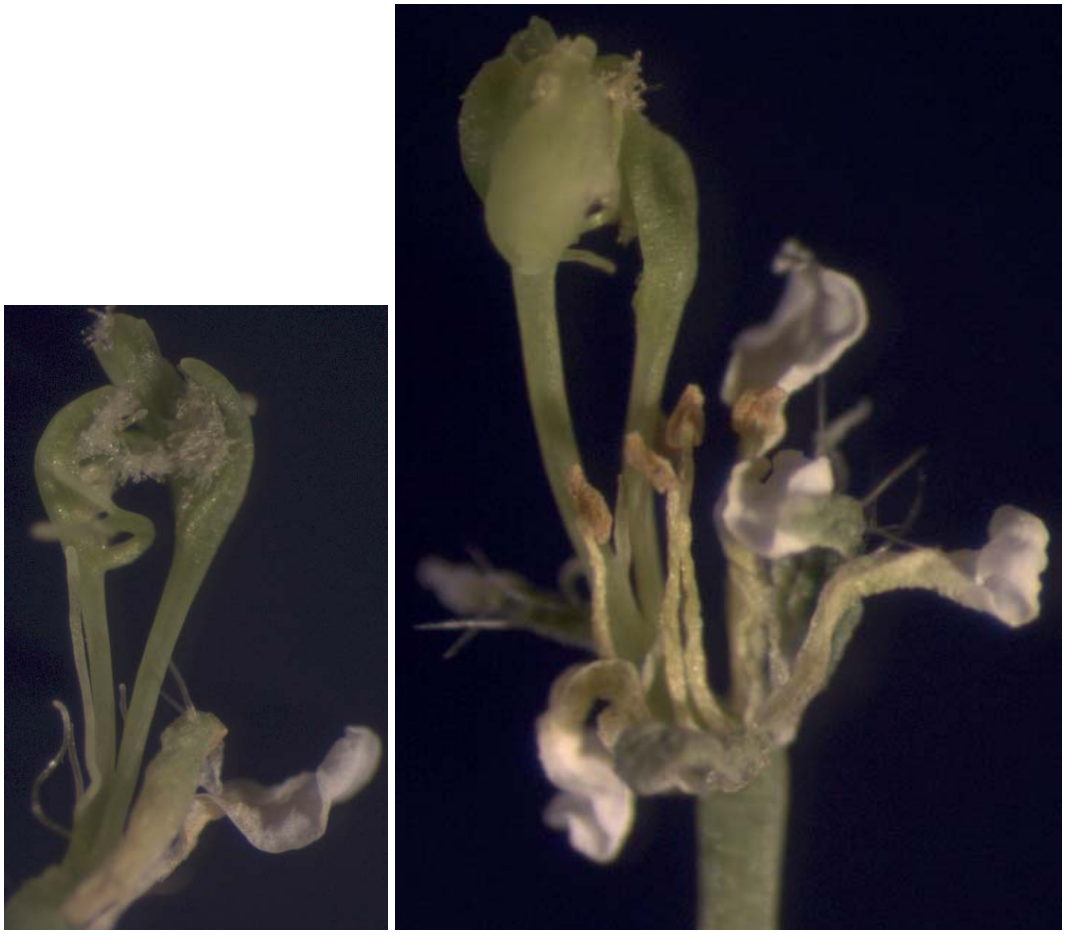
Isolated T-DNA *yuc1pid336* T2 mutant defective in floral organogenesis

yuc1pid336 double mutants do not have any obvious defects in flower structures. The figures 44-53 below show *yuc1pid336* mutant lines with abnormal flower development and/or pin-like inflorescences. ~40 seeds are plated for each T2 *yuc1pid336* screening line, ~20 plants are transplanted for each line.

A

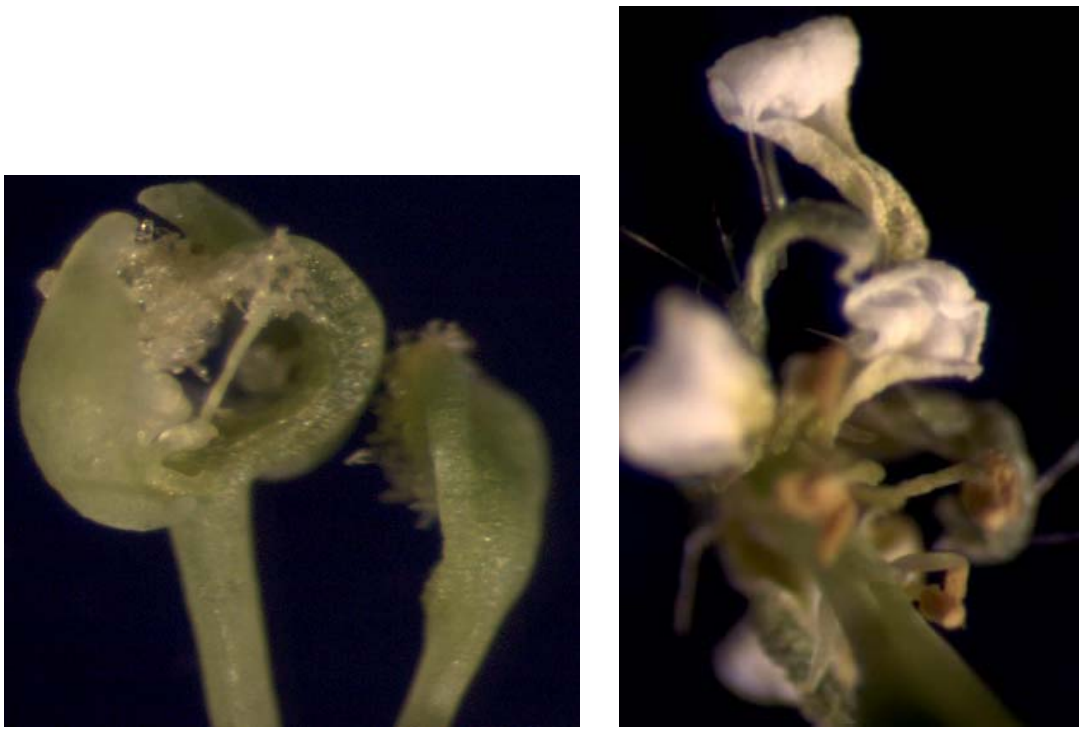
B

Figure 44: *yuc1pid336* T2-744. (A) and (B) all flowers of this mutant are abnormal and sterile. Four out of 15 plants had aberrant flowers.

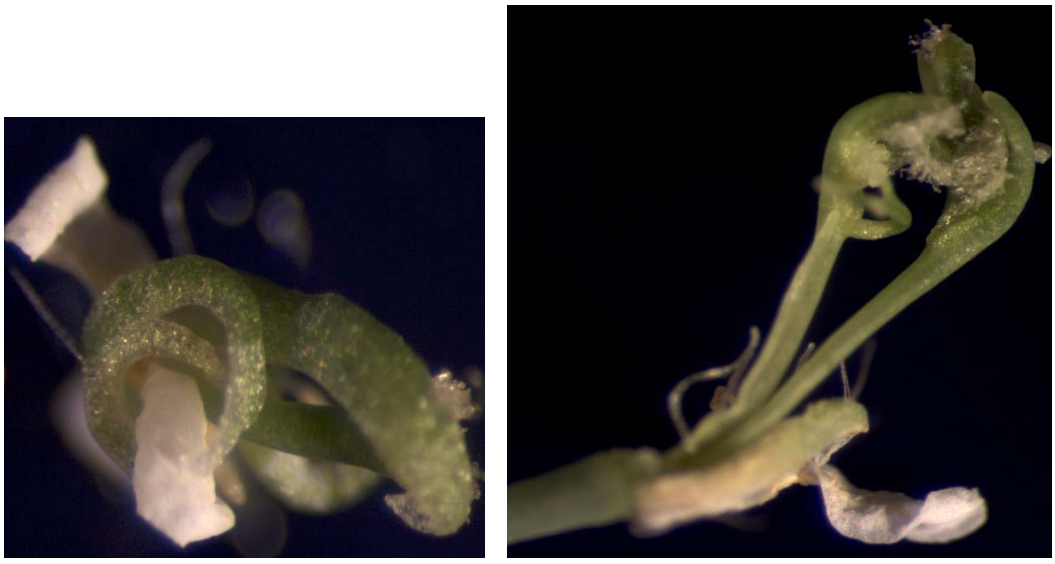


A B

Figure 45: *yuc1pid336* T2-744. (A) and (B) all flowers of this mutant are abnormal, sterile, and show great variation. Four out of 15 plants had aberrant flowers.

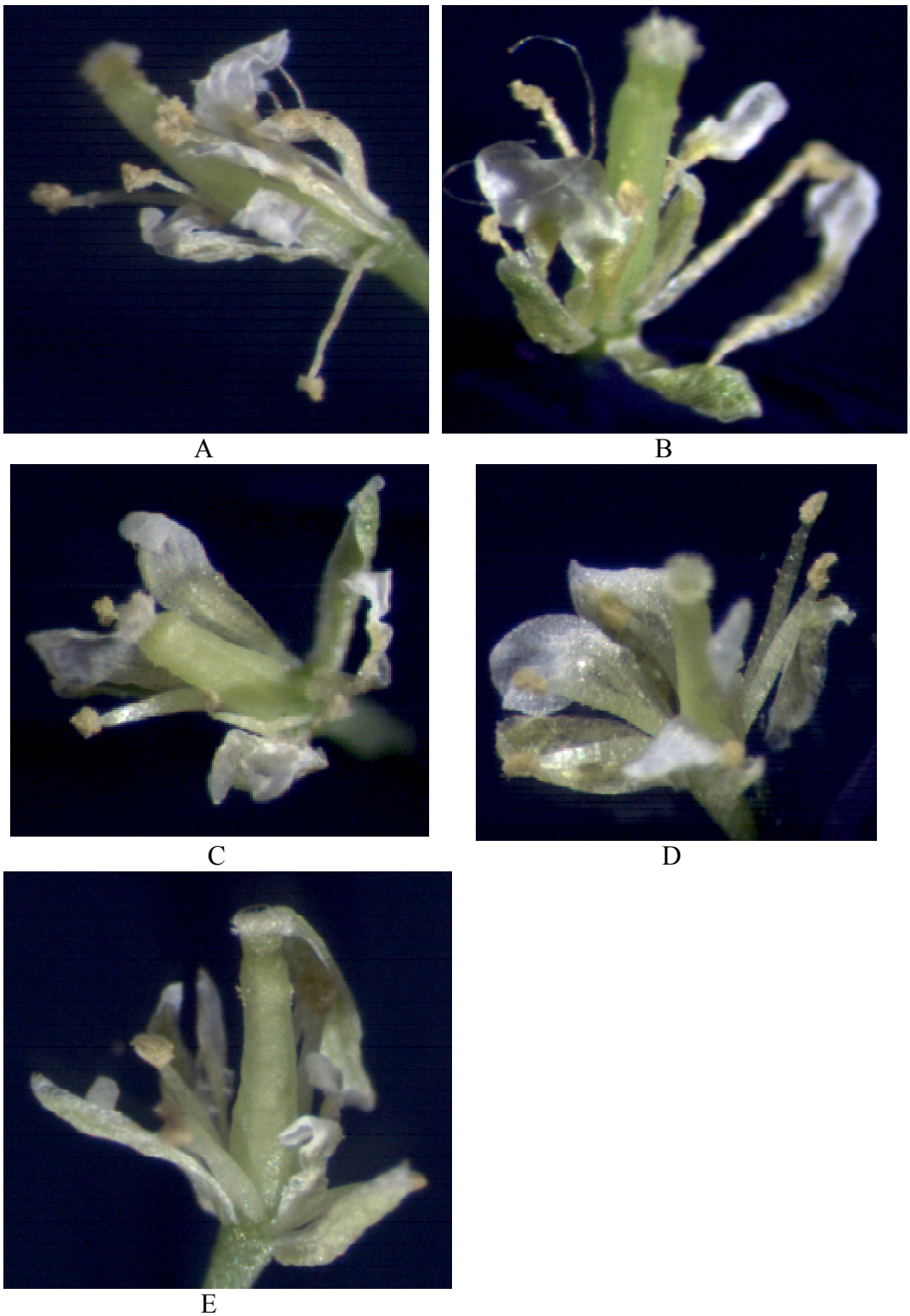


A B



C D

Figure 46: *yuc1pid336* T2-744. (A) – (D) all flowers of this mutant are abnormal, sterile, and show great variation. Four out of 15 plants had aberrant flowers.



A B C D E

Figure 47: *yuc1pid336* T2-910. (A) – (E) all flowers of this mutant are abnormal and sterile. Three out 14 plants have aberrant floral structures similar to this.

Figure 48: *yuc1pid336* T2-942. All floral structures of this mutant are abnormal; it is also sterile. 2 out 15 plants in this line lack stamen and are sterile.

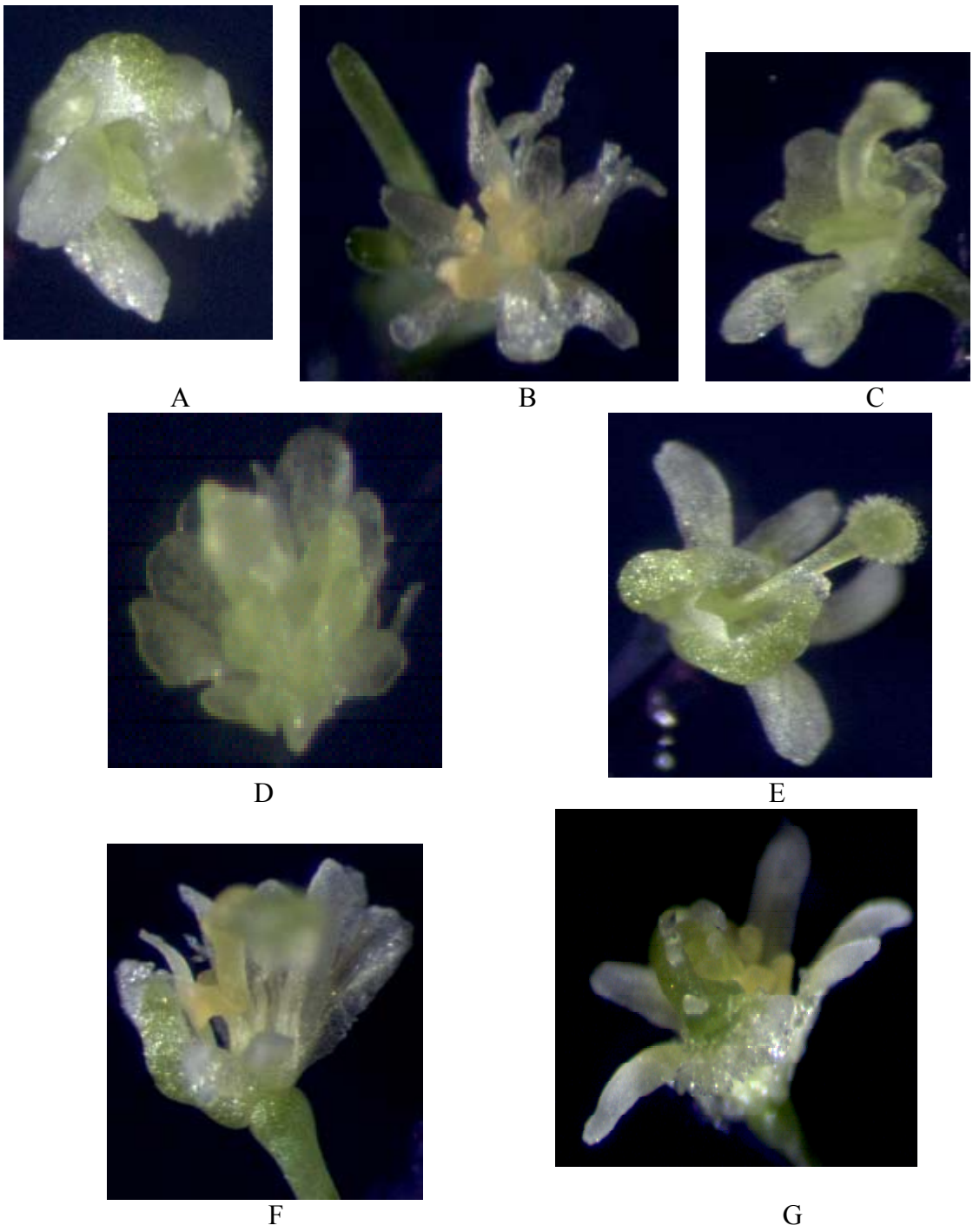
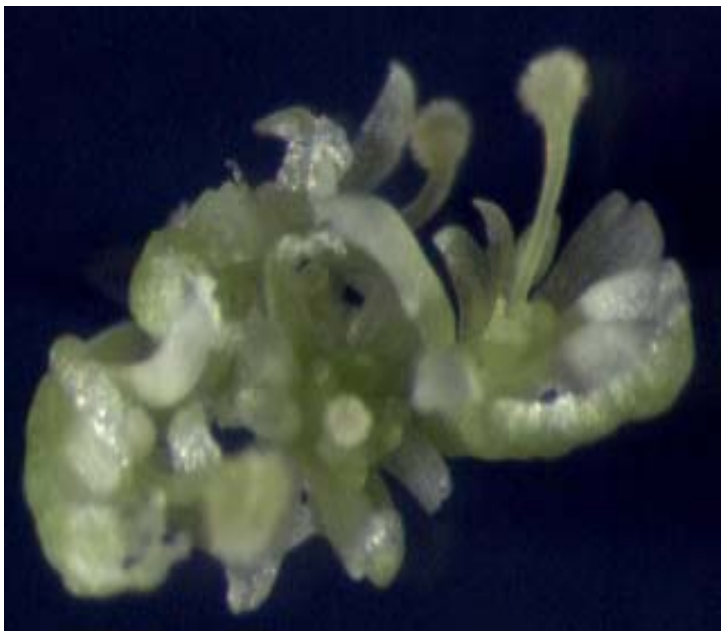


Figure 49: *yuc1pid336* T2-974. (A) – (G) all flowers of this mutant are abnormal, sterile, and show great variation. Three out of 15 plants had aberrant flowers.



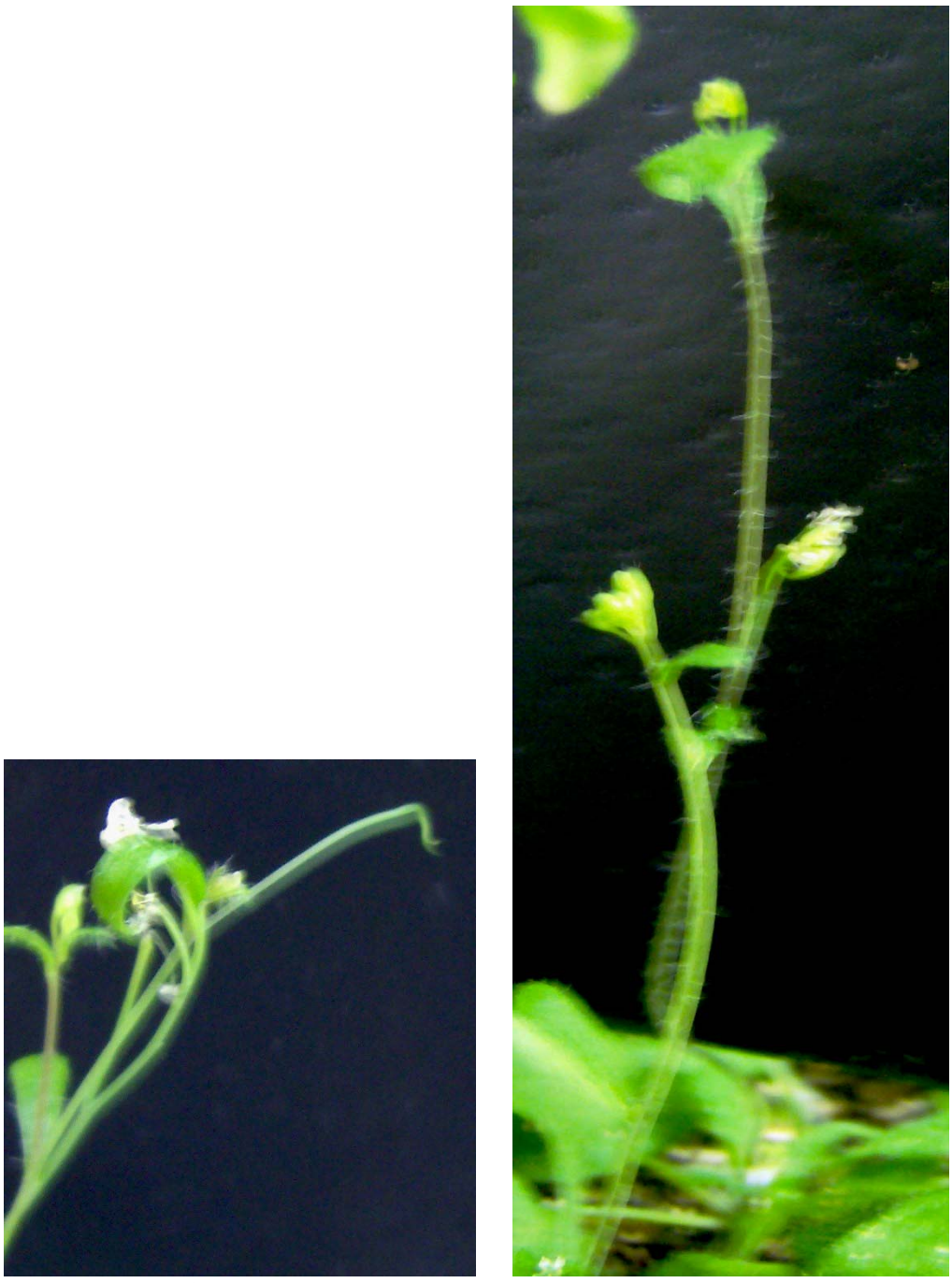
A

B

Figure 50: *yuc1pid336* T2-986. (A) and (B) different views of the same flower. All flowers of this mutant plant are abnormal and sterile. 3 out of 15 plants from this line had aberrant flowers.

A B

Figure 51: *yuc1pid336* T2-315. (A) and (B) plant at different development stage. The mutant has abnormal flowers with no petals and pollen; it forms pin-like inflorescence and is sterile. 3 out of 15 plants from this line had such phenotypes.



A B

Figure 52: *yuc1pid336* T2-660. (A) and (B) the mutants have abnormal flowers with no pollen, and abnormalities in all four flower whorls; it also forms pin-like inflorescence and is sterile. 2 out of 15 plants from this line had such phenotypes.

A B

Figure 53: *yuc1pid336* T2-667. (A) and (B) show the same mutant plant. This mutant rarely develops floral structures, it is sterile and mainly forms pin-like inflorescence. 3 out of 15 plants from this line share this phenotype.

Table 7: Summary of all the isolated *yuc1pid336* T2 lines with defective auxin-governed process phenotypes. Column A lists mutant lines with pin-like inflorescences and abnormal flowers. Column B lists mutant lines with aberrant floral structures, but without pins. On average, 2-4 plants out of 15 (1 pot) show such phenotypes. TAIL-PCR is performed for mutant lines in bold.

Isolated T2 mutant lines	Isolated T2 mutant lines
A	B
744	974
699	699
667	986
660	942
592	951
405	813
315	781
1068	963
1039	

Analysis of T3 *yuc1pid336* mutants

The next generation, T3 of the isolated *yuc1pid336* T2 lines (see table 7) are subsequently transplanted and scored to confirm the phenotypes and check segregation ratios. All isolated T2 lines are genotyped with these primers YUC1, PID336, and BAR (the Basta resistance gene carried within the T-DNA vector, and have been confirmed that they are indeed in the *yuc1pid336* background and therefore free of contamination. In each T2 mutant line there are 15-20 plants and seeds from each plant are collected. Then ~20 seeds from each of these plants are transplanted, creating a large T3 population, with ~400 T3 plants for each mutant line. Mutants with strong

phenotypes sometimes did not produce seeds so instead seeds from sister plants are collected.

In mutant lines 951, 1039, 660, 667, 744, 315, 699, 405 the mutant phenotypes (abnormal flowers / pin-like inflorescences) appear again. There are 2-3 plants out of 15 plants that show the phenotypes, averaging the segregation ratio to be 1/3, indicating T-DNA insertion in one gene, in the *yuc1pid336* background. I selected strong mutants from these lines to perform TAIL-PCR to identify the gene responsible for the phenotype, as listed in Table 8. For the rest of the mutant lines the original T2 flower and/or pin phenotypes are not recovered and thus no further experiments are performed.

Table 8: Mutants with strong abnormal flower phenotypes and pin-like inflorescences from these *yuc1pid336* lines are selected for TAIL-PCR in order to identify the location of the T-DNA insertion site and the gene responsible for auxin-deficient phenotypes.

Mutant line #
667
660
405
315
1039

Thermal Asymmetric InterLaced PCR (TAIL-PCR)

I used thermal asymmetric interlaced PCR as an efficient way to amplify unknown sequences adjacent to T-DNA insertion sites in the isolated *yuc1pid336* mutants. TAIL-PCR is simple, highly specific, efficient and sensitive. Eight shorter arbitrary degenerate (AD) primers along with three longer, nested T-DNA specific primers (SP1-3) that have differing annealing temperatures to carry out three cycles of PCR. The entire process takes approximately one week to complete. High specificity of TAIL-PCR allows for the direct sequencing of the products. The detailed background and delineation of TAIL-PCR procedure have been described in the materials and methods section.

Analysis of TAIL-PCR products

Upon the completion of TAIL-PCR cycles, the products from the secondary TAIL-PCR reaction and the tertiary TAIL-PCR reaction are subjected to regular DNA agarose gel electrophoresis (in adjacent lanes). Secondary TAIL-PCR reaction and the tertiary TAIL-PCR reaction products allows for identification of specific products because in specific products, the tertiary products are smaller in sizes compared to the secondary products (see materials and methods for detailed reasoning). The specific DNA bands are cut out, purified (according to Qiagen gel extraction kit protocol), and sequenced with specific left border T-DNA primer. Using BLAST I analyzed the sequencing results. Three types of sequencing results are generated – 1. T-DNA vector sequence only 2. T-DNA insertion within an intron 3. T-DNA insertion in the genomic exon region. That last type is the desired result and primers are designed for these

candidate genes to genotype the mutants; this allows for the identification of the corresponding gene responsible for the mutant phenotypes.

TAIL-PCR for several mutants are repeated because sequencing of DNA from specific bands did not produce ideal results, meaning that either only T-DNA vector sequences are recovered, or that after genotyping with the candidate gene specific primers, the mutant phenotypes did not link to the gene. Varying DNA and TAIL-PCR products concentrations are used when repeating TAIL-PCR to optimize results. In cases where the mutant phenotypes did not link to the candidate gene, I genotyped all the mutants from the line. I then selected plants that do not contain T-DNA insertion in the candidate gene to repeat TAIL-PCR. In this way, I improve the chance of identifying the correct T-DNA insertion site. The gels in figures 56 – 61 show TAIL-PCR results.

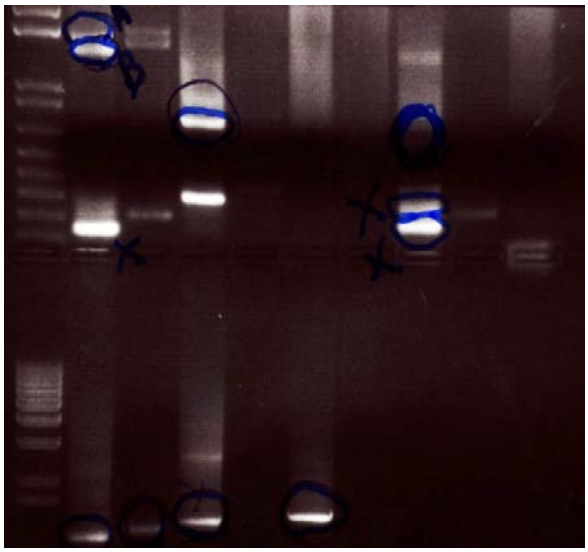
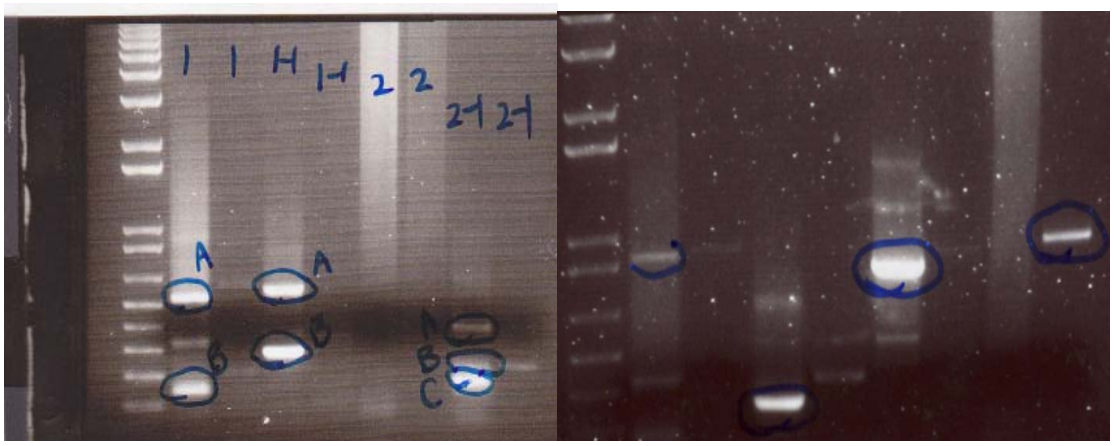
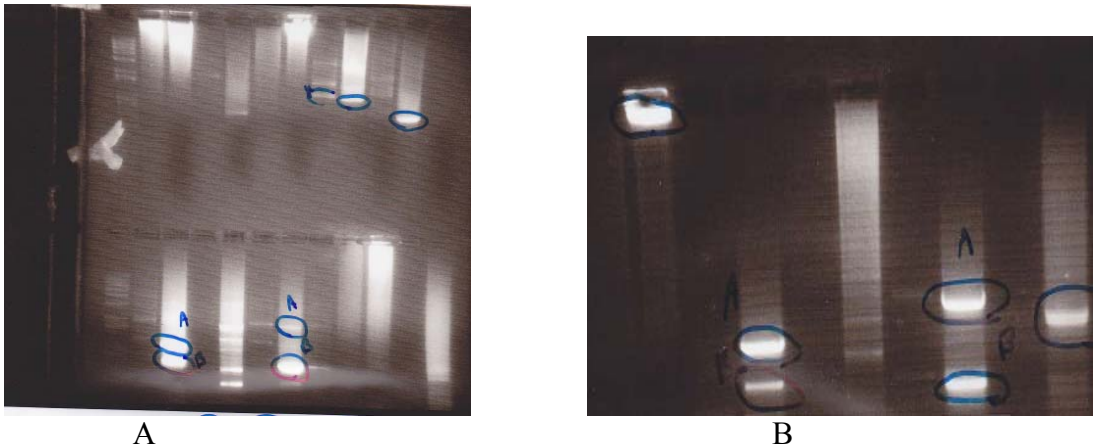


Figure 54: TAIL-PCR results for *yuc1pid336* T2-315.



A B

Figure 55: TAIL-PCR results for *yuc1pid336* T2-405 in (A) and (B).



A B

Figure 56: TAIL-PCR results for *yuc1pid336* T2-660 in (A) and *yuc1pid336* T2-667 in (B).

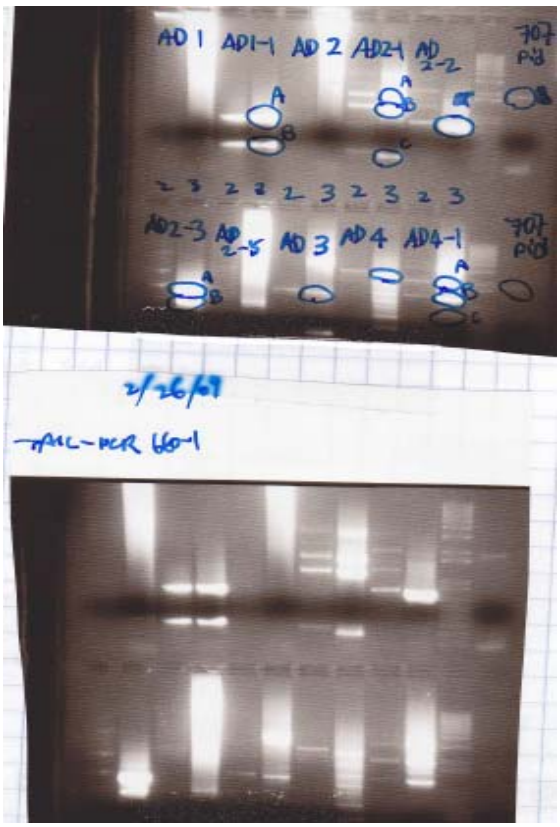
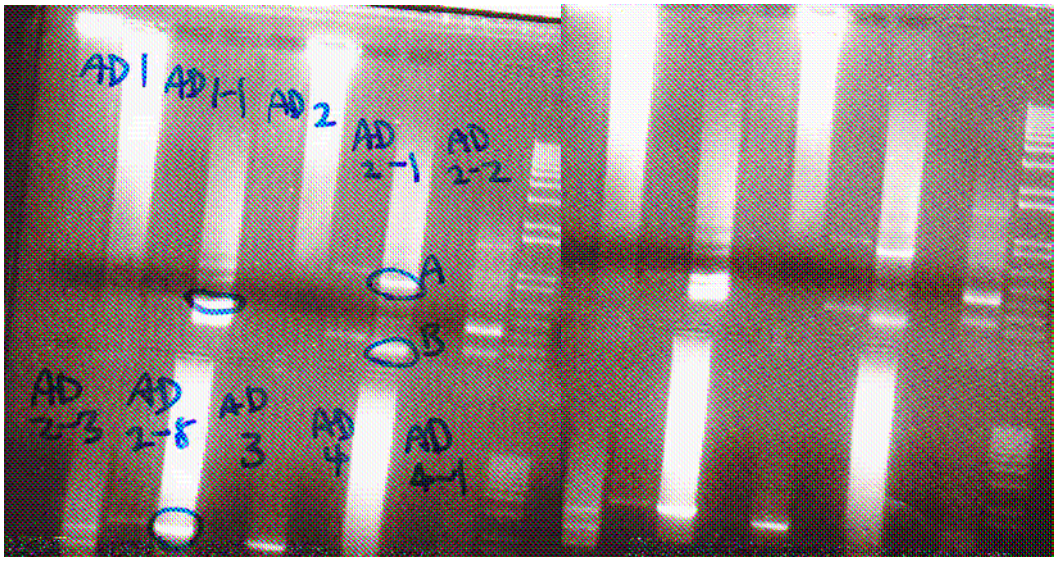
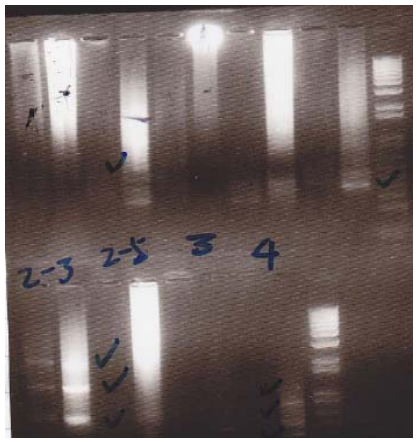


Figure 57: Repeated TAIL-PCR results for *yuc1pid336* T2-660.

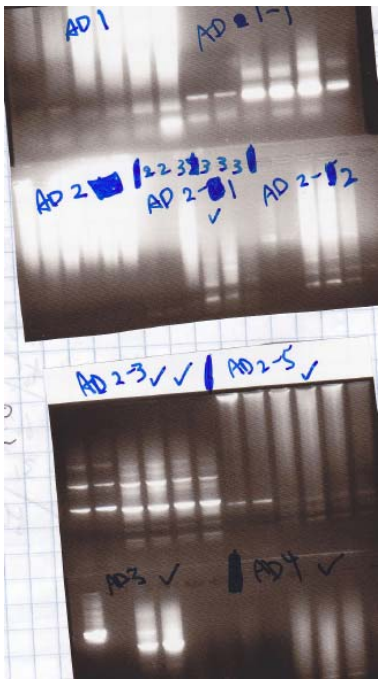


A B

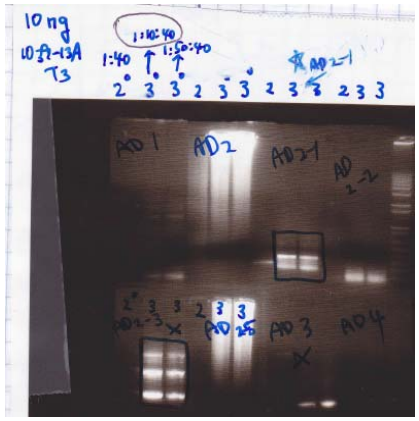
Figure 58: Reapted TAIL-PCR results for *yuc1pid336* T2-667 in (A) and *yuc1pid336* T2-667 in (B).



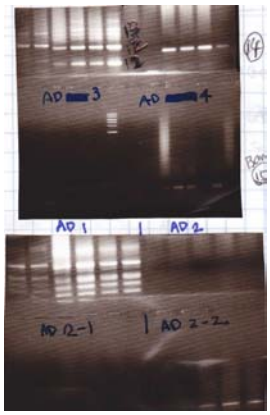
A



B

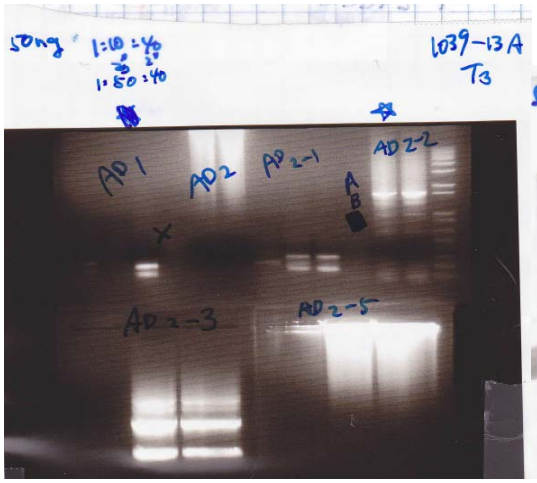


C

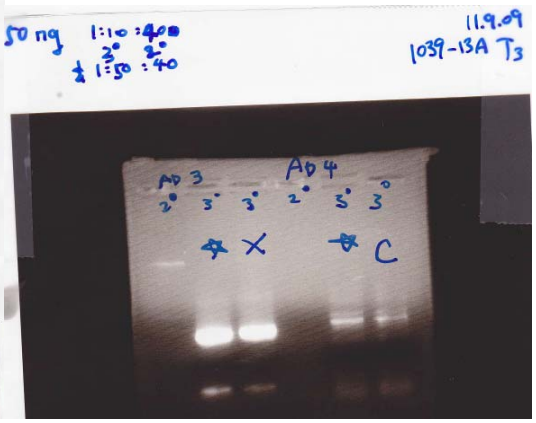


D

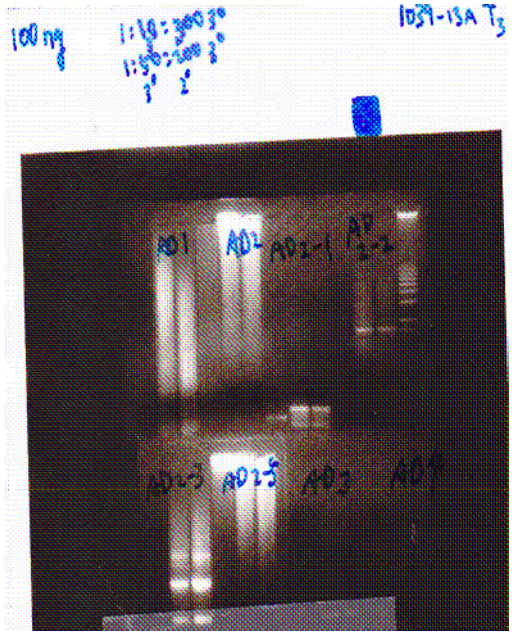
Figure 59: Many repeated TAIL-PCR results for *yuc1pid336* T2-1039 and *yuc1pid336* T3-1039 in (A) – (H). The mutants all exhibit strong aberrant floral structures and pin-like inflorescences. For 1039, the mutant phenotypes did not link to the candidate gene, and I genotyped all the mutants from the line. I then selected plants that do not contain T-DNA insertion in the candidate gene to repeat TAIL-PCR in an attempt to improve the chance of identifying the correct T-DNA insertion site. Varying DNA and TAIL-PCR concentrations are used when repeating TAIL-PCR to optimize obtaining specific DNA bands.



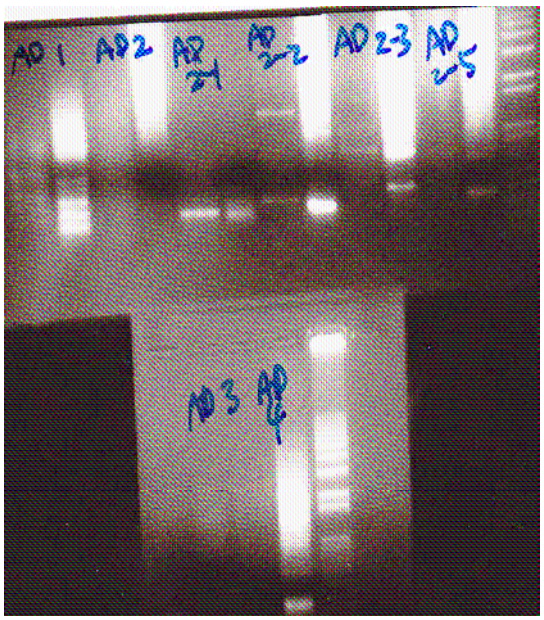
E



F



G



H

Figure 59: Many repeated TAIL-PCR results for *yuc1pid336* T2-1039 and *yuc1pid336* T3, Continued.

Although much time effort has been placed on repeated TAIL-PCRs for several mutants, sequencing and BLAST analysis reveal many T-DNA vector sequences; while genotyping analysis reveal incorrect candidate genes, not linking the genotype back to the observed mutant phenotypes.

Adapter ligation PCR

Sequencing data show T-DNA vector sequences without the flanking genomic DNA. This is most likely due to multiple (tandem) T-DNA insertions within a gene. In such cases, TAIL-PCR is unable to amplify the flanking genomic sequence and I cannot map the precise location of the insertion site. Therefore, I perused an alternative method that can circumvent this particular problem – adapter ligation PCR, a reliable method to identify the T-DNA insertion site. It has been used to screen mutant library and identify more than 150,000 T-DNA insertional mutants [O'Malley et al., 2007]. There are three basic steps – 1. restriction enzyme-mediated ligation of adapter to genomic DNA 2. PCR of the T-DNA, genomic DNA junction using primers specific to the adapter and T-DNA 3. sequencing of T-DNA, genomic junction to map the reference genome (see figure 62) [O'Malley et al., 2007]. Sequencing usually continues to the T-DNA border, thus allowing the precise location of the T-DNA insertion. My experimental design follows principles delineated in the paper by O'Malley et al., but using different adapters and primers due to different T-DNA vectors. However due to limited time, this experiment has been designed but not yet been carried out.

My Adapter ligation PCR design

I. Choosing restriction enzymes

The three restriction enzymes must not have cut site withins T-DNA vector, otherwise the adaptor will ligate within the T-DNA sequence and the desired T-DNA/gDNA junction will not be recovered [O'Malley et al., 2007.] *Eco*RI, *Hind*III, and *Ase*I are the enzymes used in O'Malley et al, but all three have cut sites within the pSKI015 vector. Examining the sequence where T-DNA junctions with g-DNA, (assuming a complete vector insertion) I have:

```
  1 cagcttttgt tccctttagt… …
    … …cgaggggggg cccggtac   10138 end
```

*Nco*I:
5′...C▼CATGG...3′
3′...GGTAC▲C...5′
*Bsi*WI:
5′...C▼GTACG...3′
3′...GCATG▲C...5′
*Sal*I:
5′...G▼TCGAC...3′
3′...CAGCT▲G...5′

The three restriction enzymes are *Nco*I, *Bsi*WI, and *Sal*I.

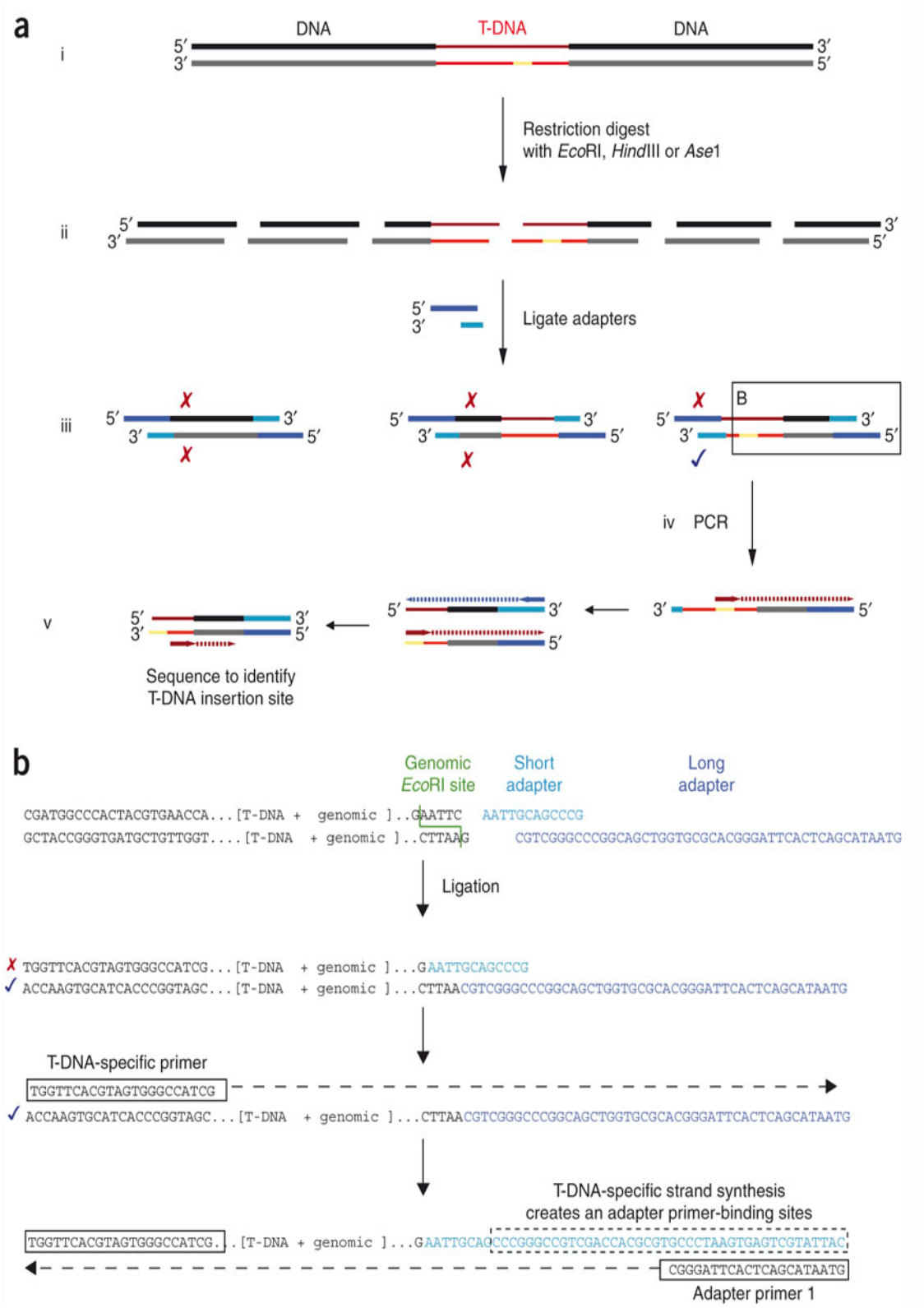


Figure 60: An adapter ligation-mediated PCR method for high-throughput mapping of T-DNA inserts in the Arabidopsis genome. From O'Malley et al., 2007.

II. Oligonucleotides required

Short strand of adapter NcoI:

- 5’ phosphate – CATG**CTAGGACT –amino C7-3’**
- 5’ phosphorylated and 3’ C7 amino modification. HPLC purified

Short strand of adapter BsiWI:

- 5’ phosphate – GTAC**CTAGGACT– amino C7-3’**
- 5’ phosphorylated and 3’ C7 amino modification. HPLC purified

Short strand of adapter SalI:

5’ phosphate – TCGA**GTAGGACT– amino C7-3’**

- 5’ phosphorylated and 3’ C7 amino modification. HPLC purified

Long strand of adapter 1: NcoI + BsiWI

5’ *ACCTCCTCGTAGAACGAGTC*ACGCGGTAGTCGAGGTC**AGTCCTAG** 3’

[AP1 bind site after T-DNA synthesis]

[AP2 bind site after T-DNA primer synthesis, adapters anneal site]

Long strand adapter 1 is used with short strand NcoI and BsiWI adapters

Long strand of adapter 3: SalI

5’ *ACCTCCTCGTAGAACGAGTC*ACGCGGTAGTCGAGGTC**AGTCCTAC 3’**

[AP1 bind site after T-DNA synthesis]

[AP3 bind site after T-DNA primer synthesis, adapters anneal site]

Long strand adapter 3 is used in combination with short strand of SalI adapter

Adapter Primer 1 (AP1)

5' *ACCTCCTCGTAGAACGAGTC* 3' GC% = 55, Tm = **53.1**

LBa1 (T-DNA specific left border a1):

5' -> ATGTGCCAACTGAGCCG 3'

At 9068 / 10138, 17bp, **Tm 54.9**, GC% 58.8

Primer for 1st PCR for *Nco*I + *Bsi*WI + *Sal*I

Expected product size: 1070bp from T-DNA + ____ bp from gDNA

LBa1 + AP1 → 1st PCR

LBb1 + AP2, AP3 → 2nd nested PCR

LBb1 is also sequencing primer

Adapter Primer 2 (AP2)

5' GTAGTCGAGGTC**AGTCCTAG 3'** GC% = 55, Tm = **48.3**

Primer for 2nd nested PCR, used with NcoI and BsiWI adapters

Adapter Primer 3 (AP3)

5' GTAGTCGAGGTC**AGTCCTAC** 3' GC% = 55, Tm = **48.0**

Primer for 2nd nested PCR, used with SalI adapter

LBb1 (T-DNA specific left border b1):

5' -> GATGGAGCCGAGACG 3'

At 9940 / 10138, 15bp, Tm 48.2, GC% 66.7

Primer for 2nd nested PCR with AP2, AP3. Also Sequencing primer.

Expected Product size: 198 bp from T-DNA + ____ bp from gDNA

Two different T-DNA primers, LBa1 and LBb1, are used in two rounds of PCR for adapter ligated gDNA. The LBa1 and AP1 primer pair ised used in the 1st PCR, and the LBb1 and AP2,3 pair is used in a 2nd, nested PCR. The LBb1 primer is used in all cases for the final sequencing of the T-DNA/gDNA junction.

DISCUSSION

Auxin is a widely studied plant hormone that governs all aspects of plant growth and development. Regulation of auxin levels is indispensible in mediating developmental processes. Multiple mechanisms contribute to establishing and maintaining auxin concentrations – 1. biosynthesis 2. metabolism (ie. de/conjugation, degradation) 3. transport 4. perception and response.

Bartel and Woodward propose a model stating auxin is synthesized from tryptophan in four different pathways and may be characterized by their intermediates – IPA, IAM, TAM, and IAOx. However, the genes involved in these pathways have not all been identified and characterized. To date, only the TAM and IPA pathways represent the defined and physiologically important pathways in plant development.

The YUC genes convert TAM, a derivative of tryptophan, to N-hydroxyl-tryptamine, a precursor of IAOx that can be used to make auxin. The YUCCA genes encode rate-limiting enzymes in the TAM pathway [Zhao et al., 2001]. They have overlapping expression patterns and redundant functions – when multiple YUC genes are disrupted, there are impairments throughout all developmental stages, including defects in embryo patterning, vascular formation, and floral organogenesis [Cheng et al., 2006; Cheng et al., 2007]. The identification of the YUC genes and the analysis of

partially auxin deficient *yuc* mutants give a new approach to the study of the molecular mechanisms of auxin actions.

I performed forward EMS screening in a *yuc1yuc4* background for ~700 mutant lines. The foundation of my project uses the *yuc* mutants – *yuc1yuc4* as starting materials. This provided a sensitized background for isolating genes important for auxin-mediated development. *yuc1yuc4* forward EMS screening offered several advantages. In this sensitized mutant background, the endogenous auxin level is decreased, in contrast with previous studies where screens were done by adding exogenous auxin. Applying exogenous auxin is not helpful for investigating auxin mechanisms for a couple of reasons. First, exogenous auxin may not affect the actual cellular auxin level. Second, when excessive auxin is added, normal seedlings cannot elongate their roots, and so only root mutants may be isolated [Zhao, book chapter 2010]. This is problematic since auxin clearly influences all developmental stages. However, my EMS forward genetic screening in the sensitized *yuc1yuc4* background makes up for the deficiencies in older screening approaches.

By conducting EMS screening in the *yuc1yuc4* background, I have isolated mutants and attempted to identify genes important throughout all developmental stages – from seedlings to growing to mature adult plants. The *yuc1yuc4* plants have defects in floral organs but still can make flowers. My isolated *yuc1yuc4* EMS mutants are either enhancers or suppressors. They have more severe defects in flowers, vasculature, and seedlings than *yuc1yuc4* plants alone – mutants have complete

abolishment of all floral structures and form pin-like inflorescences. Other times, they suppress *yuc1yuc4* defects and produce plants phenotypically similar to wildtype. The identification and characterization of genes from this screen will elucidate mechanisms of auxin biosynthesis and auxin mediated development. They help to fill in the gaps of our current understanding of auxin actions in plant growth and development.

Previous studies have used similar genetic screening approaches in the *yuc1yuc4* background and identified the Naked Pins in Yucca (NPY) gene. The *npy1* mutant by itself shows no defects in flowers, while the *yuc1yuc4* plants have defects in floral organs but still can make flowers [Cheng et al., 2007]. The *npy1yuc1yuc4* triple mutant forms strong pins and is phenotypically similar with respect to auxin transport and signaling mutants (e.g., *pin1, pinoid,* and *monopteros*) [Cheng et al., 2007]. NPY1 belongs to large gene family containing 32 members, and it is homologous to the founding member of the family – NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3). NPH3 is a BTB / POZ protein working together with Phototropin 1 (PHOT1) to mediate phototropic responses [Motchoulski and Liscum 1999]. NPY1 is shown to work with the protein kinase PID (homologous to PHOT1) to govern auxin-dependent plant development [Cheng et al., 2007]. In addition, genetic analysis of NPY1 homologs and PID homologs show that the NPY genes PID genes both participate in auxin-mediated organogenesis [Cheng et al. 2008]. The genetic interactions among YUCs, PIDs, and NPYs suggests a linear pathway that controls auxin action in *Arabidopsis* organogenesis [Cheng et al. 2008]. The identification and analysis of the

NPY genes prove the power of a forward genetic screening approach in the sensitized *yuc* mutant background.

Some scientists in the past have held the notion that plant development is mainly governed through auxin gradients established by polar auxin transport, but it is clear now that both auxin biosynthesis and polar auxin transport together control developmental processes. Thus, polar auxin transport does not mediate plant growth by itself. The serine / threonine kinase protein PINOID regulates polar PIN localization, contributing to directional auxin transport and distribution in plant tissues and influencing developmental processes [Friml et al., 2004]. The *336 pinoid* (*pid336*) weak allele contains a single base pair mutation at position 336 of the PID gene.

Since both auxin biosynthesis and auxin polar transport are important in coordinating developmental processes, I used a combined inactivated auxin biosythesis YUC gene (YUC1) and inactivated auxin transport gene PINOID weak allele 336 to conducted T-DNA activation tagging screen (in *yuc1pid336* background). I attempted to uncover novel components in auxin-mediated processes. *yuc1pid336* plants are similar to wildtype and have no obvious developmental defects. Using T-DNA activation tagging I screened ~1,300 plants in the *yuc1pid336* background, and I have isolated several mutants with severe defects throughout development – e.g., abnormal seedling growth and aberrant floral organogenesis. Subsequently I employed TAIL-PCR as a technique to map the location of T-DNA insertion and to identify the corresponding gene responsible for the mutant phenotypes.

Although TAIL-PCR has many advantages including simplicity, high specificity and efficiency, speed, direct sequencing, and high sensitivity (Liu & Whittier, 1995), the problem of multiple (tandem) T-DNA insertions within a gene disallows TAIL-PCR to amplify out the flanking genomic sequence. This problem prohibits mapping the precise location of the insertion site. Therefore, I perused an alternative method – adapter ligation PCR, which is a reliable method to identify the T-DNA insertion site because it can overcome the problem of tandem T-DNA insertions. It has been used to screen mutant libraries and identified more than 150,000 T-DNA insertional mutants [O'Malley et al., 2007]. The experimental design of adapter ligation PCR has been laid out, but due to time limitation, the actual experiment has not been conducted. Alternative methods to identify the flanking genomic sequences include: inverse PCR, plasmid rescue, or T-DNA probe in the cDNA library. When the corresponding genes have been identified from the isolated *yuc1pid336* mutants, we will uncover new factors in auxin-mediated processes; in addition, we will elucidate the genetic interactions between components of auxin biosynthesis and polar transport.

Previous studies on loss of function mutants in both *yuc* and *pin1* backgrounds provide evidence for synergistic interactions between YUC-mediated auxin biosynthesis and PIN-regulated polar auxin transport. In *pin1-5* weak allele mutants, flowers have few defects and are fertile [Cheng et al., 2007]. Combining *yuc1yuc4* loss of function mutants *with pin1-5* alleles, the plants form pin-like inflorescences

and have much smaller stature [Cheng et al., 2007]. In addition, the triple mutant does not make leaves and shows more severe defects than the single PIN1 loss of function plant [Cheng et al., 2007]. This indicates that PINs and YUCs interact genetically. Furthermore, it was observed that auxin synthesized by specific YUC genes in a particular organ / tissue does not make up for lack of auxin elsewhere – YUCs make auxin locally and maintain auxin maxima in a particular region [Cheng et al., 2007]. In other words, the specific temporal and spatial expressions of YUC genes mediate diverse developmental processes [Cheng et al., 2007]. These observations raise questions about the relationship between auxin synthesis and transport – to what degree does each mechanism contribute to establishing and maintaining auxin gradients? At the same time, the exact mechanisms involved in PIN and YUCCA synergistic interactions remain to be determined.

A further complication comes from the fact that auxin itself can govern its own distribution through the regulation of PIN protein at multiple levels, such as affecting PIN transcription, turnover and membrane localization [Sauer et al., 2006]. For example, it has been shown that auxin application induces PIN expression, polarization, and eventually the formation of new vascular strands [Sauer et al., 2006].

Recently, the identification of aminotransferases (TAAs) that convert tryptophan to IPA has shown the IPA pathway to be functionally relevant in auxin-mediated development. Auxin production is decreased in *taa* mutants, resulting in strong defects in embryogenesis, vascular development, and gravitropism [Stepanova

et al., 2008; Tao et al., 2008]. Diverse extrinsic cues – light quality [Tao et al., 2008] and ethylene [Stepanova, et al., 2008], both affect TAA transcription and therefore regulate auxin distribution and auxin-dependent processes.

Although both studies provide evidence for TAA1 mediated auxin biosynthesis and hormone crosstalk in plant development, there are many unanswered questions. It is clear that TAAs are important enzymes in the IPA pathway and they establish auxin gradients corresponding to environmental and developmental signals, but they are not the enzymes catalyzing the rate-limiting step in IPA dependent auxin biosynthesis. In this case we wonder which enzyme does? It is also interesting to ask why different developmental processes require different amounts of auxin. How many auxin pools are there in plants, and do they all respond to extrinsic signals? What are the molecular mechanisms by which light regulates TAA1? Because the *taa* mutants sometimes have analogous phenotypes to *yuc* mutants – it raises the question whether the IPA and TAM pathways are independent or have overlapping functions in making auxin. From these studies, it is reasonable to suspect plausible interactions between auxin biosynthetic pathways. The extent to which these pathways contribute to auxin production requires further research.

Identification and characterization of genes in the isolated mutants from my screening projects will help to answer the many questions regarding the mechanisms of auxin biosynthesis and auxin action.

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