Investigating the Function of the

*Arabidopsis thaliana INDETERMINATE DOMAIN2* gene

by

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ABSTRACT

INVESTIGATING THE FUNCTION OF THE ARABIDOPSIS THALIANA INDETERMINATE DOMAIN2 GENE

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The INDETERMINATE DOMAIN (IDD) genes in Arabidopsis thaliana encode a transcription factor family found in all higher plants. IDD2, one of 16 IDD family genes in Arabidopsis, is highly expressed in hypocotyls, shoot apices and petioles. Loss-of-function idd2 mutants show no obvious differences in growth and development under a variety of conditions, suggesting that IDD2 may function redundantly. Constitutive overexpression of IDD2 causes altered seed coat morphology and defects in early growth. 35S::IDD2 overexpression seedlings show a constitutive response to the phytohormone auxin. Elucidation of IDD2 expression in IDD2::GFP::GUS reporter plants revealed a highly specific pattern, including expression in leaf and cotyledon vasculature, petioles, hypocotyl, shoot apex, root tip and emerging lateral roots, which all correspond to areas with high levels of auxin. Taken together, analysis of loss-of-function mutants, overexpression lines and the IDD2 expression pattern suggests that IDD2 has a redundant role in auxin sensing and response.
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<th>Definition</th>
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<tbody>
<tr>
<td>35S</td>
<td>constitutive <em>cauliflower mosaic virus</em> promoter</td>
</tr>
<tr>
<td>3-PGA</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>6-BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>A. thaliana</td>
<td><em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>ABI4</td>
<td><em>ABSCISIC ACID INSENSITIVE4</em></td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid</td>
</tr>
<tr>
<td>ACCase</td>
<td>acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ADP-glucose</td>
<td>adenosine diphosphoglucose</td>
</tr>
<tr>
<td>AGPase</td>
<td>ADP-glucose pyrophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARF</td>
<td><em>AUXIN RESPONSE FACTOR</em></td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BASTA</td>
<td>glufosinate ammonium</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CDF</td>
<td><em>CYCLIN DOF FACTOR</em></td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
</tr>
<tr>
<td>CEI</td>
<td>cortex epidermal initial</td>
</tr>
<tr>
<td>CMT</td>
<td><em>COMATOSE</em></td>
</tr>
<tr>
<td>CO</td>
<td><em>CONSTANS</em></td>
</tr>
<tr>
<td>CO2</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>Col-0</td>
<td><em>Arabidopsis thaliana</em> Columbia-0 ecotype</td>
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<tr>
<td>CPC</td>
<td><em>CAPRICE</em></td>
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$CTR1$  CONSTITUTIVE TRIPLE RESPONSE1

DAG  days after germination

$DGAT1$  DIACYLGLYCEROL ACYLTRANSFERASE1

DNA  deoxyribonucleic acid

DNase  deoxyribonuclease

EDTA  Ethylenediaminetetraacetic acid

ER  endoplasmic reticulum

EtOH  ethanol

$ETR$  ETHYLENE RESISTANT1

$FD$  FLOWERING LOCUS D

$FKF1$  FLAVIN-BINDING, KELCH REPEAT, F-BOX1

Fru6P  fructose-6-phosphate

$FT$  FLOWERING LOCUS T

G3P  glyceraldehyde-3-phosphate

GA  gibberellic acid

GFP  Green Fluorescent Protein

$GI$  GIGANTEA

Glc1P  glucose-1-phosphate

$GL2$  GLABRA2

GOPOD  Glucose Determination Reagent

GUS  β-glucuronidase

$GWD1$  α-glucan water, dikinase

HXK  hexokinase

IAA  indole-3-acetic acid

$ID1$  INDETERMINATE1
<table>
<thead>
<tr>
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<th>Full Form</th>
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<tbody>
<tr>
<td>IDD</td>
<td>INDETERMINATE DOMAIN</td>
</tr>
<tr>
<td>INV</td>
<td>invertase</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NPA</td>
<td>N-1-napthylphthalemic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PIN</td>
<td>PIN-FORMED</td>
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<td>PLETHORA</td>
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</tr>
<tr>
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<td>quantitative polymerase chain reaction</td>
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</tr>
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<td>ribonucleic acid</td>
</tr>
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<td>RQ</td>
<td>relative quantity</td>
</tr>
<tr>
<td>SAIL</td>
<td>Syngenta Arabidopsis Insertion Library</td>
</tr>
<tr>
<td>SALK</td>
<td>Salk Institute Genomic Analysis Laboratory</td>
</tr>
<tr>
<td>SAM</td>
<td>shoot apical meristem</td>
</tr>
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<td>SCRAMBLED</td>
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<td>SCARECROW</td>
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<td>SUGAR DEPENDENT1</td>
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</tr>
<tr>
<td>SHR</td>
<td>SHORTROOT</td>
</tr>
<tr>
<td>STM1</td>
<td>SHOOT MERISTEMLESS1</td>
</tr>
<tr>
<td>SUS</td>
<td>sucrose synthase</td>
</tr>
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</table>
TAG    triacylglycerol
T-DNA  transfer DNA
WER    WEREWOLF
WOX    WUSCHEL-related homeobox
WRII   WRINKLED1
X-GlcA 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
Zm    Zea mays
ZT    Zeitgeber Time
Chapter 1: Literature Review and Background

1.1 Transition to flowering in Arabidopsis

The floral transition in higher plants is an important step in the plant lifecycle (Bernier et al., 1993). During this transition, the shoot apical meristem (SAM) changes from a vegetative meristem, which produces leaves, into a reproductive meristem, which produces inflorescences. After the transition, most of the energy produced by the plant is devoted to reproductive tissues and the timing of this transition is crucial, as it can affect agronomically important traits such as seed yield and biomass accumulation. Consequently, understanding the mechanisms that control this transition is an important area of plant research. We know that most plants use a combination of endogenous and exogenous signals to flower under optimal conditions (Bernier et al., 1993; Mouradov et al., 2002; Srikanth and Schmid, 2011). Plants use internal factors such as plant size, nutrient availability and plant age as indicators of overall plant health, while day length and air temperature are environmental factors that contribute to the timing of the floral transition. Different species require different environmental conditions to flower and conditions that may induce flowering in one species, such as long day lengths, may repress flowering in another species (Bernier, 1988; Srikanth and Schmid, 2011). For example, the model plant Arabidopsis thaliana (Arabidopsis) is a facultative long day plant, meaning that flowering is greatly accelerated under long day conditions (Koornneef et al., 1998), whereas Oryza sativa (rice) flowers under short day conditions (Yano et al., 2001).

While finding conditions that are favorable to flowering in various species was relatively simple, uncovering the mechanisms that control the floral transition posed a greater challenge.
Pioneering this effort in 1936, Mikhail Chailakhyan grafted leaves of a flowering tobacco plant onto a tobacco plant grown in a non-inductive photoperiod, which resulted in the second plant flowering (Chailakhyan, 1936). These grafting experiments showed that the signal to induce flowering actually originates in mature leaves and is transmitted to the SAM. This mobile inductive signal was named ‘florigen’ (Chailakhyan, 1936) and its identity remained a mystery for many decades. Finally, in 2007, the FLOWERING LOCUS T (FT) protein was identified as florigen in Arabidopsis (Corbesier et al., 2007; Mathieu et al., 2007; Jaeger and Wigge, 2007). FT travels from mature leaves through the phloem to the SAM, where it interacts with the FLOWERING LOCUS D (FD) to activate expression of genes that cause the SAM to transition to a reproductive meristem (Abe et al., 2005; Imaizumi and Kay, 2006). FT expression is controlled via a pathway that combines photoperiod information and circadian clock rhythm called the external coincidence model (Suárez-López et al., 2001).

In the external coincidence model, the circadian rhythm-regulated GIGANTEA (GI) and FLAVIN-BINDING, KELCH REPEAT, F-BOX1 (FKF1) proteins regulate expression of the CONSTANS (CO) gene through interactions with CYCLIN DOF FACTOR (CDF) family proteins (Fornara et al., 2009; Imaizumi et al., 2005; Sawa et al., 2007). CO expression levels oscillate throughout the day with peak expression levels occurring in late afternoon. The stability of the CO protein depends on the amount of light the plant receives; a longer day length leads to a more stable protein (Valverde et al., 2004). Under long day conditions, the CO expression peak and the peak in CO protein stability coincide, allowing CO to activate expression of FLOWERING LOCUS T (FT) in mature leaves (Valverde et al., 2004) so that the FT protein can travel to the SAM to induce flowering. Whereas numerous genes involved in controlling the floral transition have been characterized in Arabidopsis, very few are identified in Zea mays (maize). The first floral regulator
gene identified in maize was *INDETERMINATE1* (Colasanti et al., 1998) (hereafter referred to as *ZmID1*).

The ZmID1 protein contains an N-terminal putative nuclear localization signal (NLS) and four zinc finger domains, which make up the *INDETERMINATE DOMAIN (IDD)* (Colasanti et al., 1998). Interestingly, it was discovered through site-directed mutagenesis that the two outer zinc fingers are not involved in binding the consensus sequence, despite the finding that one of them shows typical DNA-binding characteristics. Instead, the inner zinc fingers are responsible for binding the target sequence (Kozaki et al., 2004). *Zmid1* loss-of-function mutants do not undergo the transition to flowering and continue to produce leaves long after their wild-type siblings have flowered and senesced (Colasanti et al., 1998). Recently, it was shown that *Zmid1* mutants have similar photosynthetic abilities to wild type plants but have higher levels of sucrose and other soluble sugars and lower levels of tricarboxylic acid cycle organic acids (Coneva et al., 2012). These differences in carbohydrate profile suggest that ZmID1 is involved in controlling carbon allocation in mature leaves in addition to its function as a floral regulator (Coneva et al., 2012).

Phylogenetic analysis comparing *ZmID1* to genes from maize, rice and Arabidopsis identified numerous genes showing the characteristic zinc finger pattern and putative NLS that make up the *INDETERMINATE DOMAIN* (Colasanti et al., 2006). These genes make up the *IDD* gene family, which is conserved among all higher plants (Colasanti et al., 2006). Many *IDD* genes are currently uncharacterized, however, several seem to be involved in regulating carbon flow and assimilation (Coneva et al., 2007, 2012).
1.2 *Arabidopsis* INDETERMINATE DOMAIN family genes are largely uncharacterized

The Arabidopsis *IDD* gene family contains 16 genes named *IDD1* to *IDD16* (Colasanti et al., 2006). Several members have been characterized using loss-of-function and constitutive overexpression transgenic plants and have functions related to diverse stages of development. For example, *35S::IDD1* overexpression transgenic plants show an array of phenotypes involving the seed. *35S::IDD1* mutants have altered seed coat morphology, altered mucilage deployment and delayed endosperm degradation (Feurtado et al., 2011; LeBlanc, 2012). Seeds are also able to germinate successfully on media containing the phytohormone abscisic acid (ABA), which represses germination in wild type seeds (Feurtado et al., 2011). These findings suggest that *IDD1* is involved in seed dormancy and germination and controlling carbon allocation in seeds (Feurtado et al., 2011; LeBlanc, 2012). The *idd8* loss-of-function mutant also shows a difference in carbon assimilates, measured in whole seedlings rather than in the seed (Seo et al., 2011). The *idd8* mutant has higher endogenous sucrose, glucose and starch levels and shows late flowering in inductive long day conditions compared to wild type seedlings, suggesting that *IDD8* is involved in controlling sugar transport and metabolism and regulating flowering (Seo et al., 2011). Interestingly, *IDD8* is the only *IDD* gene characterized to date that has a function related to flowering time. The *idd15* mutant exhibits a completely different set of phenotypes, including reduced gravitropic responses in the inflorescence stems, reduced circumnutation and lateral branches that emerge at an increased angle relative to the primary inflorescence compared to wild type plants (Morita et al., 2006; Tanimoto et al., 2008). Gravity sensing amyloplasts in *idd15* endodermis cells sediment more slowly and iodine/potassium iodide staining suggests that *idd15* amyloplasts contain less starch than those in wild type plants. These phenotypes suggest that *IDD15* is involved in promoting starch accumulation in amyloplasts used to sense gravity.
(Tanimoto et al., 2008). *IDD15* is closely related to *IDD14* and *IDD16*, as they clade together in phylogenetic reconstructions of the *IDD* family (Colasanti et al., 2006). Triple loss-of-function *idd14 idd15 IDD16i* transgenic plants were made and had enlarged, infertile flowers, increased angles between primary and secondary inflorescences and accumulated less auxin in leaves and inflorescences (Cui et al., 2013). Therefore, these results suggest that *IDD14, IDD15* and *IDD16* work together to regulate auxin biosynthesis and control organ development in aboveground tissues (Cui et al., 2013). As well as acting in aboveground tissues, *IDD* family genes also play a role in root development. *IDD10* controls patterning in root epidermal cells through interactions with the root cell division regulators, SHORTROOT (SHR) and SCARECROW (SCR) (Welch et al., 2007) and *IDD10* is implicated specifically in determining hair cell fate (Hassan et al., 2010).

An emerging understanding of the *IDD* family suggests that many members are involved in linking carbon assimilation with growth and development (Coneva et al., 2012). Carbon flow and the way carbon is partitioned in plants are important processes to understand, since they affect how energy is stored in different parts of the plant, which is important for agronomical traits such as yield. Overall, a better understanding of the *IDD* family could help to enhance our knowledge of carbon partitioning.

### 1.3 Oil production and hydrolysis in Arabidopsis seeds

Energy in Arabidopsis seeds is stored mainly as triacylglycerol (TAG) (Hsieh and Huang, 2004). While the seed is maturing, many genes are involved in TAG synthesis in the endoplasmic reticulum (ER) (Graham, 2008). Seeds of the *wrinkled1 (wri1)* loss-of-function mutant have reduced oil compared to wild type seeds (Focks and Benning, 1998). *WRI1* is the major regulator of oil production in seeds and is only expressed in developing seeds (Fukuda et al., 2013). *WRI1* transcriptionally regulates the expression of downstream genes encoding enzymes such as acetyl-
CoA carboxylase (ACCase), pyruvate dehydrogenase and other fatty acid synthesis genes. ACCases catalyze the first committed step of fatty acid synthesis and directly affect the amount of oil produced in the seed (Fukuda et al., 2013). Seeds with a mutation in the TAG1 locus, which codes for the DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1) enzyme, have only 55-75% of the oil that wild type seeds accumulate and contain more sucrose than wild type seeds (Katavic et al., 1995), showing that DGAT1 is an important enzyme in oil synthesis.

TAG is stored as oil bodies surrounded by a layer of phospholipids and oleosins (Figure 1.1). Oil bodies are spherical and have a diameter of approximately 0.5 µm in Arabidopsis, which is smaller than the diameter of 1 µm found in most plants (Hsieh and Huang, 2004). Oleosins are structural proteins that are important for preventing oil bodies from aggregating in the seed. The oil to oleosin ratio helps determine the size and shape of the oil bodies in a seed. The small size of Arabidopsis seed oil bodies relative to other species is due to a low oil to oleosin ratio; the increased amount of oleosin means that more surface area can be covered, thus, oil bodies are made smaller. Oil bodies are synthesized in the ER and oleosin is targeted to the ER shortly after translation. During germination, lipases can easily bind to the oil bodies because of their large surface area to volume ratio (Hsieh and Huang, 2004). At this time, the energy stored in the seed needs to be quickly converted into metabolites and transported throughout the developing seedling, as energy demands are high. It is imperative that this is done in a timely manner to ensure that the first true leaves are deployed and photosynthesis begins before energy reserves are completely depleted (Graham, 2008).
Figure 1.1: Oil body in an Arabidopsis seed. Triacylglycerol is surrounded by a single layer of phospholipids and structural proteins called oleosins. Created based on Hsieh and Huang, 2004.

Seed oil mobilization begins with the breakdown of stored oil (Figure 1.2). Lipases, such as SUGAR DEPENDENT1 (SDP1) catalyze the first step in breaking down TAG for energy. The SDP1 protein binds oleosins and hydrolyzes TAGs into fatty acids and glycerol. The sdpl loss-of-function mutants have reduced TAG hydrolysis, however, the process is not completely eliminated (Eastmond, 2006). Interestingly, once plants have reached maturity, sdpl mutants have higher TAG concentrations in stems and roots and lower TAG concentrations in leaves compared to wild type plants, suggesting opposing roles in these tissues (Kelly et al., 2013). The sdpl mutants were crossed with loss-of-function mutants for the SUGAR DEPENDENT1 LIKE (SDP1L) gene and sdpl sdp1L double mutants were viable but severely retarded in growth after germination (Kelly et al., 2011). The viability of sdpl sdp1L double mutants demonstrates that while TAG hydrolysis is a major part of germination and seedling growth, it is not essential. Together, SDP1 and SDP1L are responsible for more than 90% of the TAG hydrolysis that occurs in germinating seeds (Kelly et al., 2011).

After TAG hydrolysis, fatty acids are transported into the peroxisome by the COMATOSE (CMT) protein and undergo the β-oxidation cycle, followed by the glyoxylate cycle to produce
Oxaloacetate (Graham, 2008). Oxaloacetate along with glycerol from the cytoplasm are transported to the mitochondria, where gluconeogenesis occurs and sucrose is produced. Sucrose can then be transported throughout the seedling via the phloem to provide energy until the plant begins to photosynthesize (Graham, 2008). During early seedling growth, exogenous sugars and ABA inhibit storage oil mobilization. Exogenous sugars alter the carbon to nitrogen ratio in the developing seedling and ABA exposure causes a transcription factor called *ABSCISIC ACID INSENSITIVE4* (*ABI4*) to be expressed. The ABI4 protein is involved in regulating the ABA response and has downstream targets important in storage oil processing (Graham, 2008).

Figure 1.2 An overview of Arabidopsis post germinative seed oil hydrolysis. Triacylglycerol in oil bodies is broken down into fatty acids and glycerol by enzymes such as *SUGAR DEPENDENT1*. Glycerol is exported to the cytoplasm and then into the mitochondria, where it is processed into sucrose. In peroxisomes, fatty acids are processed into oxaloacetate, which undergoes gluconeogenesis in the mitochondria to produce sucrose. Adapted from Quettier and Eastmond, 2009 and Graham, 2008.
1.4 Seed dormancy and germination are controlled by antagonistic hormones

Seed germination refers to the process in which a dry seed takes up water, in a mechanism called imbibition, and the embryonic axis elongates (Bewley, 1997). The point at which the radicle emerges from the seed is typically used as a marker for the completion of germination since it is the first visible sign. By contrast, seed dormancy is the opposing state in which a seed is temporarily prevented from germinating even though environmental conditions are optimal. A dormant seed can undergo all of the metabolic processes that an imbibing seed goes through except that the radicle does not elongate and push through the testa. Little is known about how the embryonic axis is prevented from elongating in dormant seeds (Bewley, 1997). When a seed is imbibed, two phases of water uptake occur (see Figure 1.3). The first phase is characterized by rapid uptake of water followed by a plateau in water uptake in the second phase. During the first phase, cell membranes become leaky due to the rapid flow of water into the seed and solutes are released into the surrounding area. The cell membranes quickly regain their structural integrity, while damaged organelles and DNA are repaired in ways that are currently unknown. Respiration and other metabolic activities resume to allow the radicle to elongate and rupture the seed coat. There are three possible causes for the commencement of radicle elongation. The first is that the osmotic potential of the radicle drops and causes it to take up more water and push through the seed coat. The second is that the walls of radicle cells become looser to allow the radicle to stretch and emerge through the surrounding tissues and the third is that the seed coat around the root tip weakens and allows the radicle to push through. Evidence exists for each of these possible explanations and it is likely that a combination of the phenomena is occurring to promote radicle elongation. For the radicle to push past the endosperm and seed coat, existing radicle cells inside the seed only need to elongate. No cell division is required until the radicle is outside the seed
coat. After radicle emergence, stored energy in the form of seed oil is mobilized (Bewley, 1997) as described in Section 1.3.

![Diagram showing germination involving two phases of water uptake and radical emergence.]

Figure 1.3: Germination involves a) two phases of water uptake and b) radical emergence. There are three hypotheses for how radicle emergence occurs: the water potential of the radicle lowers and the radicle absorbs water, or the cells of the radicle become ready for elongation, or the cells of the seed coat become weak and break apart. Adapted from Kucera et al., 2005.

Plant hormones play a key role in the balance between dormancy and germination and have a profound effect on plants even at low concentrations (see Figure 1.4). ABA accumulates in seeds as they mature and desiccate, where it keeps the seed dormant and improves the desiccation tolerance of the seed (Kucera et al., 2005). ABA synthesis is associated with the carotenoid synthesis pathway and higher levels of carotenoids correspond with higher levels of ABA. When a carotenoid synthesis gene, called phytoenesynthase, was cloned into a vector driven by a seed-specific promoter and transformed into Arabidopsis, transformed plants had higher levels of carotenoids and ABA and severe delays in germination compared to control plants. This showed that dormancy was favoured by the increased ABA levels (Lindgren et al., 2003). ABA can be produced from maternal or embryonic tissues, however only embryo-derived ABA is necessary for inducing and maintaining dormancy. Maternally-derived ABA is important for other aspects of seed development (Kucera et al., 2005). Plants that are deficient in ABA synthesis genes exhibit
a phenotype called vivipary, in which non-dormant seeds germinate while still on the mother plant, lending further evidence to the role ABA plays in promoting seed dormancy (Karssen et al., 1983). ABA also inhibits endosperm rupture, water uptake after germination and seedling growth perhaps by regulating aquaporin abundance (Kucera et al., 2005).

Gibberellin (GA) has opposing functions to ABA as GA promotes dormancy release, germination and embryo growth. Active forms of GA accumulate in provascular tissues and in the cortex and endodermis of the root just before radicle emergence (Kucera et al., 2005). Since GA is synthesized in the radicle (Yamaguchi et al., 2001) and GA-responsive genes are expressed in tissues that do not synthesize GA (Ogawa et al., 2003), it is hypothesized that GA is transported from where it is originally synthesized. It is likely that GA is transported into the seed coat tissues surrounding the root tips and helps to weaken them to allow the radicle to emerge. Mutants deficient in GA biosynthesis genes require exogenous GA application during imbibition in order to germinate, indicating that GA is essential for breaking dormancy in wild type plants. In plants that do not produce ABA or have had their seed coat and endosperm removed, GA is not necessary for radicle emergence. This provides evidence that GA has a key role in softening the seed coat to allow the radicle to penetrate and that the ABA:GA ratio is probably more important for controlling germination than the actual concentrations of each of these hormones (Kucera et al., 2005).

Ethylene also acts in opposition to ABA and both ethylene synthesis and signaling are important for promoting seed germination. Treating dormant seeds with an ethylene precursor called 1-aminocyclopropane-1-carboxylic acid (ACC) can work as an antagonist to ABA and cause germination to occur earlier than normally expected. In Arabidopsis, ethylene receptors belong to the ETHYLENE RESISTANT1 (ETR1) family. The etr1 mutant is insensitive to ethylene
and hyper-sensitive to ABA compared to wild type plants because ethylene is not able to counteract the effects of ABA. The etr1 mutant seeds also have poor germination rates. The ETR1 protein activates CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) when ethylene is not present, which begins a MAPK signaling pathway, that helps link hormonal signals and environmental conditions, such as light and osmolarity, with the appropriate plant growth response (Kucera et al., 2005).

![Hormonal interactions in seed germination](image)

Figure 1.4: Hormonal interactions in seed germination. ABA promotes dormancy and inhibits germination. GA and ethylene act antagonistically to ABA and promote germination. Adapted from Kucera et al., 2005.

1.5 Root development and growth are complex processes

Early in the development of an embryo, the founder cell for root tissue, the hypophysis, divides to produce a cell that will give rise to the quiescent centre (QC) and a cell that will become the columella (Petricka et al., 2012). Transcription factors encoded by *WUSCHEL-related homeobox* (*WOX*) genes are expressed at different times and locations throughout embryogenesis and help to control the initial patterning of the root. The root apical meristem (RAM) is a collection of stem cells that is developed during embryogenesis and contains the QC and the stem cells surrounding the QC. The *PLETHORA* (*PLT*) and *SHR/SCR* pathways are responsible for controlling development of the QC. The PLT proteins induce elevated rates of cell division in the stem cells of the RAM, while SHR activates SCR expression in the QC, which is essential for maintaining QC identity. The QC releases a factor that causes the stem cells surrounding it to remain undifferentiated. Researchers suspect that this factor could be WOX5 or a downstream
target since mutants in which WOX5 expression is limited to the QC have a smaller stem cell niche (Petricka et al., 2012). The IDD10, or JACKDAW, gene is also important for RAM patterning, since it regulates SCR expression (Welch et al., 2007).

The developing root can be divided into three zones: the meristematic zone, the elongation zone and the differentiation zone (see Figure 1.5a). The meristematic zone, located at the tip of the root, is where division and differentiation occur. Cells lengthen in the elongation zone located above the meristematic zone. The differentiation zone is the rest of the root, where different types of tissues form. Root hairs only form in the differentiation zone. Each epidermal cell can become a hair cell or a non-hair cell depending on the position of the adjacent cortical cells. An epidermal cell that is in contact with only one cortex cell not develop a root hair, while an epidermal cell that touches two cortex cells develops a root hair (see Figure 1.5b). In non-hair cells, two genes called GLABRA2 (GL2) and CAPRICE (CPC) are activated by a protein complex. The GL2 protein is necessary for non-hair cell fate. The CPC protein is transported to neighboring epidermal cells, where it replaces a member of the protein complex called WEREWOLF (WER) to prevent GL2 from being expressed, which leads to hair formation. SCRAMBLED (SCM), a receptor-like kinase, aids in this pathway by repressing WER expression in hair cells (Petricka et al., 2012). It is suspected that there is a mobile signal produced in cortical cells that may be a downstream target of IDD10, since IDD10 is expressed in cortical cells (see Figure 1.5b) (Hassan et al., 2010). If an epidermal cell is adjacent to two cortical cells, it may be exposed to more signal and become a hair cell (Petricka et al., 2012).
Figure 1.5: a) Zones of a developing Arabidopsis root and b) schematic diagram of interactions between various proteins that control root hair development. Adapted from Hassan et al., 2010 (b) and Petricka et al., 2012 (a and b).

Root growth is largely controlled by two opposing hormones: auxin and cytokinin. Auxin promotes cell division while cytokinin promotes cell differentiation (Petricka et al., 2012). Auxin can act as a stimulator of root initiation or an inhibitor of root elongation, depending on its concentration. PIN-FORMED (PIN) transporters create an auxin gradient with an auxin maximum at the root stem cell niche (Petricka et al., 2012). Along the gradient, auxin maintains stem cells and controls cell division, elongation and differentiation based on its concentration (Petricka et al., 2012). Conversely, cytokinin controls root meristem size by inhibiting cell division and promotes differentiation in the transition zone between the meristematic and elongation zones (Petricka et al., 2012). Gibberellic acid mediates the auxin-cytokinin antagonism by interacting with both hormones and promotes division in the meristematic zone upstream of auxin and represses the root growth inhibition activity of cytokinin (Petricka et al., 2012). Another class of phytohormones, brassinosteroids, affect differentiation and organization of the stem cell niche by regulating the

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expression of SCR and WOX5, while ABA and ethylene regulate division in the quiescent centre of the root (Petricka et al., 2012). The interactions between brassinosteroids, ABA and ethylene are not well understood and further research needs to be done in this area.

Lateral roots are important for expanding the underground reach of the plant and securing it in an upright position in the soil. Lateral root initiation begins with a periclinal division in the pericycle adjacent to the two xylem poles (xylem are arranged in a diarch pattern, as discussed in Section 1.6) (Casimiro et al., 2001). Auxin is very important in this division. Auxin is transported basipetally from the growing root tip towards the shoot apex in order to promote lateral root primordia initiation and the first periclinal division (Casimiro et al., 2001). The daughter cells divide transversely and periclinally, eventually leading to the protrusion of the lateral root through the surface of the main root (see Figure 1.6). Treating seedlings with N-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, inhibits even the first pericycle division when applied at 10 μM, demonstrating how important auxin is for initiating lateral root development (Casimiro et al., 2001). While basipetal auxin transport is necessary for lateral root initiation, acropetal (shoot apex to root tip) auxin transport may only be required for proper lateral root development after initiation. shoot meristemless1 (stm1) mutants lack the ability to produce leaf primordia, which are the primary source of shoot-derived auxin, yet these mutants are still able to produce lateral roots. However, when the length and length pattern of their lateral roots are compared to that of wild type seedlings, mutants show shorter lateral roots that lack the usual pattern of longest towards the shoot and shortest towards the root tip. These results suggest a role for shoot-derived auxin in helping lateral roots develop properly (Casimiro et al., 2001). Overall, root development is a complicated process that requires the cooperation of many genes and the influence of several key hormones.
1.6 Vasculature formation in roots, hypocotyl and leaves follows specific patterns

Vasculature tissue consists of the xylem and phloem, which are responsible for transporting water and minerals, and sugars, respectively. The vasculature of the hypocotyl is arranged with the xylem forming a line between two poles (called a diarch pattern) and the phloem placed on either side of the line at the opposite poles (Turner and Sieburth, 2003). Vasculature is surrounded by endodermis, cortex and epidermis layers. Hypocotyl vasculature is formed from pre-existing procambium cells whose differentiation is pausing during embryogenesis. This pause occurs by an unknown mechanism but is believed to be related to the seed maturation process (Turner and Sieburth, 2003).

Vasculature in roots also follows a diarch pattern and arises from the root apical meristem, where procambium cells are formed via asymmetric divisions (Turner and Sieburth, 2003). As the root grows, vasculature extends and eventually secondary growth occurs to add girth to the root. A vascular cambium forms between the primary xylem and phloem and lays down secondary
xylem towards the centre of the root and secondary phloem towards the exterior (Turner and Sieburth, 2003).

In the rosette and inflorescence, vasculature is arranged in 5 to 8 veins around a central pith, with phloem towards the outside and xylem towards the inside (Turner and Sieburth, 2003). This vein arrangement is advantageous for connecting vasculature between shoots and lateral tissues, such as leaves and flowers. Each ring of vasculature and associated cells is surrounded by a starch sheath that is similar to the endodermis in the root and hypocotyl. The starch sheath divides to form the interfascicular region, which produces secondary phloem and lignified cells for structural support, while the vascular cambium produces secondary xylem and secondary phloem (Turner and Sieburth, 2003).

Arabidopsis leaves show a reticulate or netted vein pattern, with xylem on the adaxial side and phloem on the abaxial side (Turner and Sieburth, 2003). A primary vein runs down the middle of the leaf. Secondary veins branch off the primary vein and form loops by connecting with other secondary veins. Tertiary and quaternary veins branch from secondary and tertiary veins, respectively, and act to maximize the reach of the vascular system. Vein formation and patterning is believed to be influenced by auxin transport, as evidenced by examining vein patterns in auxin transport mutants and plants grown in the presence of auxin transport inhibitors (Turner and Sieburth, 2003). Experiments that quantify auxin suggest that areas with high levels of auxin correspond with future veins (Avsian-Kretchmer et al., 2002). There are several theories to explain how auxin maxima form in leaves. The up-the-gradient theory suggests that cells sense the auxin concentration of nearby cells and pump auxin towards the cells with the highest auxin concentrations, creating local maxima (Vernoux et al., 2010). Conversely, the canalization theory states that by being exposed to auxin, some cells develop an increased ability to transport auxin,
which leads to auxin drainage from the surrounding cells and a vein forms at the auxin maximum. Some aspects of leaf vasculature development are not explained by the canalization theory, such as the way in which minor veins form, since they are connections between major veins and do not have polarity (Turner and Sieburth, 2003). Further research is needed in this area to prove or disprove these theories.

1.7 **Leaf development is a multi-stage process**

Leaves synthesize sugars from light energy to fuel all of the energy needs of the plant and, as such, leaf development is one of the most important developmental processes. Leaf development proceeds through three stages; primordium initiation, abaxial/adaxial identity determination and further growth from a marginal meristem (Tsukaya, 2002). Leaf primordia arise from the shoot apical meristem (SAM) via local auxin maxima in a spiral phyllotaxy. Rapid cell division and elongation in the leaf primordia enlarge the leaf bulge and abaxial/adaxial identity is established. The first step in defining abaxial/adaxial identity is carried out by an unknown signal believed to originate in the SAM. There is evidence to suggest that a number of genes expressed preferentially in the future adaxial, middle and abaxial regions control the identity of these areas, however, this theory has not been proven yet. After abaxial/adaxial identity is established, cells in the basal portion of the primordia become long and parallel to eventually form the petiole, while cells in the apical portion remain small and less organized to eventually form the leaf blade. The junction between these tissues becomes the marginal meristem, which provides new cells for further development of the leaf blade and petiole (Tsukaya, 2002). Vascular development in leaves is discussed in the Vasculature section.
1.8 Starch metabolism is a highly regulated process

Starch constitutes about 50% of our daily caloric intake. Thus, it is incredibly important to understand starch metabolism and the mechanisms that control it. Plants accumulate transitory starch during the day, in order to degrade it during the subsequent night so that they can continue to grow during darkness (Streb and Zeeman, 2012). Starch accumulation must be tightly controlled to produce sufficient starch to last through the upcoming night, so rates of starch synthesis and degradation are altered in response to day and night length (Streb and Zeeman, 2012). Arabidopsis produces ellipsoid-shaped starch granules, which are 1-2 µm in diameter and are made up of the glucose polymers amylopectin and amylose. Both amylopectin and amylose have α-1,4-glucosidic bonds between glucosyl units, which form linear chains. At branch points, α-1,6-glucosidic bonds connect linear chains to form complex branched structures. Amylopectin has a highly branched structure and makes up most of the mass of a starch granule, while amylose has a more linear structure and accounts for less of the mass (Streb and Zeeman, 2012).

During the day, starch is produced largely in the spongy and palisade mesophyll cells of the leaf, but is made in lower quantities in the endodermis layer of the shoot and in columella cells of the root. Transitory starch plays an essential role in allowing plants to continue their growth during the night (Streb and Zeeman, 2012). Mutants deficient in starch synthesis or degradation display an extremely slow growth pattern when grown on a diurnal cycle but this phenotype is mostly rescued when plants are grown in constant light (Caspar et al., 1985). In general, there is a trade-off between plant growth rate and starch accumulation; mutants or accessions that grow faster have less starch, while those that grow slower contain more (Sulpice et al., 2009). Reduced starch mutants flower at a later time and developmental stage compared to wild type plants,
showing that proper starch accumulation is important for inducing the floral transition (Bernier et al., 1993; Corbesier et al., 1998).

Fixed carbon use is split between sugar synthesis and starch synthesis. In chloroplasts, carbon dioxide (CO$_2$) is fixed into triose-phosphates via the Calvin cycle, which are exported into the cytosol and converted into sucrose (Streb and Zeeman, 2012). To make starch, an intermediate of the Calvin cycle called fructose-6-phosphate (Fru6P) is converted into starch though a series of enzymatic reactions. The amount of carbon used for starch synthesis is controlled by regulation of ADP-glucose pyrophosphate (AGPase), an enzyme that activates ADP-glucose, which is the substrate for starch synthesis. AGPase is a heterotetramer with two small subunits and two large subunits encoded for by six genes and is activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate. Since 3-PGA is produced during the Calvin cycle, when carbon assimilation is occurring at high rates, starch synthesis will occur at high rates, allowing the plant to take advantage of optimal photosynthetic conditions (Streb and Zeeman, 2012).

During the night, Arabidopsis degrades almost all of the starch it has stored the previous day. Starch is degraded primarily into maltose with some glucose and glucose-1-phosphate (Glc1P). It is suggested that maltose and glucose are exported to the cytosol via membrane transporters, while Glc1P remains in the chloroplast to provide the organelle with energy (Streb and Zeeman, 2012). Starch excess (sex) mutants in Arabidopsis, also called gwd1, since they do not contain a functional α-glucan water, dikinase (GWD1), do not degrade starch as efficiently as wild type plants and gradually accumulate more starch in their leaves as the leaf ages (Zeeman and Rees, 1999). These mutants also export carbon assimilate to the roots at a slower rate compared to wild type plants (Zeeman and Rees, 1999). GWD1 is one of the first enzymes involved in initiation of starch degradation (Streb and Zeeman, 2012). GWD1 phosphorylates amylopectin
glucosyl residues on the surface of starch granules, which disrupts their structure to make them more susceptible to further breakdown. However, phosphorylated glucans are problematic for β-amylases that are also involved in breakdown, so enzymes called STARCH EXCESS 4 and LIKE SEX FOUR 2 dephosphorylate amylopectin after the granule structure has been disrupted (Streb and Zeeman, 2012). Next, α-amylases break down amylose and amylopectin by catalyzing the hydrolysis of α-1,4 glucan bonds (Karrer and Rodriguez, 1992).

Starch metabolism is tightly regulated, as evidenced by the ability of the plant to produce sufficient starch to last through the upcoming night and when photoperiods change, the plant will quickly adapt its starch synthesis and degradation rates to match the new photoperiod (Streb and Zeeman, 2012). Starch synthesis does not start as soon as the day begins and starch degradation does not start as soon as the night begins, suggesting that starch metabolism is not simply regulated by presence or absence of light. Instead, concentrations of metabolic intermediates or other signaling methods may be involved (Streb and Zeeman, 2012). As discussed earlier, the rate at which carbon is fixed into starch is largely controlled by changes in AGPase activity. Since this enzyme is mostly inactive at night, starch synthesis is halted. During the day, it is hypothesized that starch granules assume their insoluble form and are not able to be degraded, perhaps because enzymes that phosphorylate amylopectin are inactive (Streb and Zeeman, 2012). This has yet to be demonstrated. Little research has been done to explore whether hormones control starch biosynthesis and breakdown in Arabidopsis, but in cereal grains, α-amylase gene expression is enhanced by gibberellic acid (GA) and repressed by abscisic acid (ABA). For example, the α-amylase genes of barley contain GA and ABA response elements, indicating that these genes are directly controlled by these antagonistic hormones (Chrispeels and Varner, 1967; Jacobsen and
Beach, 1985; Skriver et al., 1991). Overall, starch metabolism is a highly regulated process that we do not yet fully understand.

1.9  **Sucrose is transported from source to sink**

Plants photosynthesize to produce energy in the form of various sugars that are transported from photosynthetic tissues to non-photosynthetic tissues. To begin, CO$_2$ is fixed in chloroplasts as glyceraldehyde 3-phosphate (G3P) (Ludewig and Flügge, 2013). The G3P can either be exported from the chloroplast to the cytoplasm and converted into sucrose or be converted into starch and stored in the chloroplast until night. During the night, the starch is converted to glucose or maltose and transported to the cytoplasm where it is made into sucrose as previously discussed. Although several types of sugars exist in plants, most sugars are converted to sucrose for transport. Sucrose is transported from source tissues, such as leaves, through the phloem to sink tissues, such as roots and flowers. In Arabidopsis, sucrose is usually loaded apoplastically into the phloem; sucrose is moved into vacuoles by tonoplast monosaccharide transporters and then exported into the apoplast, likely by symporters or facilitated diffusion. Members of the *SWEET* gene family transport sucrose into the phloem companion cells, where it is loaded into the phloem by energy-dependent sucrose/H$^+$ cotransporters. When the sucrose reaches the destination sink tissue, it is released from the companion cells into the apoplast and is taken up into sink tissue cells by sucrose/H$^+$ cotransporters. After this, sucrose may be transported apoplastically or symplastically until reaching its final destination (Ludewig and Flügge, 2013). Here, sucrose can be broken down into hexoses by invertases (INVs) and sucrose synthases (SUSs). These compounds are further metabolized to provide energy for the sink tissue (Ruan, 2012).
1.10 *Sugar metabolism enzymes have a signaling role*

Hexoses and their metabolic products are important for several functions including providing energy, making up structural compounds like starch and cellulose and reducing sucrose concentrations in certain tissues to allow for facilitated diffusion (Ruan, 2012). When sucrose reaches a sink tissue, it must be cleaved into hexoses before further metabolism can occur, a reaction that is done by INVs and SUSs. INV converts sucrose into fructose and glucose, whereas SUS cleaves sucrose into uridine diphosphate (UDP) glucose and fructose (Ruan, 2012). In addition to their enzymatic roles, there is evidence to suggest that INV and SUS also play a signaling role. For example, in tomato, repressing the INV that localizes to the cell wall caused an increase in petal and sepal number that could not be explained by the altered hexose levels, suggesting that loss of INV activity led to the loss of a signaling pathway affecting division. When the activity of a similar INV in maize was suppressed, seeds lacked a fully developed endosperm and were much smaller than wild type seeds. This seed phenotype could not be rescued by treating the plants with exogenous glucose or fructose or a combination of both, indicating that it was not a reduction in glucose or fructose levels that caused the phenotype but rather a result of the lack of cell wall INV activity (Ruan, 2012). Cell wall INV mediates glucose signaling, which is implicated in controlling cell division and auxin biosynthesis. Additionally, cell wall INV-mediated signaling also affects leaf development and aging by regulating the effects of cytokinin and ABA on the leaf (Ruan, 2012). Research regarding INV signaling is still in its preliminary stages and many questions remain unanswered, such as how plants sense extracellular glucose and
fructose produced by cell wall INVs and how the auxin biosynthesis and glucose signaling pathways are linked. Further research needs to be done in order to explore these questions.

Once sucrose is converted into hexoses, these hexoses must be phosphorylated by hexokinases (HXKs) and fructinases before the plant can metabolize them. HXKs are able to phosphorylate glucose, fructose, mannose and glucosamine but not galactose. There is also evidence that HXKs have a signaling role that allows them to control gene expression and the interactions of phytohormones during development (Granot et al., 2013). When maize protoplasts were exposed to sugars that are substrates of HXKs, expression of photosynthetic genes was repressed, while upon exposure to sugars that are not substrates for HXKs, no repression was observed (Jang and Sheen, 1994). Plants were also treated with a glucose analog called 2-deoxyglucose that has its 2’OH group replaced with a hydrogen atom such that it can be phosphorylated and activate HXK signaling but not be metabolized. Treatment with 2-deoxyglucose led to reduced expression of photosynthetic genes, showing that HXKs play a signaling role. HXKs help the plant sense the amount of sugar present and downregulate photosynthesis genes accordingly (Jang and Sheen, 1994). An Arabidopsis mutant called glucose insensitive2 has been engineered such that the main HXK enzyme is catalytically inactive and is unable to phosphorylate hexoses. These mutants were still able to regulate expression of photosynthetic and growth-related genes without any phosphorylation activity, implying that HXKs have a role in signaling separate from their catalytic function (Moore et al., 2003). The way in which this signal is transported through the plant and how gene expression is controlled remain to be elucidated.
1.11 Hypothesis

This project aims to investigate three main hypotheses.

Hypothesis One: Loss of IDD2 function will result in differences in growth and development compared to wild type plants.

Hypothesis Two: The IDD2::GFP::GUS reporter plants show that IDD2 is expressed in diverse tissues throughout the plant.

Hypothesis Three: IDD2 has a function related to auxin or gibberellic acid response.

1.12 Objectives

Stable, homozygous IDD2 loss-of-function T-DNA insertion lines will be obtained and examined when grown under various conditions, including in the presence of exogenous sugars, in the presence of exogenous hormones and in the dark. 35S::IDD2 overexpression transgenic plants will be created and analyzed for morphological differences from Col-0, as well as differences in their response to hormones, sugars and darkness compared to Col-0. IDD2::GUS::GFP reporter transgenic plants will be created and analyzed throughout the plant lifecycle to visualize the expression pattern of IDD2. These objectives will help to elucidate the function of IDD2.
Chapter 2: Materials and Methods

2.1 idd2 Mutant Lines

Two T-DNA insertion mutants with insertions in *IDD2* were ordered from the SALK institute (Alonso et al., 2003) and one was ordered from the SAIL/GARLIC project (Sessions et al., 2002). These mutants were genotyped and backcrossed to wild type Col-0 plants at least three times. Inserts were sequenced so their exact location could be mapped and this information along with SALK and SAIL identification numbers is shown in the Appendix in Table 0.3 and in the schematic diagram shown in Figure 2.1.

![Figure 2.1: Schematic diagram showing *IDD2* gene structure and T-DNA insertions for *idd2-1*, *idd2-2* and *idd2-3* lines. Boxes represent exons and lines represent introns.](image)

2.2 Plant Growth Conditions

The Arabidopsis ecotype Columbia-0 (Col-0) was used as a wild type control in all experiments. For plants grown on plates, seeds were sterilized using the following protocol. To a 1.5 ml microfuge tube containing about 100 seeds, 1 ml of 10% bleach and 0.1% Triton X solution was added and incubated for 5 minutes, mixing by inversion three times throughout. The solution was removed and 1 ml of 70% ethanol was added and tubes were mixed by inversion for two minutes. Four rinses with ddH$_2$O were done. Sterilized seeds were placed on plates using a P1000
pipette. Six to eight seeds each of wild type and mutant plants were placed near the top of each plate for root growth experiments and plates were oriented vertically. Many seeds were scattered throughout the plate for selection plates and plates were oriented horizontally. Plates were wrapped in parafilm or micropore tape, covered with foil and kept at 4°C for two days to imbibe the seeds. Plated Arabidopsis seedlings were grown in a growth chamber at approximately 21°C and a light intensity of 45 µmol/m²s on a schedule of 16 hour days and 8 hour nights.

Plants to be grown in soil were placed on damp filter paper in petri dishes, wrapped in foil and kept at 4°C for 2-3 days to imbibe the seeds. Seeds were planted in SUNSHINE MIX soil in 3” pots and placed in a growth chamber in the University of Guelph Phytotron facility at 23.0°C. Clear plastic lids were placed on trays for 5 days to increase humidity to promote germination. Sprouted seedlings were thinned so that each pot held a single seedling. For normal, long day treatment, growth chambers were kept at a light intensity of 130 µmol/m²s on a schedule of 16 hour days and 8 hour nights. For short day treatments, the chambers had 10 hour days and 14 hour nights and for low light treatments, chambers were kept at a light intensity of 40 µmol/m²s. Plants were watered every 3-4 days by adding water to trays underneath pots to allow the soil in the pots to absorb the water. Fertilized water was used approximately once weekly.

2.3 Media

Plant media consisted of 2.2% Murashige and Skoog powder (Murashige and Skoog, 1962) (Caisson Labs), 0.5% 2-(N-morpholino)-ethanesulfonic acid monohydrate (BioShop) and 0.1% phytgel (Sigma) or 0.8% agar (Fisher). All media was adjusted to a pH of 5.7 to 5.8 using 3 M KOH and autoclaved for 20 minutes to sterilize before it was poured into sterile petri plates. Plant media for sugar response experiments was made with either 1% to 8% sucrose (Fisher), 1% to 8% glucose (Fisher), or no added sugar. Plant media for hormone response experiments was made the
same as sugarless plant media above supplemented with filter-sterilized 1 µM GA (Sigma), 10 µM GA, 1 µM ABA (Sigma), 10 µM ABA, 1 µM IAA (Sigma), 10 µM IAA, 1 µM 6-BAP (Sigma) or 10 µM 6-BAP after autoclaving when media cooled to approximately 50°C. Plant media for selecting transgenic lines was made the same as sugarless media above supplemented with 15 µg/L glufosinate ammonium (Bayer Crop Science) after autoclaving when media cooled to approximately 50°C.

For *E.coli* growth, Luria Broth (LB) media with appropriate antibiotics added was used. 1L of LB contained 10 g NaCl (Fisher), 10 g tryptone (Fisher), 5 g yeast extract (Fisher) and 15 g agar (Fisher), if making plates. Concentrations of antibiotics used for various selections are shown in Table 2.1.

For *Agrobacterium tumefaciens* growth, 2xYT media with appropriate antibiotics was used. 1L of 2xYT contained 5 g NaCl (Fisher), 16 g tryptone (Fisher), 10 g yeast extract (Fisher) and 8 g agar (Fisher), if making plates. Concentrations of antibiotics used for various selections are shown in Table 2.1.
Table 2.1 Antibiotic selection criteria for constructs used. The appropriate antibiotic(s) was/were added to LB or 2xYT media for growing each construct. All constructs were from Gateway (Life Technologies) (Karimi et al., 2002).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Purpose</th>
<th>Antibiotic Selection</th>
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<tr>
<td></td>
<td></td>
<td><strong>E. coli</strong></td>
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<tr>
<td>pDONR 221</td>
<td>entry vector for all constructs</td>
<td>Kanamycin – 50 µg/µl</td>
</tr>
<tr>
<td>pBGWFS7</td>
<td>destination vector for reporter constructs</td>
<td>Spectinomycin – 50 µg/µl</td>
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<tr>
<td>pB7WG2D</td>
<td>destination vector for overexpression constructs</td>
<td>Spectinomycin – 50 µg/µl</td>
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2.4 *Use of Gateway® Technology to make Recombinant DNA Constructs*

The Gateway® cloning method (Invitrogen) was used to create reporter constructs for *IDD1* and *IDD2* and an overexpression construct for *IDD2*. AtIDD1pro-F and AtIDD1pro-R primers were used to amplify 2518 bp of the *IDD1* promoter. AtIDD2proB1-F and AtIDD2proB1-R primers were used to amplify 1787 bp of the *IDD2* promoter. AtIDD2OE-F and AtIDD2OE-R primers were used to amplify the *IDD2* coding region (1885 bp) from a BAC called U67269. Primers contained recombination sites for the Gateway BP reaction which uses specific recombinases to put the amplified sequences into the pDONR221 entry plasmid. To do this, 15-75 ng of PCR-amplified, gel-extracted fragment was added to 150 ng purified pDONR221 vector and 1X TE buffer pH 8.0 (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) up to 8 µl and 2 µl BP Clonase II. Samples were incubated at 25°C for 1 hour and enzyme was deactivated by adding 1 µg Proteinase K and incubating at 37°C for 10 minutes. Plasmids were then transformed into
Subcloning Efficiency DH5α Competent Cells (Invitrogen). Next, 5 μl of reaction product was added to 50 μl of competent cells and cells were kept on ice for 30 minutes, then heat shocked for 30 seconds in a 42°C water bath and returned to ice for 2 minutes. 950 μl pre-warmed SOC media was added to each tube of cells and tubes were incubated at 37°C for one hour before being plated on pre-warmed LB plates with 50 μg/μl kanamycin and placed at 37°C overnight. Colony PCR was done on surviving colonies with AtIDD1proF and AtIDD1proR for pDONR221/IDD1pro, AtIDD2pro SF and AtIDD2pro SF primers for pDONR221/IDD2pro and AtIDD2For1 and AtIDD2Rev2 for pDONR/IDD2. The same colonies were also grown in liquid media overnight and plasmids were extracted using the GeneJET Plasmid Miniprep kit (Thermo Scientific) according to manufacturer’s instructions.

After all plasmids were checked by sequencing with the above primers at University of Guelph Lab Services, LR reactions were performed to recombine fragments into desired destination vectors: pBGWFS7 for reporter constructs and pB7WG2D for the overexpression construct. To do this, 10 fmoles of pDONR221 with desired fragment transformed into it were added to 20 fmoles of destination vector and 1X TE buffer pH 8.0 up to 8 μl, 2 μl LR Clonase II was added and samples were incubated for 16 hours at 25°C. Reaction was stopped by adding 1 μg Proteinase K and incubating at 37°C for 10 minutes. 2 μl of reaction product was transformed into DH5α competent cells as previously described, except that plasmids were plated on LB plates with 50 μg/μl spectinomycin. Plasmids were sequenced again with the same primers as mentioned before to confirm that reaction was successful.

Plasmids with confirmed inserts were transformed into Agrobacterium tumefaciens. Then, 1 μg of purified plasmid was added to 50 μl competent A. tumefaciens GV3101 cells (Invitrogen), tubes were frozen in liquid nitrogen for 45 seconds and heated at 37°C for 5 minutes. One ml of
pre-warmed LB was added to cells and they were incubated for 4 hours at 28°C. Next, 500 μl of this media was plated on 2xYT media with 50 μg/μl spectinomycin, 50 μg/μl gentamycin and 25 μg/μl rifampicin and placed at 28°C for 2 days. Colony PCR was done with primers mentioned above to double-check for plasmid presence and colonies were transferred to liquid culture and grown at 28°C for 2 days.

2.5 Floral Dip Agro-infiltration to Create Transgenic Plants

The floral dip method was used to introduce reporter and overexpression constructs into Arabidopsis plants (Clough and Bent, 1998; Desfeux et al., 2000) 50 ml of liquid 2xYT media was inoculated with A. tumefaciens containing the construct of interest and grown for about 2 days to stationary phase (OD₆₀₀ > 2.0). Cells were pelleted and resuspended in a 5% sucrose solution containing 500 μl Silwet/1 L solution to an OD₆₀₀ of about 1.5. Approximately 48 plants were dipped for each construct. Siliques were removed from plants, then plants were inverted and dipped into the sucrose and bacteria solution. The shoots were fully submerged and swirled for 10 seconds. After all plants were done, they were tilted on their side to prevent sucrose from dripping onto leaves and placed in low light for one day. The next day, plants were returned to normal light and upright position. A second dip was done 5 days later in the same way except that no siliques were removed, as these siliques could contain transformed seeds. Seeds were harvested when mature and planted in soil. Transformants were selected by spraying with 200 g/L glufosinate ammonia (BASTA, Bayer Crop Science) at 10 days after germination. This spraying was repeated every 2 days for 3 more sprayings. In later generations, BaRF and BaRR primers were used to genotype reporter and overexpression plants if spraying needed to be avoided.
2.6 Genetic Crosses of Arabidopsis Plants

The *idd2* loss-of-function mutants were backcrossed to wild type Columbia plants and *idd2-1, idd2-2 and idd2-3* mutants were crossed to each other. Crossing was done by emasculating closed buds on the mother plant, brushing pollen from the father plant on to the stigma of the mother plant and isolating the exposed stigma using pieces of Saran wrap. Reciprocal crosses were always done. The *idd2-2 and idd2-3* mutants were backcrossed to Col-0 plants three times.

2.7 DNA Extraction

DNA was extracted from Arabidopsis plants using the following protocol. A leaf piece of about 1 cm$^2$ was taken from a mature leaf and placed in a 1.5 ml tube. Tubes were either placed in liquid nitrogen and tissue was homogenized with a chilled mini-pestle or a chrome bead was added to each tube along with 100 µl of PCR extraction buffer without detergent (200 mM Tris-Hcl pH 7.5, 250 mM NaCl and 25 mM EDTA pH 8) and tissue was homogenized with a Beadbeater MM301 (Retsch) for 30 seconds at a frequency of 1/30s. For the Beadbeater method, 300 µl of PCR extraction buffer (200 mM Tris-Hcl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8 and 0.5 % SDS) was added after homogenization and tubes were inverted to mix. For the mini-pestle method, 400 µl of PCR extraction buffer was added after homogenization. Samples were spun down at 15 000 rpm in a table-top centrifuge for 5 minutes and the supernatant was drawn off and placed in a new tube. To this new tube, 400 µl of 100% isopropanol was added and the tube was inverted 6 times. The tube was centrifuged at 15 000 rpm for 10 minutes and the supernatant was poured off. Then, 500 µl of 70% ethanol was added and the tube was flicked to mix the contents. The tube was centrifuged at 15 000 rpm for 1 minute and the supernatant was poured off. The sample was allowed to air-dry for several minutes and then resuspended in 50 µl ddH$_2$O. Samples were stored at -20°C for up to one year.
2.8  **PCR Amplification and Product Analysis**

Most PCR was done using GOTaq Green Master Mix (Promega). For a 20 µl reaction, 10 µl of master mix, 0.2 µl of 10 µM forward and reverse primer, 1 µl of DNA template and 8.6 µl of nuclease-free water were added to a PCR tube, vortexed briefly and collected at the bottom of tubes using a small centrifuge. Next, the following protocol was run in a Bio-rad T100 thermo cycler: an initial denaturation step of 95°C for 3:00, 35 cycles of 95°C for 0:30, annealing temperature (45-60°C) for 0:30 and 72°C for 1:20 and a final elongation step of 72°C for 5:00. In instances where a high fidelity product was needed, such as for sequencing or cloning, Phusion High-Fidelity DNA Polymerase (New England Biolabs) was used. For a 25 µl reaction, 5 µl of 5X Fusion HF buffer, 0.5 µl of 10 mM dNTPs (Invitrogen) 1.25 µl of 10 µM forward and reverse primers, 0.25 µl of Phusion DNA polymerase, 1 µl of DNA template and 15.75 µl of nuclease-free water were added to a PCR tube, vortexed briefly and collected at the bottom of tubes using a small centrifuge. Then, the following protocol was run in a Bio-rad T100 thermo cycler: an initial denaturation step of 98°C for :30, 35 cycles of 98°C for 0:10, annealing temperature (45-60°C) for 0:30 and 72°C for :30 and a final elongation step of 72°C for 5:00.

PCR products were run on 1.5% agarose (BioShop) gels in 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) with a 1 kilo base pair DNA ladder (Fermentas). Gels were analyzed using a Bio-rad Gel doc and Image Lab software version 2.01 (Bio-rad).

To extract PCR products from gels, a Purelink Quick Gel Extraction kit (Invitrogen) was used according to manufacturer’s instructions. The quality of the extracted PCR product was analyzed using a Nanodrop 2000 (Thermo Scientific). If the product needed to be sequenced, it was sequenced by the Genomics Facility at the University of Guelph.
2.9 RNA Extraction and cDNA Synthesis

To prepare for quantitative polymerase chain reaction (qPCR), RNA was extracted from Arabidopsis leaves, quantified, standardized, treated with DNase and synthesized into cDNA.

Four mature leaves from each plant were harvested in liquid nitrogen and the EZ-10 Spin Column Plant RNA Minipreps kit (Biobasic) was used to extract RNA. Frozen tissue was ground using one chrome bead per tube and the Beadbeater MM301 (Retsch). Next, 450 µl Buffer R Lysis-PG extraction buffer was added to lyse cells. Samples were centrifuged and supernatant was transferred to a new tube and mixed with 100% ethanol to precipitate RNA. The supernatant and ethanol solution was transferred to a column and centrifuged, then two washes with 500 µl of Universal GT solution, then Universal NT solution were done. Samples were centrifuged a final time to remove residual ethanol. RNA was eluted in 50 µl RNase-free water and stored at -80°C if necessary, for no longer than 2 weeks.

Extracted RNA was quantified using a NanoDrop 2000 machine (Thermo Scientific). For each sample, either 500 ng or 1 µg of RNA was treated with DNase I (Fermentas) by mixing sample with 1 µl DNase I, 1 µl reaction buffer and RNase-free water up to a total volume of 10 µl. Samples were incubated at 37°C for 30 minutes. 1 µl of 50 mM EDTA (Invitrogen) was added to each sample and samples were incubated at 65°C for 10 minutes to stop the reaction. This DNase-treated RNA was transformed into cDNA using the qScript cDNA SuperMix kit (Quanta Biosciences). The entire 11 µl was mixed with 4 µl of cDNA SuperMix and 5 µl nuclease-free water. Samples were gently flicked, briefly spun to collect solution and incubated at 25°C for 5 minutes, 42°C for 30 minutes and 85°C for 5 minutes. cDNA samples were stored at -20°C and used within 2 weeks of extraction.
2.10 Quantitative PCR

Quantitative PCR (qPCR) was used to quantify expression of $IDD_2$ and $IDD_1$ in wild-type and mutant plants. A reaction mix with SYBR green as the fluorescent dye and Rox as an inert control called PerfeCTa SYBR Green Supermix with Rox (Quanta Biosciences) was used. SYBR is a dye that produces a strong fluorescent signal when it binds to double-stranded DNA. Fluorescence is measured at each PCR cycle, as the amount of product increases exponentially. Eventually, one reagent will become limited and the reaction will plateau. In the exponential phase, the cycle at which the fluorescence level passes a certain threshold is measured and is called the Ct. Rox is also a fluorescent dye in the reaction mix but its fluorescence does not change as amplification occurs. Instead, Rox controls for slight well-to-well variation that can arise from differences in illumination or condensation amount. The fluorescence of SYBR is divided by the fluorescence of Rox at each cycle to make the amplification curve and find the Ct. 7300 System SDS software (Applied Biosystems) was used for these analyses.

Primers were designed and optimized for the target gene, $IDD_2$, and an endogenous control house-keeping gene, $ACTIN7$. These primers, their optimal dilutions and their expected amplicon size can be found in the Appendix in Table 0.2. For each reaction, 5 μl cDNA, 12.5 μl PerfeCTa SYBR Green Supermix, 1 μM each forward and reverse primer (1 μl of each of 10 μM stock) and 5.5 μl nuclease-free water were mixed for a 25 μl reaction volume. Samples were loaded into a 96-well plate and qPCR was done in 7300 Real-Time PCR system (Applied Biosystems). Data was analyzed with the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). This method involves the following equation:
\[ RQ = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct \text{ mutant} - \Delta Ct \text{ wildtype})} \\
\quad -((\Delta Ct \text{ mutant, target} - \Delta Ct \text{ mutant, endo}) - (\Delta Ct \text{ wildtype, target} - \Delta Ct \text{ wildtype, endo})) \]
\[ = 2 \]

where RQ (relative quantification) is expression of the target gene (IDD2) in the mutant compared to the expression of the target gene in the wild-type after taking into account the expression of the endogenous control. Since RQ is related to Ct in an exponential way, this complicates how standard deviations (s) are calculated. The exponential transformation means that the standard deviations above and below the mean will not be equal; the standard deviation above the mean will be larger than the standard deviation below the mean. To calculate the standard deviations, an interval of \( \Delta\Delta Ct \pm s \) was transformed into an interval of (RQ – s lower, RQ + s upper). To make results more intuitive, RQ values were converted to percentage of expression in wild-type Col-0 by multiplying RQ by 100%.

### 2.11 Starch, Glucose and Sucrose Extraction and Quantification

Starch, glucose and sucrose were extracted from mature leaves pre- and post-floral transition to investigate differences in concentrations of these compounds. Tissue of interest (usually 3 mature leaves) was collected into pre-weighed screw-cap tubes containing a metal ball at ZT0, ZT8 and ZT16, frozen in liquid nitrogen and stored at -80°C until needed. Starch and sugars were extracted the Total Starch Assay kit (Megazyme) with a modified protocol. Tissue was weighed and freeze-dried overnight in a FreeZone 6 Plus freeze-dryer (Labconco) and a CentriVap Concentrator (Labconco) and ground with the Beadbeater for 30s at 30s⁻¹. 1 mL of 80% EtOH with 3 mM HEPES (Sigma) and lightly vortexed to mix. Tubes were stored on ice and metal balls were removed with a magnet. Samples were shaken at 800 rpm and 50°C for 15 minutes, then centrifuged at 14 000 g for 5 minutes. Supernatant was transferred to a 15 ml Falcon
Another 1 ml of ethanol and HEPES was added and subsequent steps were repeated until 4 ml of supernatant has been transferred. Supernatant was stored at -80°C for sugar extraction at a later date.

To extract starch, the following protocol was used. The pellet from above was freeze-dried for 2 hours, 400 μl of ddH2O was added and tubes were lightly vortexed to break up pellet before being shaken at 99°C at 800 rpm for 20 minutes. Tubes were stored on ice and 400 μl of α-amylase and MOPS solution (1 ml α-amylase mixed with 14mL 50 mM MOPS with 5 mM calcium chloride, pH 7.0) was added. Tubes were shaken at 99°C for 10 minutes. 400 μl of sodium acetate buffer (200 mM, pH 4.5) and 26.6 μl amylloglucosidase was added to tubes and samples were vortexed and shaken at 50°C and 800 rpm for 5 minutes. To quantify starch, 50 μl of solution was mixed with 450 μl water and 500 μl GOPOD. A blank sample with 500 μl water and 500 μl GOPOD was also made. Samples were shaken at 50°C and 800 rpm for 30 minutes. Three technical replicates of 200 μl each were taken from each sample and placed into a 96-well plate. Absorbance at 510 nm was measured using a Multiskan GO Microplate spectrophotometer (Thermo Scientific) and the SkanIt software (Thermo Scientific). Technical replicates were examined and averaged and absorbance of the blank sample was subtracted from all sample absorbances. A standard curve with the following equation was used to convert absorbance to glucose.

\[
\text{Glucose (μg)} = \frac{\text{Absorbance at 510 nm} - 0.0083}{0.0244}
\]
Glucose was then converted to starch concentration (measured in mg glucose per g fresh weight) using the following equation:

\[
Starch \ (\text{in glucose (mg/g)}) = \left( \frac{\text{glucose (}\mu\text{g)} \times 1226.6 \mu\text{l} \times \frac{1 \text{mg}}{1000 \mu\text{g}}}{\text{fresh weight (g)}} \right)
\]

Sugar extraction was done using the supernatant mentioned earlier. After vortexing, 2.25 ml of supernatant was transferred to a new 15 ml tube and 1.5 ml of chloroform was added. Samples were incubated in a 37°C water bath for 10 minutes. 3.45 ml of ddH_2O was added and tubes were vortexed for 15 seconds, then centrifuged at 3000 rpm for 7 minutes. 2 ml of supernatant was transferred to a microfuge tube, freeze-dried overnight and re-hydrated with 200 \mu\text{l of ddH}_2\text{O}. Both glucose and sucrose were quantified from this solution.

To measure glucose, the same procedure as that of starch was used, except that after converting absorbance to glucose using equation 1, a different equation was used to convert glucose to glucose concentration:

\[
Glucose \ (mg/g) = \left( \frac{\text{glucose (}\mu\text{g)} \times 200 \mu\text{l} \times \frac{5 \text{ml}}{2 \text{ml}} \times \frac{4 \text{ml}}{2.25 \text{ml}} \times \frac{1 \text{mg}}{1000 \mu\text{g}}}{\text{fresh weight (g)}} \right)
\]

To measure sucrose, 50 \mu\text{l of sugar solution was added to 20 \mu\text{l of invertase and 30 \mu\text{l ddH}_2\text{O and shaken at 50°C for 30 minutes to convert sucrose to glucose and fructose. Next, 500 \mu\text{l GOPOD and 400 \mu\text{l of ddH}_2\text{O were added to this and absorbances were measured as before.}}}}
The value from the conversion of absorbance to glucose actually represents both the glucose in the sample and the sucrose that was converted to glucose, so to obtain the amount corresponding to the sucrose, the glucose value from the glucose measurement for the same plant is subtracted from the glucose value for the sucrose measurement. Then, equation 3 was used to obtain the amount of glucose in mg/g leaf tissue that corresponded to sucrose. Finally, equation 4 was used to account for the molecular weight difference between sucrose and glucose.

\[
\text{Sucrose (mg/g)} = \frac{\text{glucose (mg/g)} \times 342.2965 \times g_{mol} \text{ sucrose}}{180.16 \times g_{mol} \text{ glucose}}
\]

**2.12 GUS Assay for Gene Expression Analysis**

β-glucuronidase (GUS) assays are useful to visualize gene expression in plants transformed with a reporter construct (Jefferson et al., 1987). When tissues are treated with X-GlcA, GUS cleaves X-GlcA to produce a dichloro-di-bromo-indigo precipitate that is water insoluble and blue-coloured. Plant samples at various stages of growth were prepared according to a protocol modified from Jefferson et al. (1987). Briefly, samples are covered with Standard GUS staining solution (50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA pH 8.0, 0.1% (v/v) Triton X-100 and 0.5 mg/ml X-GlcA) and placed under a weak vacuum for 5 minutes to encourage tissue penetration. Samples are then incubated at 37°C for approximately 24 hours. Stain is removed and samples are covered with 70% ethanol and incubated at room temperature for at least 2 hours to clear the tissue and remove residual staining solution. Samples are viewed, with a microscope if necessary, and pictures are taken.
2.13 Mucilage Staining

Ruthenium red stain (Sigma) was used to view mucilage extruded from soaked seeds. Individual seeds were placed in wells of a 96-well plate with 10 μl dH2O and incubated at room temperature for 10 minutes to allow seeds to take up water. Then, 200 μl of 0.001% ruthenium red stain (pH 6.5-7.5) was carefully added to the side of each well to avoid disrupting the emerging mucilage and samples were incubated at room temperature for 25 minutes. Seeds and mucilage were viewed under a dissecting microscope.

2.14 Qualitative Comparison of Relative Seed Oil Concentrations

A seed sedimentation assay using solutions with different densities was used to determine whether seeds had different oil concentrations compared to Col-0 (adapted from Focks and Benning, 1998). Solutions with various ratios of 1,6-dibromohexane to 1-bromohexane to mineral oil were carefully layered in order of density into 2 ml microfuge tubes. The ratios, from most dense to least dense, were 1:1:0, 0:1:0 and 0:1:1. Approximately 10 to 20 seeds were added to each tube and tubes were centrifuged at 16 000 g for 2 minutes. Sedimentation patterns were compared to Col-0 seeds, which settled at the bottom of the middle layer. If seeds were in the top layer, this suggested that they had more oil than wild type seeds and if seeds were in the bottom layer, this suggested that they had less oil than wild type seeds.

2.15 Image Capture and Analysis

Macroscopic images were captured using a Nikon D5000 camera or Canon Rebel 2ti. Microscopic images were captured using a Q Imaging Retiga 1300R camera or Canon Rebel 2ti with a microscope adapter. Microscopes used in this study were a Leica MZ6, a Leica MZ FLIII and a Hitachi Tabletop Scanning Election Microscope. Images were analyzed using Image J software.
2.16 Statistical Tests

Student’s t-tests with \( \alpha = 0.05 \) were used to compare measurements of various properties of wild type and mutant plants for experiments in which one time point or set of data was collected. Experiments with repeated measurements of root or hypocotyl lengths were analyzed using repeated measures multivariate analysis of variance (MANOVA) in Statistical Analysis System (SAS) 9.2 (SAS Institute Inc.).

All significant differences are shown with means and p-values from a Student’s t-test, unless otherwise noted. In experiments where multiple comparisons were done, a modified p-value is reported to reflect the increased chance of committing a Type I error. The Bonferroni method for reducing family-wise error in multiple comparisons involves dividing the original \( \alpha \) by \( k \), where \( k \) is the number of comparisons done (Dunn, 1961). For example, if 5 comparisons are done, \( \alpha^* = \frac{\alpha}{k} = \frac{0.05}{5} = 0.01 \), so p-values would be compared to 0.01 instead of the standard 0.05. To avoid having to keep track of different \( \alpha^* \) values throughout this manuscript, original p-values have been multiplied by \( k \) and will be compared to the standard \( \alpha \) of 0.05. This is equivalent to the above correction.
Chapter 3: Results

3.1 IDD2 expression in transgenic plants

3.1.1 Analysis of putative idd2 loss-of-function mutants

T-DNA insertion mutants for IDD2 were obtained from the SALK institute (Alonso et al., 2003) and the SAIL/GARLIC project (Sessions et al., 2002) and referred to as idd2-1, idd2-2 and idd2-3. Information about SALK and SAIL numbers and insertion locations is found in Table 0.3. To check whether each mutant had reduced IDD2 expression, RNA was extracted from leaves and qPCR was done to detect IDD2 expression levels.

RNA was extracted from leaves 4, 5 and 6 of Col-0, idd2-1, idd2-2 and idd2-3 plants that were grown under normal conditions except that they were given 40 µmol/m²s of light instead of 130 µmol/m²s to induce growth of the petiole and leaf blade. IDD2 expression is reported to be highest in the petiole (Winter et al., 2007), therefore, RNA was extracted from the petiole and leaf blade to ensure that wild type expression would be high enough to prove knock-out in the mutant lines. qPCR was done with six biological replicates for each mutant line and seven biological replicates for Col-0 and three technical replicates for each biological replicate. Each sample was amplified with an endogenous control set of primers for ACTIN7 and two sets of primers in IDD2; one spanning Intron 2 that is upstream of all inserts and one within Exon 4, which is downstream of the insert in idd2-2 and upstream of the inserts in idd2-1 and idd2-3 (see Figure 3.1). The idd2-1 mutants did not have significantly different expression for either primer set. The idd2-2 mutants had an IDD2 expression level of 533% relative to Col-0 for the first primer set amplifying between Exon 2 and 3 and an expression level of 5% relative to Col-0 for the second primer set in Exon 4 (p=0.00248 and p=0.0348, respectively), suggesting that idd2-2 produces increased amounts of a
truncated transcript. The *idd2*-3 mutants had an expression level of 0.37% relative to Col-0 for the first primer set and an expression level of 1% relative to Col-0 for the second primer set (p=0.0333 and p=0.0202, respectively), suggesting that *idd2*-3 is an *idd2* loss-of-function mutant. See Figure 3.2 and Figure 3.3 for expression data.

Figure 3.1: Schematic diagram showing *IDD2* gene structure, T-DNA insertions for *idd2*-1, *idd2*-2 and *idd2*-3 mutant lines and qPCR primer locations. Boxes represent exons and lines represent introns. Primers labelled 1 and 2 are AtIDD2E2F and AtIDD2E3R, respectively, and primers labelled 3 and 4 are AtIDD2E4F and AtIDD2E4R, respectively.

Figure 3.2: *IDD2* expression in *idd2*-1, *idd2*-2 and *idd2*-3 mutants relative to expression in Col-0. Six biological replicates were used for each line and *IDD2* expression in Col-0 is shown by the dashed line at 100%. Primers used were in exon 2 and exon 3 of *IDD2*. Stars indicate a significant difference in expression from Col-0.
Figure 3.3: IDD2 expression in *idd2*-1, *idd2*-2 and *idd2*-3 mutants relative to expression in Col-0. Six biological replicates were used for each line and IDD2 expression in Col-0 is shown by the dashed bar at 100%. Primers used were in exon 4 of IDD2. Stars indicate a significant difference in expression from Col-0.

### 3.1.2 Analysis of IDD2 overexpression transgenic plants

To investigate the effects of overexpressing IDD2 at all developmental stages and in all tissues, transgenic plants expressing IDD2 under the control of a constitutive cauliflower mosaic virus 35S promoter were created using Gateway cloning and the floral dip procedure. The *IDD2* cDNA sequence was amplified and cloned into the pB7WG2D vector from Gateway (Life Technologies) (see Figure 3.4). The finished vector was transformed into *Agrobacterium tumefaciens* and into Arabidopsis via the floral dip method. Potential positive transformants were selected by spraying seeds from floral dipped plants with glufosinate ammonium (BASTA) (Bayer CropScience). Seed was collected from potential positive plants and planted out. These plants were sprayed with BASTA to identify plants with the insert and seed was collected. Finally, this seed was planted out to determine the zygosity of the corresponding parent.
Figure 3.4: *IDD2* overexpression construct containing the *IDD2* ORF constitutively driven by a 35S promoter. This construct was created using Gateway cloning with pB7WG2D as a destination vector (Karimi et al., 2002)

Sixteen potential overexpression lines were isolated. To ensure that these lines were expressing elevated levels of *IDD2* transcript, qPCR was done to quantify *IDD2* expression. For lines that were homozygous, RNA was extracted from leaves 5, 6 and 7 from three biological replicates for each line. qPCR was done on these replicates, as well as three biological replicates for Col-0 that was grown with these plants. Three technical replicates were done for each biological replicate. Each sample was amplified with an endogenous control set of primers for *ACTIN7* and the primers in *IDD2* exon 2 and 3 described above and in Figure 3.1. Twelve of the sixteen potential overexpressing lines showed overexpression of *IDD2* compared to Col-0, as shown in Figure 3.5. Since only three replicates of each line were used and 16 comparisons were done, t-tests are somewhat unreliable. Therefore, p-values are given without a Bonferroni correction first. The lines with significantly higher expression are as follows: 35S::*IDD2* 1-4, 1-6, 5-4, 5-6, 8-6, 9-4, 15-1, 15-3, 19-1, 19-4, 21-4 and 22-3 (with p-values of 0.00374, 0.000496, 0.000874, 0.001752, 0.00122, 0.000675, 0.00121, 0.00636, 0.00352, 0.000521 and 0.0173,
respectively). These lines are considered overexpression transgenic plants from this point forward and used in various phenotypic analyses. After Bonferroni corrections, only 35S::IDD2 1-6, 5-4, 5-6, 9-4 and 21-4 remained significant (with p-values of 0.00793, 0.0140, 0.0280, 0.0195 and 0.00833, respectively, shown by double stars in Figure 3.5). These lines were used preferentially in further analysis but the other lines were not discounted because only three replicates were used and the mean expression levels of these lines were so much higher than Col-0. The IDD2 expression of the 12 lines ranged from 1052% to 10375% of Col-0 expression and the average expression among the twelve lines was 4961% of Col-0 expression. The lines that did not have significantly different expression were 35S::IDD2 1-1, 9-4, 15-2 and 22-4 and these lines were not used after this point.
Figure 3.5: *IDD2* expression in 35S::*IDD2* lines relative to expression in Col-0. Three biological replicates were used for each line and primers used were in exons 2 and 3 of *IDD2*. Stars indicate a difference in expression from Col-0 and are considered overexpression mutants from this point forward.

3.2 *IDD2* expression throughout development

*IDD2* expression patterns were studied using the β-glucuronidase (GUS) assay to pinpoint where *IDD2* is expressed and help elucidate its function. A reporter construct with the *IDD2* promoter driving expression of a GFP:GUS fusion protein (see Figure 3.6) was used to visualize *IDD2* expression patterns throughout development.
Figure 3.6: *IDD2* reporter construct contains the *IDD2* promoter up to 1.8 kb upstream of the start codon driving expression of a GFP:GUS fusion reporter. This construct was created using Gateway cloning with pBGWFS7 as a destination vector (Karimi et al., 2002).

A total of seven *IDD2∷GFP:GUS* lines from independent insertion events and wild-type Col-0 plants were examined at 3, 6, 10, 15, 21, 30, 35, 37 and 49 days after germination (DAG). Figure 3.7 shows two lines at each of the developmental time points studied. Col-0 plants never showed any staining, so only one picture of Col-0 is included. Pictures from the 49 DAG time point were not included since none of the lines showed any staining in the senesced leaves.

In vegetative tissues, reporter lines showed *IDD2* expression in leaf vasculature, hypocotyls, petioles and emerging lateral roots. In reproductive tissues, reporter lines show expression in developing seeds, in the funiculus, and in branch points where pedicels join inflorescences and siliques.
**Observations:** Staining is very dark at the shoot apex and in petioles of leaves. Major and minor veins are stained in young and older leaves. Scale bars represent 2 mm.

**Observations:** Staining appears in early vasculature of cotyledons, hypocotyl and at nodes and growing tip of the root. An image of control Col-0 seedling is shown on the right. Scale bars represent 0.5 mm.

**Observations:** Same as 3 DAG. Staining also appears at nodes of the root where lateral roots will emerge. Scale bars represent 0.5 mm.

**Observations:** Staining appears very dark in petioles of emerging true leaves, as well as in leaf vasculature and in tips of newly formed lateral roots. Scale bars represent 2 mm.

**Observations:** Staining is very dark at the shoot apex and in petioles of leaves. Major and minor veins are stained in young and older leaves. Scale bars represent 2 mm.
Observations: Staining appears in vasculature and petioles as noted before. Scale bars represent 10 mm.

Observations: Staining appears at nodes where individual flowers emerge from the inflorescence, where the pedicel joins the silique, as well as in developing flowers and some developing seeds. Scale bars represent 2 mm.
Figure 3.7: GUS staining of different tissues of *IDD2::GFP:GUS* reporter plants at various developmental time points. Images of two or three separate lines of reporters are shown here, however seven lines were used in the analysis and trends were the same in all lines. Col-0 seedlings were stained at each step to check for non-specific staining. No staining was ever observed in Col-0 and one control image is included at 3 DAG. Some final images have been stitched together from multiple pictures.
Later, *IDD2::GFP:GUS* were grown on ½ MS media as before and looked at just 1.5 DAG to examine early growth (see Figure 3.8). Staining appears in cotyledons, at the shoot apical meristem, in vasculature of the hypocotyl and at the root tip.

**Observations:** Staining appears in the cotyledons, at the shoot apex, in the vasculature of the hypocotyl and at the root tip. Scale bar represents 0.5 mm.

Figure 3.8: GUS staining of *IDD2::GFP:*GUS reporter seedlings early in development. Images of two separate lines of reporters are shown here, however seven lines were used in the analysis and trends were the same in all lines. Col-0 seedlings were stained to check for non-specific staining and no staining was observed.

Expression of *IDD2* was also examined when *IDD2::GFP:*GUS lines were grown on ½ MS media with 1 μM IAA (indole-3-acetic acid or auxin) to see if the hormone had an effect on *IDD2* expression patterns (see Figure 3.9). Seedlings were smaller and had more lateral roots at 7 DAG due to the effect of exogenous auxin, but staining patterns resemble those observed on ½ MS media in Figure 3.7.
Figure 3.9: GUS staining of *IDD2::GFP:*GUS reporter seedlings grown on 1 μM IAA. Six lines were used in this analysis and trends were similar in all lines. Col-0 seedlings were stained at each step to check for non-specific staining. No staining was ever observed in Col-0. Some final images have been stitched together from multiple pictures.

It is important to note that when these reporter lines were examined for GFP fluorescence, no fluorescence was observed.

### 3.3 *IDD1* expression throughout development

Expression patterns of *IDD1* were also studied using the β-glucuronidase (GUS) assay to see if *IDD1* expression patterns overlapped with *IDD2* expression patterns, since *IDD1* and *IDD2* are closely related (Colasanti et al., 2006). A reporter construct with the *IDD1* promoter driving expression of a GFP:GUS fusion protein (see Figure 3.10) was used to visualize *IDD1* expression patterns throughout development.
Figure 3.10: *IDD1* reporter construct contains the *IDD1* promoter up to 2.5 kb upstream of the start codon driving expression of a GFP:GUS fusion reporter. This construct was created using Gateway cloning with pBGWFS7 as a destination vector (Karimi et al., 2002).

A total of seven *IDD1∷GFP:GUS* lines from independent insertion events and wild-type Col-0 plants were examined at 3, 6, 10, 15, 21, 30, 35, 37 and 49 days after germination (DAG). Figure 3.11 shows two lines at each of the developmental time points studied. Col-0 plants never showed any staining, so only one picture of Col-0 is included in Figure 3.7. Pictures from the 49 DAG time point were not included since none of the lines showed any staining in the senesced leaves.

In vegetative tissues, reporter lines showed *IDD1* expression throughout cotyledons, at the root tip, at the SAM and in midveins of leaves. In reproductive tissues, reporter lines show expression in flowers and inflorescences, in developing seeds, and where siliques join pedicels.
Observations:
Diffuse staining throughout the cotyledons and at the root tip. Scale bars represent 0.5 mm.

Observations:
Staining appears in cotyledons and root tips. Vasculature appears to be staining in the cotyledons. Scale bars represent 0.5 mm.

Observations:
Staining appears in leaves, SAM and root tips. Scale bars represent 2 mm.

Observations:
Staining is darkest in SAM and midvein of the leaf. Scale bars represent 2 mm.

Observations:
Staining appears to be darkest in the midvein of leaves and is also spread diffusely throughout leaves. Scale bars represent 10 mm.
Figure 3.11: GUS staining of different tissues of *IDD1::GFP:GUS* reporter plants at various developmental time points. Images of two separate lines of reporters are shown here, however seven lines were used in the analysis and trends were the same in all lines. Col-0 seedlings were stained at each step to check for non-specific staining. No staining was ever observed in Col-0. Some final images have been stitched together from multiple pictures.
3.4 Root and Hypocotyl Growth Assays

3.4.1 idd2 mutant plants do not show a difference in germination or root growth

Since GUS staining revealed that IDD2 is expressed in hypocotyls and cotyledons early in development, idd2 mutants were examined for differences in germination and early growth. Twelve seeds each of Col-0, idd2-1, idd2-2 and idd2-3 were plated on ½ MS media with 2% sucrose and photographed every 10 minutes for 72 hours. The photos were made into a time-lapse video with 6 frames/second, such that 1 second of video is 1 hour of pictures. There was no difference in germination times for any of the mutants compared to Col-0 (see Figure 3.12) but the first root hairs of idd2-1 emerged significantly slower (p=0.00000587) as shown in Figure 3.13.

![Figure 3.12: Time to germination of Col-0, idd2-1, idd2-2 and idd2-3 seedlings. Seeds were sown on 1/2 MS media with 2% sucrose and pictures were taken every 10 minutes for 72 hours. Photos were examined and a time-lapse video was created with 1 second of video equal to 1 hour in real time. Error bars represent standard deviation. Stars indicate a statistically significant difference from Col-0.](image-url)
Figure 3.13: Time to first root hair emergence of Col-0, idd2-1, idd2-2 and idd2-3 seedlings. Seeds were sown on 1/2 MS media with 2% sucrose and pictures were taken every 10 minutes for 72 hours. Photos were examined and a time-lapse video was created with 1 second of video equal to 1 hour in real time. Error bars represent standard deviation. Stars indicate a statistically significant difference from Col-0.

To investigate whether loss of IDD2 expression affected root growth, wild type and idd2 mutant seedlings were grown on various concentrations of agar, sucrose, glucose and sorbitol. Col-0, idd2-1, idd2-2 and idd2-3 seeds were sown on ½ MS media made with either 1%, 2% or 3% agar. Plates and seeds were imbibed at 4°C for 2 days and grown under standard conditions in a vertical orientation. Root length was measured daily for 5 days starting one day after germination. Root growth rates of mutants were compared to that of the wild-type by testing for a type*time interaction with repeated measures ANOVA at each treatment. The idd2-1 plants grew slower than Col-0 plants at 1%, 2% and 3% agar (p=0.00960, p=0.0156 and p=0.00120, respectively). The growth rates for idd2-3 did not differ from Col-0 at any of the agar treatments and only the growth rate on 3% agar differed between idd2-2 and Col-0 (p=0.0174) with the
idd2-2 plants growing slightly faster. See Figure 3.14, Figure 3.15 and Figure 3.16 for visual representations.

Figure 3.14: Root growth of Col-0, idd2-1, idd2-2 and idd2-3 on 1/2 MS media made with 1% agar. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.
Figure 3.15: Root growth of Col-0, idd2-1, idd2-2 and idd2-3 on 1/2 MS media made with 2% agar. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

Figure 3.16: Root growth of Col-0, idd2-1, idd2-2 and idd2-3 on 1/2 MS media made with 3% agar. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.
Col-0, *idd2-1*, *idd2-2* and *idd2-3* seeds were sown on ½ MS media either without supplemental sugar, with 2% sorbitol, with 5% sorbitol, with 2% sucrose, with 5% sucrose or with 2% glucose. Sorbitol treatments were included to alter the osmotic pressure of the media without providing the seedlings with an energy source. Plates and seeds were imbibed at 4°C for 2 days and grown under standard conditions in a vertical orientation. Root length was measured daily for 5 days starting one day after germination. Root growth rates were compared between mutants and wild-type on each sugar treatment by testing for a type*time interaction term using repeated measures ANOVA. On ½ MS media without supplemental sugar, the growth rates of *idd2-1*, *idd2-2* and *idd2-3* were not significantly different from Col-0 (data not shown). On ½ MS media with 2% sorbitol, only *idd2-2* had a significantly different growth rate (p=0.0342. However, overall root growth on sorbitol was almost negligible, so this finding was not interesting (data not shown). On ½ MS media with 5% sorbitol, the growth rates of *idd2-1*, *idd2-2* and *idd2-3* were not significantly different from Col-0 (data not shown). On ½ MS media with 2% sucrose, *idd2-1* seedlings grew significantly slower than Col-0 (p<0.0001), while the growth rates of *idd2-2* and *idd2-3* were not significantly different (see Figure 3.17). On ½ MS media with 5% sucrose, *idd2-1* seedlings grew slower than Col-0 (p<0.0001) while *idd2-3* growth rates did not differ from Col-0 (see Figure 3.18). On ½ MS media with 2% glucose, *idd2-1* and *idd2-2* seedlings grew slower than Col-0 (p<0.0001 and p<0.0001, respectively) while the growth rate of *idd2-3* did not differ from Col-0 (see Figure 3.19).
Figure 3.17: Root growth of Col-0, *idd2-1*, *idd2-2* and *idd2-3* on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

Figure 3.18: Root growth of Col-0, *idd2-1*, *idd2-2* and *idd2-3* on 1/2 MS media with 5% sucrose added. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.
Figure 3.19: Root growth of Col-0, idd2-1, idd2-2 and idd2-3 on 1/2 MS media with 2% glucose added. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

To investigate the dominance of the idd2-1 phenotype, seeds from a cross between Col-0 and idd2-1 were sown on ½ MS media with 2% sucrose. Root growth was examined to see if a plant that was heterozygous for the idd2-1 allele would still have the slower growth phenotype as previously observed.

Col-0, idd2-1 and Col-0 x idd2-1 cross seeds were sown on ½ MS media with 2% sucrose. Plates and seeds were imbibed at 4°C for 2 days and grown under standard conditions in a vertical orientation. Root length was measured daily for 5 days starting one day after germination. Root growth rates were compared between mutants and wild-type by testing for a type*time interaction term using repeated measures ANOVA. The idd2-1 seedlings’ roots grew slower compared to those of Col-0 (p<0.0001), while Col-0 x idd2-1 seedlings’ roots grew at the same rate as Col-0 (see Figure 3.20), meaning that the idd2-1 allele is recessive.
Figure 3.20: Root growth of Col-0, *idd2-1*, and Col-0 x *idd2-1* crosses on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting one day after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

3.4.2 **Allelism test shows that the *idd2-1* allele phenotype is due to a possible second, linked insertion**

Mutants with the *idd2-1* allele show slower root growth compared to Col-0 under a variety of growth conditions, but do not have a difference in *IDD2* expression. We suspected that the *idd2-1* mutants might have a second insertion located close to the *IDD2* gene, such that the second insertion was not lost during backcrossing. An allelism test was done by crossing *idd2* mutants to each other and testing for a difference in root growth rates.

Col-0, *idd2-1, idd2-2, idd2-3, idd2-1 x idd2-2, idd2-1 x idd2-3* and *idd2-2 x idd2-3* seeds were sown on ½ MS media supplemented with 2% sucrose. Crosses were included to see whether they showed the slower root growth phenotype previously observed in *idd2-1*. Plates and seeds were imbibed at 4°C for 2 days and grown under standard conditions in a vertical orientation. Root length was measured daily for 5 days starting one day after germination. Root growth rates were
compared between mutants and wild-type by testing for a type*time interaction term using repeated measures ANOVA. The *idd2-3* seedlings only had 4 days of measurements collected as the plate they were growing on became contaminated. Only *idd2-1* showed a difference in root growth rate (p<0.0001). Since crosses showed normal growth and *idd2-1* did not show a difference in *IDD2* expression, this indicates that the phenotype of the *idd2-1* mutant is likely due to a second T-DNA insertion close to the insertion in the 3’-UTR of *IDD2*.

In addition, when F2 progeny from a cross between Col-0 and *idd2-1* were genotyped, some plants that did not have the *idd2-1* insertion did have the KanR gene (included in the T-DNA insert), indicating that there is likely a second, linked T-DNA insertion. A second insertion close to the insertion in *IDD2* may not have been removed during back crossing, if it was near the *IDD2* insertion, which was selected for at each generation. The second insertion is in an unknown gene with an unknown function and it is likely that the slow root growth phenotype observed in *idd2-1* plants is due to disruption of expression of the unknown gene rather than *IDD2*.
Figure 3.21: Root growth of Col-0, *idd2-1*, *idd2-2* and *idd2-1 x idd2-2* on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting two days after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

Figure 3.22: Root growth of Col-0, *idd2-1*, *idd2-3* and *idd2-1 x idd2-3* on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting two days after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.
Figure 3.23: Root growth of Col-0, *idd2-2*, *idd2-3* and *idd2-2 x idd2-3* on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting two days after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

### 3.4.3 IDD2 overexpression does not affect hypocotyl elongation or root growth

Recently, it was reported that a 35S::*IDD2* line created by another lab showed longer hypocotyls especially when grown on gibberellic acid (GA) in normal light conditions (Fukazawa et al., 2014). To see if this phenomenon occurred in the overexpression lines created here, Col-0, *idd2-2*, *idd2-3* and 35S::*IDD2* 15-1 seeds were sown on 1/2 MS media with 0, 1 or 10 µM GA and placed in the growth chamber with light for three days after germination. Later, Col-0, 35S:IDD2 5-4, 19-1 and 21-4 seeds were sown on the same media but left to grow for 6 days instead of 3 days. In both experiments, no significant differences in hypocotyl length were observed (see Figure 3.24).

Additionally, Col-0, *idd2-2*, *idd2-3*, 35S::*IDD2* 5-4, 15-1, 19-1 and 21-4 seeds were grown on 1/2 MS media with 0, 1 or 10 µM GA in the growth chamber in darkness for six days after germination to examine whether darkness exaggerated any hypocotyl phenotype. Seedlings from
one 35S::IDD2 line (15-1) had significantly shorter hypocotyls compared to Col-0 at 1 and 10 µM of GA (p=0.0297 and 0.000220, respectively) (see Figure 3.25). For all other 35S::IDD2 lines, no differences in hypocotyl lengths in dark grown plants were observed, therefore, this is not considered a true phenotype for IDD2 overexpression plants.

During these experiments, another phenotype was observed. 35S::IDD2 seedlings seemed to be weaker compared to Col-0, idd2-2 and idd2-3. Many 35S::IDD2 seedlings did not progress past their cotyledons opening and did not see full cotyledon expansion or root elongation. While there are often a few seedlings in any line that will experience this failure to continue growth, in the case of the overexpression seedlings, almost all of the seedlings showed this phenotype. When 35S::IDD2 5-4, 15-1, 19-1, and 21-4 seedlings were grown on ½ MS media, a significantly higher proportion of 35S::IDD2 15-1 and 19-1 seedlings showed difficulty in postgerminative growth compared to Col-0 seedlings (p=0.00497 and p=0.0451, respectively) (Figure 3.27). Photos are shown in Figure 3.26.
Figure 3.24: Hypocotyl lengths at 3 days after germination of Col-0, idd2-2, idd2-3 and 35S::IDD2 seedlings grown on ½ MS media supplemented with 0, 1 or 10 µM gibberellic acid. Error bars indicate standard deviation and a star shows a significant difference from Col-0 at the same treatment.

Figure 3.25: Hypocotyl lengths of Col-0, idd2-2, idd2-3 and 35S::IDD2 seedlings grown on 1/2 MS media 0, 1 or 10 µM gibberellic acid (GA) in the dark for 6 days after germination. This experiment was grown at two separate times; Col-0, idd2-2, idd2-3 and 35S::IDD2 15-1 were grown together in Experiment 1 and Col-0, 35S::IDD2 5-4, 19-1 and 21-4 were grown together in Experiment 2. Error bars represent standard deviation and stars indicate a significant difference from Col-0 at the same treatment and in the same experiment.
Figure 3.26: Col-0, idd2-2, idd2-3 and 35S::IDD2 15-1 seedlings grown on seedlings grown on ½ MS media supplemented with 0, 1 or 10 µM gibberellic acid for 6 days after germination. Column A) shows 0 µM GA, column B) shows 1 µM GA and column C) shows 10 µM GA. Col-0, idd2-2, idd2-3 and 35S::IDD2 seedlings are shown in Rows 1, 2, 3 and 4, respectively. Note weakness of 35S::IDD2 seedlings at all treatments.
Figure 3.27: Proportion of Col-0, *idd2*-3, *35S::IDD2* 5-4, 15-1, 19-1 and 21-4 experiencing normal or delayed growth on ½ MS media for 6 DAG. Proportion of seedlings with normal growth is in blue, while proportion of seedlings with delayed growth is in red. Stars indicate a difference in proportion compared to Col-0 seedlings.

After observing the stunted growth of many *35S::IDD2* seedlings, overexpression seedlings were grown on media containing sucrose to see whether sucrose would help the seedlings to progress past the cotyledon stage. The Col-0, *idd2*-1, *idd2*-2, *idd2*-3 and *35S::IDD2* 15-1 seeds were sown on ½ MS media supplemented with 2% sucrose. Plates and seeds were imbibed at 4°C for 2 days and grown under standard conditions in a vertical orientation. Root length was measured daily for 5 days starting one day after germination. Root growth rates were compared between mutants and wild-type by testing for a type*time interaction term using repeated measures ANOVA. The *idd2*-1 seedlings had a significantly slower root growth rate compared to Col-0 (p<0.0001), while the root growth rate *idd2*-2, *idd2*-3 and *35S::IDD2* seedlings did not (see Figure 3.28). The *35S::IDD2* seedlings were able to grow normally and did not experience the reduced growth observed on ½ MS media, suggesting that sucrose was able to rescue the stunted growth of the overexpression seedlings.
Figure 3.28: Root growth of Col-0, *idd2-1, idd2-2, idd2-3* and *35S::IDD2* seedlings on 1/2 MS media with 2% sucrose added. Root lengths were measured over 5 days starting two days after germination. Standard deviation bars are shown. Stars represent a statistically significant difference in root growth rate compared to Col-0.

3.5 **Morphology of idd2 and 35S::IDD2 plants**

3.5.1 **Petiole lengths and leaf size in idd2 plants do not differ from Col-0**

Since GUS assays showed high IDD2 expression in the petioles, possible differences in petiole lengths, leaf lengths and leaf emergence angles were investigated. The Col-0, *idd2-2* and *idd2-3* seeds were sown on soil and grown under a low light condition of 40 μmol/m²s to exacerbate any possible petiole phenotypes. Petiole lengths, leaf lengths and leaf emergence angle were measured at 10 DAG. No differences were observed between *idd2-2* or *idd2-3* compared to Col-0 (data not shown).

3.5.2 **Lateral roots of idd2 and 35S::IDD2 plants do not differ from Col-0**

Since *IDD2::GFP::GUS* reporter plants showed an interesting *IDD2* expression pattern in lateral root primordia, loss-of-function and overexpression mutants were examined for differences
in lateral root growth. It was hypothesized that there would be a difference in the number or length of lateral roots in knockout or overexpression mutants. The Col-0, idd2-3, 35S::IDD2 5-4, 19-1 and 21-4 seeds were sown on ½ MS media and grown for 10 days. Lateral roots per plant were counted and the longest lateral root was measured for each seedling. No significant differences between mutants and Col-0 plants were observed (data not shown).

3.5.3 35S::IDD2 plants have pale leaves

The Col-0, idd2-2, idd2-3, 35S::IDD2 5-4, 19-1 and 21-4 seeds were sown on ½ MS media supplemented with 0 or 10 µM indole-3-acetic acid (IAA), a plant-derived auxin. At 8 DAG, seedlings were examined for differences in their response to auxin, particularly their leaf colour (see Figure 3.29). Col-0 seedlings have green cotyledons and appear to have abundant root growth on ½ MS media and have yellow cotyledons on media with auxin. 35S::IDD2 seedlings appear to have smaller, yellow cotyledons and reduced root growth compared to Col-0 and often did not progress past the cotyledon stage on ½ MS media. On auxin media, 35S::IDD2 seedlings look similar to Col-0.
Figure 3.29: Col-0, 35S::IDD2 5-4 and 35S::IDD2 19-1 seedlings grown on 0 and 10 μM IAA for 8 DAG. White arrowheads point to examples of seedlings that have failed to progress past the cotyledon stage. Scale bars represent 1 cm.

3.5.4 35S::IDD2 seeds have altered morphology

Since 35S::IDD2 lines seem to have trouble progressing past the cotyledon stage of growth, Col-0, idd2-3, 35S::IDD1, 35S::IDD2 5-4, 15-1 and 19-1 seeds were examined with a scanning electron microscope to see if there were any differences in seed morphology (see Figure 3.30). 35S::IDD1 seeds were included as it was determined previously that they showed altered seed coat patterning (LeBlanc, 2012). The idd2-3 seeds did not show any visible differences compared to
Col-0. The $35S::IDD2$ lines showed altered seed coats with some cells appearing crumpled.

![Figure 3.30: Col-0, idd2-3, 35S::IDD1, 35S::IDD2 5-4, 15-1 and 19-1 seeds viewed with scanning electron microscope. Scale bars represent 300 μm.]

To investigate whether the altered seed coat in $35S::IDD2$ seeds was due to differences in mucilage deposition, imbibed seeds were stained with ruthenium red stain. $35S::IDD2$ seeds did not appear to have more or less mucilage compared to Col-0 (data not shown).

We also hypothesized that the altered seed coat in $35S::IDD2$ seeds might be due to a difference in seed oil content, so a sedimentation assay was done (adapted from Focks and Benning, 1998). The assay sediments seeds based on their oil content in layers of varying ratios of 1,6-dibromohexane, 1-bromohexane and mineral oil (described in Materials and Methods section). The $35S::IDD2$ overexpression seeds sedimented to the same layer as Col-0 seeds, indicating that there was no difference in seed oil content (data not shown). Siliques of
35S::IDD2 plants were also examined for differences in morphology that might explain the altered seed phenotype, but no differences from Col-0 siliques were found (data not shown).

### 3.6 Leaf Starch, Sucrose and Glucose Concentrations

Several members of the IDD gene family have been implicated in regulating carbon flow and assimilation (Coneva et al., 2007, 2012), thus we wondered whether IDD2 also played a role in controlling carbon flow. Starch, glucose and sucrose were quantified from leaves of idd2 loss-of-function mutants and 35S::IDD2 overexpression lines before and after the floral transition to see whether differences were present or not.

#### 3.6.1 Pre-floral transition leaf starch, sucrose and glucose concentrations do not differ in idd2 mutants

Starch, glucose and sucrose were extracted from three plants each of Col-0, idd2-1, idd2-2 and idd2-3 lines. The plants were grown under standard conditions and above-ground tissue was collected just before the floral transition. Samples were collected when the day began and the lights in the growth chamber turned on (ZT0) and when the night began and the lights turned off sixteen hours later (ZT16). The idd2-1, idd2-2 and idd2-3 did not show any significant differences from Col-0, at the same time point, in pre-floral transition leaf starch concentrations (Figure 3.31).

Glucose was extracted from the same plants as mentioned above. The idd2-1, idd2-2 and idd2-3 did not show any significant differences from Col-0, at the same time point, in pre-floral transition leaf glucose concentrations (Figure 3.32).

Sucrose was extracted from the same plants as mentioned above. The idd2-1, idd2-2 and idd2-3 did not show any significant differences from Col-0, at the same time point, in pre-floral transition leaf sucrose concentrations (Figure 3.33).
Figure 3.31: Starch concentration in Col-0 and *idd2* Arabidopsis leaves harvested prior to the floral transition at dawn (ZT0) and nightfall (ZT16). Aboveground tissue was harvested for each of three biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at that time point.

Figure 3.32: Glucose concentration in Col-0 and *idd2* Arabidopsis leaves harvested prior to the floral transition at dawn (ZT0) and nightfall (ZT16). Aboveground tissue was harvested for each of three biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at that time point.
Figure 3.33: Sucrose concentration in Col-0 and *idd2* Arabidopsis leaves harvested prior to the floral transition at dawn (ZT0) and nightfall (ZT16). Aboveground tissue was harvested for each of three biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at that time point.

### 3.6.2 *idd2-3* mutants sometimes show reduced post-floral transition leaf starch and glucose concentrations

Starch was extracted from six plants each of Col-0, *idd2-1*, *idd2-2* and *idd2-3* lines. The plants were grown under standard conditions and three mature leaves were collected just after the floral transition when inflorescences were just visible (19 days after germination). Samples were collected when the day began and the lights in the growth chamber turned on (ZT0), 8 hours later (ZT8) sixteen hours later when the night began (ZT16).

At ZT0, *idd2-1*, *idd2-2* and *idd2-3* did not show significant differences in starch concentration compared to Col-0. At ZT8, *idd2-3* had a lower leaf starch concentration compared to Col-0 (p=0.0139) and *idd2-1* and *idd2-2* were not significantly different from Col-0. At ZT16, none of the lines were significantly different from Col-0. Data is shown in Figure 3.34.

At ZT0, *idd2-1*, *idd2-2* and *idd2-3* did not show significant differences in glucose concentration compared to Col-0. At ZT8, *idd2-3* had a significantly lower glucose concentration
compared to Col-0 (p=0.0155), while *idd2-1 and *idd2-2 were not significantly different. At ZT16, none of the lines were significantly different from Col-0 (Figure 3.35). At ZT0, ZT8 and ZT16, leaf sucrose concentrations of the mutants were not different from Col-0 (Figure 3.36).

Figure 3.34: Starch concentrations in Col-0 and *idd2 Arabidopsis leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of six biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point. Starch is measured as mg glucose/ g leaf fresh weight.

Figure 3.35: Glucose concentrations in Col-0 and *idd2 Arabidopsis leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of six biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point.
Figure 3.36: Sucrose concentrations in Col-0 and idd2 Arabidopsis leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of six biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point.

3.6.3 idd2 and 35S::IDD2 plants do not show differences in post-floral transition leaf starch, glucose and sucrose concentrations

Since a previous experiment showed a difference in leaf starch and leaf glucose at the ZT8 time point, another experiment to measure these compounds was done to confirm the previous result and to investigate whether 35S::IDD2 lines had altered starch, glucose and sucrose levels. Starch, glucose and sucrose were extracted from ten plants each of Col-0, idd2-3, 35S::IDD2 5-4 and 35S::IDD2 21-4 lines and eight plants from a 35S::IDD2 x idd2-3 cross. The plants were grown under standard conditions and three mature leaves were collected after the floral transition when inflorescences were just visible (19 days after germination). Samples were collected at dawn when the lights in the growth chamber turned on (ZT0), 8 hours later (ZT8) sixteen hours later when the night began (ZT16).

At ZT0, the starch concentrations of all of the lines were not different from Col-0. At ZT8, the 35S::IDD2 x idd2-3 cross had significantly higher starch compared to Col-0 (p=0.00165).
However, the magnitude of this difference in starch concentrations was quite small, so the biological relevance of this difference is questionable. At ZT16, there was no difference in starch concentrations between the lines and Col-0 (Figure 3.37).

At ZT0, ZT8 and ZT16, the glucose concentrations of all of the lines were not different from Col-0 (Figure 3.38). At ZT0, ZT8 and ZT16, the sucrose concentrations of all of the lines were not different from Col-0 (Figure 3.39).

Figure 3.37: Starch concentrations in Col-0, idd2-3, 35S::IDD2 5-4, 35S::IDD2 21-4 and 35S::IDD2 x idd2-3 leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of ten biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point. Starch is measured as mg glucose/ g leaf fresh weight.
Figure 3.38: Glucose concentrations in Col-0, idd2-3, 35S::IDD2 5-4, 35S::IDD2 21-4 and 35S::IDD2 x idd2-3 leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of ten biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point. Glucose is measured as mg glucose/ g leaf fresh weight.

Figure 3.39: Sucrose concentrations in Col-0, idd2-3, 35S::IDD2 5-4, 35S::IDD2 21-4 and 35S::IDD2 x idd2-3 leaves harvested after the floral transition at ZT0, ZT8 and ZT16. Three mature leaves were harvested for each of ten biological replicates and three technical replicates per biological replicate were used for absorbance readings. Error bars indicate standard deviation of the biological replicates and a star indicates a significant difference from Col-0 at the corresponding time point. Sucrose is measured as mg sucrose/ g leaf fresh weight.

In this experiment, idd2-3 mutants did not have reduced leaf starch and glucose concentrations at ZT8, as previously observed, suggesting the difference observed was due to random chance and is not due to loss of IDD2 expression.
3.7 **Flowering Time**

3.7.1 **idd2 mutants do not differ in long day flowering time**

Since the founding member of the *IDD* gene family is a floral regulator in *Zea mays* (Colasanti et al., 1998), we wondered whether IDD2 had a role in flowering time regulation. To investigate this possibility, time to flowering was recorded for *idd2* mutants and wild type plants by counting both the number of leaves to be produced before inflorescence emergence and the number of days from germination until inflorescence emergence. These plants were grown under standard conditions and 16-hour days. Mutants did not differ from wild type plants in days to flowering or leaf number at flowering (data not shown).

3.7.2 **idd2 and 35S::IDD2 mutants do not differ in short day flowering time**

A recent study reported *35S::IDD2* plants exhibiting an early flowering phenotype under short day conditions, both in days to flowering and leaf number at flowering (Fukazawa et al., 2014). This study used only one overexpression line and did not quantitatively test an *idd2* loss-of-function line. To examine flowering time in *idd2* mutants and *35S::IDD2* lines, 16 seeds each of Col-0, *idd2*-2, *idd2*-3, *35S::IDD2* 5-4, 8-6, 9-4, 15-1, 19-1, 21-4, and 22-3 were sown on soil and grown under standard conditions except that they received 10 hours of light and 14 hours of darkness each day. Days to flowering was considered the day that an inflorescence bud appeared and was measured in days after germination (DAG). Rosette leaves were counted at this point as well. Flowering time did not differ from Col-0 for *idd2*-2, *idd2*-3 for any of the *35S::IDD2* lines tested.
Chapter 4: Discussion

4.1 IDD2 is expressed at auxin maxima

Examining \textit{IDD2}:GFP:GUS reporter lines revealed a highly specific and interesting expression pattern corresponding with areas of high auxin concentrations. Staining was observed in the hypocotyl, petioles of all leaves, leaf vasculature, the shoot apex, the root tip, lateral root primordia, the pedicel where it joins the inflorescence and siliques, developing seeds and the funiculus (see Figure 4.3 for a schematic representation). Auxin is produced or transported to each of these tissues to promote division or elongation. For example, in the roots, auxin is transported from the root tip to the two lateral root founder cells of the pericycle to promote divisions required to form the lateral root primordia (Casimiro et al., 2001). This maxima matches the pattern observed in the \textit{IDD2}:GFP:GUS lines, which showed intense staining in the growing root tip and the lateral root primordia as early as 6 DAG before the lateral root primordia broke through the surface of the main root (Figure 3.7). While auxin promotes division in roots, it promotes hypocotyl elongation in the shoot. When seedlings are treated with the auxin transport inhibitor NPA, hypocotyl elongation is severely compromised (Jensen et al., 1998). The \textit{IDD2} reporter lines showed staining in hypocotyls at 3, 6 and 10 DAG, suggesting that \textit{IDD2} expression matches the auxin maximum in the hypocotyl. Just above the hypocotyl, the SAM also has high auxin concentrations, especially in the central zone and primordia (Vernoux et al., 2010). The \textit{IDD2}:GFP:GUS reporter lines showed staining in the shoot apex, although seedlings were too small to determine whether staining localized specifically to the central zone and primordia or not. In leaves, auxin is involved in vasculature development with auxin maxima marking future vein locations (Avsian-Kretchmer et al., 2002; Turner and Sieburth, 2003) and while auxin has not been proven to be involved in petiole growth, \textit{auxin resistance1} mutants do not respond to auxin stimuli.
and have shorter petioles compared to wild type plants, suggesting a role in petiole growth (Lincoln et al., 1990). The \textit{IDD2} reporter lines showed staining in both petioles and leaf vasculature, corresponding with these auxin maxima. Lastly, auxin is involved in post-fertilization fruit growth. Once fruit is mature, valve margins (between valves and replum; see Figure 4.1) require a reduction in auxin levels or response in order for dehiscence and seed dispersal to occur (Sorefan et al., 2009). \textit{IDD2} expression was observed in some areas at the top and bottom of siliques while seeds were developing but signal reduced as development progressed, similar to the way auxin would become reduced in these areas. During seed development, expression was also present in another sique structure called the funiculus, which attaches seeds to the sique septum (Figure 4.1). While this was not discussed in literature, it is logical to suppose that the funiculi would have auxin present while seeds are maturing and then also experience an auxin minimum to allow seeds to separate from the replum. Overall, it is evident that \textit{IDD2} expression patterns correspond with areas of high auxin concentration.

![Immature and mature siliques showing various tissues](image)

**Figure 4.1:** Immature and mature siliques showing various tissues. Created based on Dardick and Callahan, 2014.
4.2 IDD2 expression patterns differ from previous reports

Examination of the IDD2::GFP:GUS reporter lines showed a similar expression pattern to the patterns obtained from microarrays on the Arabidopsis eFP Browser from the BioAnalytical Resource, shown in Figure 4.2 (Winter et al., 2007), but also revealed several key differences (Figure 4.3). The eFP Browser shows highest IDD2 expression in the hypocotyl, petioles of a full-grown stage 7 leaf and the vegetative shoot apex, while rosette leaves, cauline leaves, senescent leaves, cotyledons, roots and the root apex during and after the floral transition are reported to have moderate expression. The IDD2::GFP:GUS reporter lines examined in this study provide a more detailed look at IDD2 expression in different tissues throughout plant development. High expression was observed in hypocotyl, petioles of all leaves and the shoot apex, as previously reported, but expression was also high in leaves, specifically in vasculature, and in roots at the root tip and lateral root primordia. Staining was not observed in senescing leaves, contrary to previous reports, but was observed in floral organs such as the pedicel where it joins the inflorescence and siliqua, developing seeds and the funiculus. These observations were useful in pinpointing an expression pattern to give a more specific idea of where IDD2 acts in the plant. It is important to remember that this expression pattern should be confirmed with a more reliable method, such as mRNA *in situ* hybridization, since creating a reporter construct relies on the assumption that the promoter region is chosen to drive the reporter is the correct promoter length. The expression pattern observed with GUS staining provides a good idea of tissues that might be altered in *idd2* loss-of-function and 35S::IDD2 overexpression lines.
Figure 4.2: Developmental expression heat map for *IDD2* from BioAnalytical Resource (Winter et al., 2007). Yellow indicates low expression, while red indicates high expression.
Figure 4.3: Developmental expression heat map for *IDD2* created from examining *IDD2::GFP::GUS* reporter plants. Darker blue corresponds to darker staining in original experiments.

### 4.3 Tissues with high *IDD2* expression are not phenotypically different in *idd2* loss-of-function mutants

Based on the expression patterns observed with the reporter lines, *idd2* knockout mutants and *35S::IDD2* overexpression lines were tested for phenotypic differences in the areas of high *IDD2* expression. Petioles were examined for differences in length and the angle between leaves but no differences were found. No differences were observed in germination times, root hair
emergence or root growth rates. Since high \textit{IDD2} expression was observed in lateral root primordia, loss-of-function and overexpression mutants were examined for differences in lateral root numbers and lengths, but no consistent differences were observed. Some overexpression lines had less lateral roots compared to Col-0 but the phenotype was not consistent. Overexpression lines also showed delayed postgerminative growth compared to wild-type plants. When grown on $\frac{1}{2}$ MS plates, they would often arrest growth after cotyledons expanded, however, normal growth was observed when seedling were grown on $\frac{1}{2}$ MS media supplemented with 2% sucrose, indicating that providing an alternative carbon source can complement the retarded growth.

4.4 \textbf{IDD2 and IDD1 show similar expression patterns}

\textit{IDD2} and \textit{IDD1} are both members of the \textit{INDETERMINATE DOMAIN} family and make up the ENHYDROUS clade in phylogenetic reconstructions of the family (Colasanti et al., 2006). When \textit{IDD2} and \textit{IDD1} nucleotide sequences are aligned using the EMBOSS Water pairwise sequence alignment tool (www.ebi.ac.uk/Tools/psa/emboss_water/nucleotide.html), they show 59.6\% identity and similarity with 27.7\% gaps, while their protein sequences show 68.1\% identity, 75.8\% similarity and 12.5\% gaps. \textit{IDD1} also shows a similar expression pattern to \textit{IDD2} as evidenced by the GUS assays performed (Figure 4.4 and Figure 4.3). \textit{IDD1} was expressed in cotyledons, root tips and developing seeds similar to \textit{IDD2}. There were some differences in the expression patterns though, as \textit{IDD1} was expressed in inflorescences and flowers much more than \textit{IDD2}. \textit{IDD1} also seemed to have more diffuse expression throughout leaves, whereas \textit{IDD2} was expressed strictly in vasculature and petioles. In addition, \textit{IDD1} did not have expression in lateral root primordia in the same way that \textit{IDD2} did. Nevertheless, expression patterns were generally similar. This expression overlap combined with the relatively similar protein sequences lead to a speculation that \textit{IDD2} and \textit{IDD1} may have similar functions and that one may be able to
complement the functions of the other in loss-of-function mutants. This would explain why *idd2* knockout mutants do not have obvious phenotypes. It is suspected that an *idd1 idd2* double loss-of-function mutant would have more obvious phenotypes, as this was observed in the *idd14 idd15 IDD16i* triple mutant. Each single mutant had a subtle phenotype, but the triple mutant had more obvious deficiencies (Cui et al., 2013). An insertional mutant with a T-DNA insertion within the coding region of *IDD1* is not available, so an *IDD1i* RNA interference knockdown construct was created by a previous graduate student and was transformed into Col-0, *idd2-2* and *idd2-3* plants. Positive transformants were obtained, however, when *IDD1* expression was quantified by qPCR, *IDD1* was still being expressed at wild type levels. Thus, a double mutant could not be made successfully in this project. Fukazawa et al. (2014) state that they have an *idd1 idd2* double mutant (under the name *idd1 gaf1*) but the *idd1* mutant has a T-DNA insertion in its promter and only has partially reduced expression levels. Little information is given as to which tissues expression was tested in as well. The double mutant discussed in Fukazawa et al. (2014) had a late flowering time phenotype and had slightly shorter hypocotyls when grown on GA and the researchers concluded that *IDD1* and *IDD2* are involved in GA signaling. A critical review of this study is presented in the next section.
Figure 4.4: Developmental expression heat map for *IDD1* created from examining *IDD1::GFP:GUS* reporter plants. Darker blue corresponds to darker staining in original experiments.

### 4.5 Comparisons to a recent IDD2 paper suggest inconsistencies

Recently, Fukazawa et al. (2014) published a paper about the association of DELLA proteins and GAI-ASSOCIATED FACTOR1 (GAF1), also known as IDD2, and their role in gibberellic acid homeostasis and signaling. They show that IDD2 associates with GIBBERELLIC ACID INSENSITIVE (GAI) through bimolecular fluorescence complementation (BiFC) in Arabidopsis culture cells. They also show a single *IDD2::GFP:GUS* reporter line showing *IDD2* expression in root tips, shoot apices and petioles, however, the pattern does not match the distinctive expression at auxin maxima pattern that was observed in this study (Figure 3.7). A
possible explanation for this is that Fukazawa et al. (2014) used the region 1.5 kb upstream of the start codon for the promoter in their reporter construct, whereas this study used 1.8 kb upstream of the start codon, which might more accurately capture the entire promoter. In addition, the researchers only presented one reporter line at 3, 6 and 10 DAG, whereas this study used seven lines from independent insertion events and looked at expression throughout development. Fukazawa et al. (2014) also looked at flowering time of an idd2 mutant, which they report to be a transcriptional knockout. The mutant line they are using is the same as the idd2-2 line used in this study, which was shown to have a higher amount of a truncated transcript rather than having very low transcript levels like the idd2-3 line. The researchers show that the idd2-2 line flowers slightly later than Col-0 under short day conditions and that their one 35S∷IDD2 overexpression transgenic plant flowers much earlier than Col-0 (Fukazawa et al., 2014). When this experiment was redone in this study with Col-0, idd2-2, idd2-3 and seven 35S∷IDD2 lines from independent insertion events, none of the lines had a significant difference in flowering time compared to Col-0. Lastly, the paper reported that the 35S∷IDD2 line had longer hypocotyls than Col-0 when grown on 1 and 10 μM GA. When four 35S∷IDD2 lines were grown on 0, 1 and 10 μM GA, no significant differences in hypocotyl length were observed. When seedlings from the same lines were grown on the same media in the dark, one of the 35S∷IDD2 lines had shorter hypocotyls at 1 and 10 μM GA, but this phenotype was not observed in the other three lines, so it has been ignored. Overall, it is suggested that findings from Fukazawa et al. (2014) be re-investigated.

### 4.6 IDD family members may function redundantly

In the IDD gene family, several members are able to self-regulate, as well as regulate other transcription factors. For example, the IDD3 and IDD10 proteins are involved in regulating the transcription factors SCARECROW (SCR) and SHORTROOT (SHR), which are involved in
creating proper root structure (Ogasawara et al., 2011; Welch et al., 2007). IDD3 also shows feedback inhibition in Arabidopsis cell culture experiments. When the IDD10 protein is present, transcriptional activity from the *IDD3* promoter increases but when the IDD3 protein is added, transcriptional activity returns to basal levels (Ogasawara et al., 2011), indicating that IDD3 regulates its own expression. *IDD* family genes are also able to work together to regulate gene expression as shown in the *SHR/SCR* pathway, for example. *SCR* is expressed in cortex/endodermal initial cells (CEI), endodermal cells and the quiescent centre where it helps define endodermal identity by preventing differentiation in adjacent cells (Di Laurenzio et al., 1996). *SHR* is expressed in the stele and then moves outward to the endodermis. IDD3 and IDD10 help control where SCR and SHR move (Welch et al., 2007). *IDD3, IDD10* and *SCR* are all expressed in the endodermis and CEI cells and IDD3, IDD10, SHR and SCR proteins are found together in these cells, even though *SHR* is only expressed in the stele (Ogasawara et al., 2011). The four transcription factors work together to activate *SCR* (Ogasawara et al., 2011), showing an example of *IDD* proteins working together to regulate gene expression. It is possible that IDD2 could work with IDD1 and/or other *IDD* family proteins to perform its regulatory function. This would explain the lack of phenotypes observed in *idd2* loss-of-function mutants; the other transcription factor(s) might be performing their function(s) without IDD2, although perhaps to a lesser degree (Figure 4.5).

If IDD2 is working in concert with other transcription factors, this would mean that a double or triple mutant would be needed to show a phenotype. This is common in transcription factor families (Riechmann and Ratcliffe, 2000) and has been previously observed in the *IDD* family in several cases. The *idd3* mutant and even the *idd3* *idd8* double mutant show no obvious root phenotypes, but reducing *IDD3* expression in an *idd10* mutant background reveals that IDD10
and IDD3 might compete for the same binding site in the SHR-SCR complex. IDD3 seems to redundantly facilitate the role the SHR-SCR complex plays in promoting asymmetric cell division in the root (Welch et al., 2007). Thus, an idd3 single mutant does not show a phenotype since SHR and SCR can function without IDD3. In another example mentioned earlier, single loss-of-function mutants idd14 and idd15 show some phenotypic differences, but these phenotypes are greatly exacerbated in the idd14 idd15 IDD16i triple mutant (Cui et al., 2013). The triple mutant has enlarged floral organs, reduced fertility, decreased auxin content and decreased auxin transport ability but the single mutants do not show any of these phenotypes. Through phenotypic analysis of the triple mutants, Cui et al. (2013) concluded that IDD14, IDD15 and IDD16 work together to regulate auxin biosynthesis and transport. It appears that when one of the proteins in this trio is missing, the other two are able to carry out most of the functions of the missing protein, since the single loss-of-function mutants have subtle or no phenotypic differences.

Overall, the aforementioned findings support the theory that IDD2 functions in concert with one or more other genes, possibly from the IDD family. Future research should include creating double loss-of-function mutants with other IDD family genes. This was attempted by crossing the idd2-2 and idd2-3 mutants with an IDD1i line, however the IDD1i line did not have reduced IDD1 expression, so this was unsuccessful. Further attempts to create an IDD1i idd2 double mutant should be pursued.

4.7 Is IDD2 involved in auxin sensing or regulating seed oil hydrolysis?

Examination of IDD2 expression patterns using IDD2::GFP::GUS reporter lines and determining that IDD2 is expressed at auxin maxima raised the possibility that the IDD2 protein might be involved in synthesizing or sensing auxin or in regulating the auxin response.
To check whether auxin could be regulating *IDD2* or if the *IDD2* protein could be regulating *AUXIN RESPONSE FACTOR* family genes (*ARF*), promoter analysis was done with Athamap (Steffens et al., 2005) to look for potential transcription factor targets. The *IDD2* promoter contained 2 auxin response elements (with the sequence TGTCTC) that were 458 bp and 354 bp upstream of the start codon, suggesting that it is possible for the presence of auxin to regulate *IDD2*. However, when *IDD2::GFP::GUS* seedlings were grown on media with 1 μM IAA, the staining pattern did not change compared to seedlings grown on ½ MS media, which suggests that *IDD2* expression is not directly regulated by auxin. It is still possible that auxin might change the shape or binding ability of the *IDD2* protein so that it might have a role in sensing auxin, however, further experiments are warranted to investigate this possibility. The *IDD* proteins are hypothesized to bind to the 11-bp (TTTGTCGTATT) consensus sequence to which the founding member of the family, *ID1*, binds (Kozaki et al., 2004). Of the 23 *ARF* genes in Arabidopsis, four contained between one and four *ID1* binding sites (summarized in Table 4.1). It is possible that *IDD2* targets auxin response genes and regulate the auxin response pathways by binding to these sites or that *IDD2* forms a complex with other transcription factors that bind auxin response genes.

Table 4.1: Summary of *ID1* binding sites found in *AUXIN RESPONSE FACTOR* family genes using Athamap (Steffens et al., 2005).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Atg #</th>
<th>ID1-binding site(s)</th>
<th>Relative distance(s) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF5</td>
<td>At1g19850</td>
<td>1</td>
<td>-37</td>
</tr>
<tr>
<td>ARF9</td>
<td>At4g23980</td>
<td>3</td>
<td>-1395, -1054, -19</td>
</tr>
<tr>
<td>ARF18</td>
<td>At3g61830</td>
<td>4</td>
<td>-1389, -1287, -915, -54</td>
</tr>
<tr>
<td>ARF19</td>
<td>At1g19220</td>
<td>2</td>
<td>-509, -862</td>
</tr>
</tbody>
</table>

Several experimental observations also support the theory that *IDD2* might be involved in sensing auxin and regulating auxin response pathways (proposed model in Figure 4.5). When
IDD2::GFP:GUS seedlings were grown on 1 µM auxin (Figure 3.9), the expression pattern of IDD2 did not change; IDD2 was still expressed in the shoot apex, vasculature, hypocotyl, lateral root primordia and root tip. If IDD2 were being directly regulated by the presence of auxin, its expression would likely change when exogenous auxin was added. In addition, 35S::IDD2 seedlings resemble Col-0 seedlings when grown on 10 µM auxin; they both have yellow cotyledons and reduced root growth (Figure 3.29). However, on ½ MS media without auxin, 35S::IDD2 seedlings still had yellow leaves and reduced root growth and looked very similar to Col-0 seedlings grown on auxin, suggesting that overexpression seedlings could be sensing auxin in the absence of auxin. The model proposed in Figure 4.5 suggests that IDD2 is part of a complex of other transcription factors that are involved in sensing auxin and regulating auxin response genes. Perhaps high levels of auxin enhance the ability of IDD2 to bind with the transcription factor complex and turn on genes associated with the auxin response, but when auxin levels are low, IDD2 could be outcompeted by an alternative transcription factor and auxin response genes would be turned off. In 35S::IDD2 seedlings exposed to high auxin levels, IDD2 would bind with the transcription factor complex as in wild-type seedlings, and the auxin response phenotype would be shown. However, when low levels of auxin are present, perhaps IDD2 is still able to outcompete the alternate transcription factor simply because there is such a high amount of IDD2 protein present and the seedlings exhibit an auxin response phenotype even though the auxin level is low. In idd2 seedlings where IDD2 is not present, it is possible that a transcription factor in the IDD family or otherwise binds the complex instead of IDD2 when auxin levels are high, leading to an auxin response and is outcompeted when auxin levels are low, leading to normal growth. To validate this model, the expression of auxin regulated genes should be quantified in 35S::IDD2 plants using qPCR, when plants are grown with and without exogenous auxin. Chromatin
immunoprecipitation could also be done to see whether the IDD2 protein binds auxin regulated genes.

The 35S::IDD2 seeds have altered seed coat patterning and appear crumpled when viewed under the SEM (Figure 3.30). It is possible that the embryo inside is affected by this difference and this is why so many of the 35S::IDD2 seedlings have difficulty progressing past the cotyledon stage. If IDD2 is involved in auxin sensing, too much or mis-expressed IDD2 might cause the plants to react as if there is a lot of auxin present when it is not actually there (Figure 4.5). Interestingly, 35S::IDD2 seedlings do not show this difficulty growing past the two cotyledon stage when grown on ½ MS media supplemented with 2% sucrose. Perhaps, even though the 35S::IDD2 seedlings are sensing excess auxin, which would normally stunt growth, the extra energy provided by the sucrose allows the roots to grow to normal lengths anyway. Recent research has led to speculation about auxin and sugars both being involved in signaling for various processes, such as bud burst (Van den Ende, 2014), so the sugars might overcome the auxin sensing happening in 35S::IDD2 seedlings, if sugar signaling is upstream of auxin signaling. Additionally, since reduced auxin is required in the valve margins for seed dehiscence (Sorefan et al., 2009), perhaps the 35S::IDD2 seedlings are still sensing auxin when there is an auxin reduction and causing a difference in the amount of time the seeds stay in the silique, which might lead to the crumpled seed phenotype. Future research should include sectioning 35S::IDD2 seeds and looking for differences in morphology, specifically in the outer and inner integument and the endosperm.
Figure 4.5: Proposed model for the role of IDD2 in auxin sensing and regulating auxin responses.
An alternate explanation for the difficulty that many 35S::IDD2 overexpression seedlings experience in growing past the cotyledon stage is a reduction in β-oxidation or seed oil hydrolysis activity, which could be linked to auxin sensing in an unknown way. Loss-of-function mutants for SUGAR-DEPENDENT genes, which encode patatin-like TAG lipases (Eastmond, 2006), show a very similar postgerminative growth phenotype to 35S::IDD2 seedlings. The sdp1 loss-of-function mutants are able to germinate properly but growth is severely delayed. At five days after germination, sdp1 seedlings have small, pale cotyledons and short roots compared to Col-0 seedlings that have large, green cotyledons and long roots (Figure 4.6). When sdp1 seedlings are grown on media with 1 % sucrose, the mutants resemble wild type seedlings (Eastmond, 2006). The growth behaviour of sdp1 seedlings is very similar to that of 35S::IDD2 seedlings, which also show retarded growth that is rescued by providing sucrose as an alternate carbon source, raising the possibility that 35S::IDD2 plants could have a reduced ability to hydrolyze seed oil upon germination. In order to confirm a reduction in oil hydrolysis, 35S::IDD2 seedlings would need to be examined five days after germination when most oil bodies should be degraded in wild type plants (Quettier and Eastmond, 2009). Since this has not been done, the following model is largely speculative and requires experimental validation.
Figure 4.6: a) Col-0 and sdp1 loss-of-function mutant seedlings at 5 DAG and b) Col-0 and 35S::IDD2 seedlings at 6 DAG grown on media with and without sucrose. Note the similarities between sdp1 and 35S::IDD2 seedlings on media with and without sucrose. In this figure, a) is from Eastmond, 2006 and b) is from this study.

A model for a role IDD2 might play in regulating postgerminative β-oxidation or oil hydrolysis is proposed in Figure 4.7. In this model, we hypothesize that the presence of sucrose enhances the ability of IDD2 to bind a transcription factor complex that regulates genes involved in β-oxidation or oil hydrolysis. In wild type seedlings grown on media without sucrose, IDD2 might be outcompeted in binding the complex by an alternate transcription factor, which would cause the complex to activate hydrolysis genes, leading to normal seedling growth. When wild type plants are treated with sucrose, either IDD2 or the alternate transcription factor could bind the complex, since oil mobilization would not be needed; seedlings would use the exogenous sucrose as an alternative energy source. In 35S::IDD2 overexpression seedlings, whether treated with sucrose or not, IDD2 would outcompete the alternative transcription factor simply because there is a large amount of IDD2 present. Expression of genes involved in β-oxidation or oil hydrolysis would be reduced and seedlings grown without sucrose would resemble sdp1 mutants and have severely delayed growth. Seedlings grown with sucrose would use the sucrose as an energy source.
and grow normally. In *idd2* loss-of-function seedlings, the alternative transcription factor would bind the complex in the presence or absence of sucrose and hydrolysis genes would be expressed, leading to normal seedling growth. Expression of genes important in postgerminative oil hydrolysis should be quantified with qPCR in Col-0, *idd2* and *35S::IDD2* seedlings to help validate this hypothesis. Chromatin immunoprecipitation could also be done to see if the IDD2 protein binds genes involved in seed oil hydrolysis. As there are many possible causes for difficulty in postgerminative growth, such as nutrient deficiency, chloroplast defects and differences in seed protein or starch levels, it would be important to consider these alternate explanations in future studies.
Figure 4.7: Proposed model for IDD2 in regulating the expression of seed oil lipase genes.
4.8 Conclusions

During this project, many discoveries about IDD2 were made. Firstly, IDD2 is expressed at auxin maxima, including the shoot apex, root tip, leaf vasculature, lateral root primordia and nodes in the stem. The idd2-3 T-DNA insertion transgenic plants are transcriptional loss-of-function mutants for IDD2, as confirmed by qPCR. Despite their lack of IDD2 expression, idd2-3 transgenic plants do not show any obvious phenotypic differences from Col-0 plants. No phenotypic differences were observed between idd2-3 and Col-0 in the following areas: germination rate or timing; root growth on various concentrations of sucrose, glucose, sorbitol, auxin, cytokinin, gibberellic acid or abscisic acid; number of lateral roots; length of the longest lateral root; petiole length; leaf blade length; leaf starch, sucrose and glucose concentrations; and flowering time in long and short day conditions. It is suspected that this lack of obvious phenotypic differences is because IDD2 functions redundantly, as is common in transcription factor families (Riechmann and Ratcliffe, 2000). Lastly, 35S::IDD2 overexpression transgenic plants experience a constitutive auxin response and experience difficulty in postgerminative growth, however, this difficulty is rescued by the addition of exogenous sucrose. Also, 35S::IDD2 seeds have crumpled seed coats, however, the reason for this has not been identified.

4.9 Perspectives

While idd2 mutants have been thoroughly examined and models for IDD2 function have been suggested, characterization of IDD2 is far from complete. The IDD2::GFP:GUS reporter lines were made and IDD2 expression patterns were examined throughout the life cycle of the plant, but further work could focus on quantifying IDD2 expression in various tissues with qPCR or using mRNA in situ hybridization to verify the presence of IDD2 mRNA in the tissues stained in the GUS assay. In addition to the reporter lines, 35S::IDD2 lines were also made and several
aspects of growth and development have been examined under various conditions. Future characterization of these 35S::IDD2 plants could include dissecting seeds to check for morphological differences and quantifying starch, sugars and oil in seeds to try to explain the altered seed coat phenotype observed in this study. The activity of various seed oil lipases, such as SUGAR-DEPENDENT1 should be examined to determine whether the 35S::IDD2 seedlings have a reduction in oil hydrolysis ability. Since the model proposed in this study suggests that IDD2 works in concert with another transcription factor, double mutants with other transcription factors of interest should be made to see if any obvious phenotypes present themselves. IDD1 is an obvious choice, but other possible choices could include various auxin response factor (ARF) genes, which might provide insight into the possible auxin sensing function of IDD2.

In Arabidopsis, it is common for the members of a transcription factor family to function redundantly (Riechmann and Ratcliffe, 2000), however, we might ask ourselves why are multiple transcription factors required to perform the same function? Why has IDD2 remained in the Arabidopsis genome when loss-of-function idd2 mutants do not show any phenotypic differences or deficiencies? There are several possible explanations for this phenomenon. Firstly, perhaps IDD2 and a redundant transcription factor have not evolved separate functions yet and are actually functioning redundantly. Second, maybe the loss of IDD2 function creates a very subtle phenotype that we have been unable to observe in idd2 mutants so far. Third, it is possible that IDD2 functions in response to a particular environmental condition that we have not yet grown the idd2 mutants under in the laboratory, but that Arabidopsis might encounter in its natural habitat, which would justify the presence of IDD2 in the genome. Further investigation into the IDD family and other transcription factor families will help to explore these possibilities.
Appendix

References


**PCR Primers**

Table 0.1: Names, sequences and annealing temperatures for primers used in polymerase chain reactions (PCR).

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Annealing Temperature (°C)</th>
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<tr>
<td>AtIDD2OE-F</td>
<td>GGGGACAAGTTTGTACAAAAAGCAGGCTATGCCGGATTTTAGATAACT</td>
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<tr>
<td>AtIDD2OE-R</td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGGTATTTATGATTTTCTCTCTACTAATGTC TTTCC</td>
<td>55</td>
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<tr>
<td>AtIDD1pro-F</td>
<td>GGGGACAACCTTTGTATAGAAAAAAGTTGAGGATTTTCGTATGATAAAATAA</td>
<td>48</td>
</tr>
<tr>
<td>AtIDD1pro-R</td>
<td>GGGACTGCTTTTTGTACAAACTCGTGTTTCAAGATACATAATTCAC</td>
<td>48</td>
</tr>
<tr>
<td>AtIDD1proF</td>
<td>GGGGACAAGTTTGCAATGTGTAAGGA</td>
<td>48</td>
</tr>
<tr>
<td>AtIDD1proR</td>
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<td>48</td>
</tr>
<tr>
<td>AtIDD2-For1</td>
<td>GGATAGTTTTATAACGCAATACAGGTTAAGATTACACAGGCTACTACCTACAAAAGAAGGGG</td>
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<tr>
<td>AtIDD2-Rev2</td>
<td>TCTCACAACCTACCTATGGAAC</td>
<td>49</td>
</tr>
<tr>
<td>AtIDD2proB1-F</td>
<td>GGGGACAAGTTTGTACAAAAAGCAGGCTACCTACCTACAAAAGAAGATTACACAGGCTACTAC</td>
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<tr>
<td>AtIDD2proB2-R</td>
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<tr>
<td>AtIDD2pro SR</td>
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</tr>
<tr>
<td>AtIDD2pro SF</td>
<td>CAACAAATAACACGATCTACAT</td>
<td>42</td>
</tr>
<tr>
<td>Salk/Lba1</td>
<td>TGGTTCACGTAGTTGGGCTACCTACATTCGACCATATGACAGGCCATCCCTAAAGATCTACAT</td>
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<tr>
<td>LB3</td>
<td>TAGCATCTGAAATTTCAAAACATCTCGATACAC</td>
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<td>M13F</td>
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<td>BaRR</td>
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qPCR Primers

Table 0.2: Names, sequences, optimal dilutions and expected amplicon sizes for primers used in quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Optimal Dilution</th>
<th>gDNA/mRNA size</th>
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<tbody>
<tr>
<td>qrtATIDD1F</td>
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<td></td>
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<tr>
<td>qrtATIDD1R</td>
<td>AAAAGGCTCTATGCGTTATAAAGC</td>
<td>1/16</td>
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<tr>
<td>AtIDD2E2F</td>
<td>ACCCTTCACGTGCTTTAGGA</td>
<td>1/16</td>
<td>328/245</td>
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<tr>
<td>AtIDD2E3R</td>
<td>TCTCGCATTTTCTTCAGCCA</td>
<td>1/16</td>
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<tr>
<td>AtIDD2E4F</td>
<td>AGCAGCTTCTTCAGGAGGT</td>
<td>1/16</td>
<td>224/224</td>
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<tr>
<td>AtIDD2E4R</td>
<td>TTCCGTTACAAACCGCTCTA</td>
<td>1/16</td>
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<td>Actin7F</td>
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<td>1/16</td>
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<tr>
<td>Actin7R</td>
<td>TGAGGGATGCAAGGATTGATC</td>
<td>1/16</td>
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Mutant Line Information

Table 0.3: Gene number, names and insert information for T-DNA insertion mutants. Location in base pairs is given as the base pair where the insert starts counting from the beginning of the AtIDD2 open reading frame.

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<tr>
<th>Mutant Line</th>
<th>Gene #</th>
<th>Official Name</th>
<th>SALK/SAIL #</th>
<th>Insert Location</th>
<th>Confirmed?</th>
<th>Location (bp)</th>
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<tr>
<td>2S3</td>
<td>At3g50700</td>
<td><em>idd2-1</em></td>
<td>SALK 059 657</td>
<td>3’ end of exon 4</td>
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<td>2420</td>
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<tr>
<td>2S14</td>
<td>At3g50700</td>
<td><em>idd2-2</em></td>
<td>SALK 070 916</td>
<td>Intron 3</td>
<td>yes</td>
<td>1690</td>
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<tr>
<td>2G11</td>
<td>At3g50700</td>
<td><em>idd2-3</em></td>
<td>CS873095/</td>
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<td>yes</td>
<td>2375</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>SAIL 315G02</td>
<td>exon 4</td>
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