CACTIN is an Essential Nuclear Protein in Arabidopsis and may be Associated with the Eukaryotic Spliceosome

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Chapter 1: Eukaryotic Splicing: Focus on Plants

Abstract

Introns are 'commercial breaks' within genomic DNA that interrupt genes. Like removing outtakes from film strips, introns are spliced out of mRNA so that contiguous, complete proteins can be translated from it. The majority of eukaryotic genes contain introns. The spliceosome is a macromolecular nuclear complex that is responsible for identifying and removing the introns from between the coding exons. Although one to two billion years have elapsed since the last common eukaryotic ancestor lived, many aspects of splicing are the same between plants and animals. Therefore, it is believed that the genome of the eukaryotic common ancestor contained introns that were spliced out by a spliceosome that was very similar to today's eukaryotic spliceosome. Splicing is a ubiquitous aspect of all gene expression, but it is also a point of regulation for many alternatively spliced genes. The prevalence and consequences of alternative splicing are now becoming clear due to new research tools like transcriptome sequencing. Previously, plants were thought to use less alternative splicing than animals, but new findings show this is also an expansive form of gene regulation in flora. In this chapter we introduce the eukaryotic spliceosome with a special focus on plants.

Why do eukaryotic genes contain introns?

Eukaryotic introns are likely derived from bacterial group-II-like introns

Most eukaryotic genes are interrupted by non-coding sequences, called introns, which are spliced out of transcripts by a large complex called the spliceosome. Most of

the research into mRNA splicing has been conducted in animal cells. However, as we will discuss in this section, the common ancestor of plants and animals already had a sophisticated spliceosome and likely had an intron-dense genome. Thus many of the findings about this ancient complex translate well between distant eukaryotes.

The modern eukaryotic spliceosome is an essential nuclear complex consisting of approximately 300 proteins and five catalytic RNA molecules (Jurica and Moore, 2003; Valadkhan, 2007). All eukaryotic genomes contain introns, ranging from 5% of genes in yeast to 93% of genes in humans (Fedorova and Fedorov, 2005; Narsai et al., 2007). Each of these introns require the spliceosome in order to be removed from transcripts so that mRNAs can be translated into full-length proteins and noncoding RNAs can contain the proper sequence. Within this context, the question of intron-origin versus spliceosome-origin presents a chicken-and-egg conundrum. A significant clue comes from self-sufficient bacterial self-splicing group II introns. Some prokaryotic genes encode catalytically active RNAs and contain an intron that can splice itself out of the larger mRNA without a spliceosome. In these bacterial genes, this intron goes on to encode a reverse transcriptase protein (Martínez-Abarca and Toro, 2000). The exon/intron junction sequence and the chemistry of the excision are very similar to eukaryotic intron splicing, which is strongly suggestive of shared origins. Bacteria also contain another type of self-splicing intron (group I), but in contrast to group II introns, the exon-junction sequences and chemistry don't resemble eukaryotic introns.

The modern eukaryotic nucleus is derived from an archaea-like cell, and modern archaea do not contain introns (Lambowitz and Zimmerly, 2011). Approximately 25% of prokaryotes contain group II introns, and mitochondria and plastids are derived from this group. Indeed, some genes in the genome of modern mitochondria and plastids (such as those in Arabidopsis) contain group II introns. Thus, the first introns in the archaea-derived nuclear genome may actually be derived from mitochondria via gene-transfer (Rogozin et al., 2012). These ancestors to eukaryotic introns were able to autocatalytically self-excise, like modern group II introns. Likely, over time other RNAs and proteins found a role in facilitating this process until eventually the accessory RNAs and proteins were required and became the first spliceosome (Rogozin et al., 2012).

Not only did the eukaryotic common ancestor have a sophisticated spliceosome complex, but it also had a secondary minor spliceosome complex as well. In both plants and animals, less than half of one percent of genes contain non-conventional introns that are spliced by a different protein and RNA complex (Levine and Durbin, 2001; Zhu, 2003). This minor spliceosome (also called the U12 spliceosome), is like the doppelganger of the major spliceosome, and they share structural and catalytic concepts. However, most of the core constituents are different RNAs and proteins from the major spliceosome and the sequences that define an U12 spliceosome intron are different from regular introns. The rationale behind eukaryotes maintaining a separate spliceosome that is used on so few genes is unclear, but it is important in plants and animals because mutants in its components are lethal (Otake et al., 2002; König et al., 2007; Kim et al., 2010). The rest of this chapter will focus on the major spliceosome.

Introns are very widespread in most eukaryotic genomes

Even bacterial group II introns are only found in certain RNA-coding genes which function as reverse transcriptase-dependent mobile genetic elements (Martínez-Abarca and Toro, 2000). Bacterial introns are not found in conventional genes. This contrasts to eukaryotic cells, where introns are widespread and found in the majority of genes. There are multiple possible explanations for this expansion of intron prevalence in eukaryotes.

First, introns may facilitate successful mutational gene shuffling. Genome rearrangements can swap and reorder domains within a protein-coding gene, thus creating new proteins. Without introns, rearrangements that successfully create new proteins require that the donor coding sequences be placed next to each other in frame. On the other hand, introns tend to occur between codons (phase 0) and not within them (Rogozin et al., 2012). Therefore, if the donor domains are located on exons with flanking intron sequence, they need only be placed generally near each other to frequently make a successful mRNA. However, prokaryotes show signatures of abundant gene shuffling despite lacking widespread introns, and the junctions between eukaryotic shuffling events rarely coincide with intron/exon boundaries (Conant and Wagner, 2005).

A second explanation for the abundance of introns in eukaryotic cells is that splicing allows for increased complexity. As we will discuss in more detail later, some genes can be spliced into multiple forms resulting in different proteins that may include

alternative domain organization or truncations. The ability to produce different forms of the same gene may help cells respond to changes in the environment or to differentiate into specialized cell types. For example, Drosophila differentiate into two different sexes based on chromosome-number-determined differential splicing of the *SEX-LEATHAL* gene (Bell et al., 1988). One spliceoform of this gene encodes a protein that causes the cell to take on female characteristics and the other spliceoform results in maleness (Bell et al., 1988). Perhaps early eukaryotes benefited from the plasticity and regulation possible with having abundant introns. Although the prevalence of introns within the genomes of modern eukaryotes is highly variable, in general single-celled eukaryotes have fewer introns per gene than multicellular organisms (Rogozin et al., 2012).

On the other hand, the eukaryotic common ancestor may have already accumulated a high density of introns before it became multicellular. This is supported by the observation that 25%-30% of intron positions in conserved genes are shared between plants and animals (Fedorov et al., 2002; Rogozin et al., 2003). This type of data was used in computer modeling programs to predict that the eukaryotic common ancestor likely had similar intron density as modern plants and animals (within one order of magnitude) (Rogozin et al., 2003, 2012). Although the lifestyle and biology of the eukaryotic common ancestor is hard to envision, it is often thought of as a single-celled organism. Perhaps it still required the adaptability that making multiple proteins from the same gene allows, and thus benefitted from numerous alternatively spliced genes.

The third possible reason for the high prevalence of introns in eukaryotes is that introns allow for higher levels of gene expression (Carmel et al., 2007; Niu and Yang, 2011). Splicing takes time, and thus the observation that introns increase gene expression is unlikely an indirect effect (Alexander et al., 2010). Because splicing is already a ubiquitous part of gene expression in eukaryotes, it has become coupled to other processes like transcription, mRNA export to the cytoplasm, and mRNA stability (Narsai et al., 2007; Alexander et al., 2010). The coupling of splicing to these other processes is like an express bus route, helping mRNAs to be translated faster. Although this might be a reason for modern genes to retain introns, introns probably did not increase gene expression when they were new and rare.

Finally, perhaps the best supported of the four hypotheses presented here for the abundance of introns in modern eukaryotic genomes is neutral evolution (Lukeš et al., 2011). Once a spliceosome has evolved to help splice out a handful of introns, there may not be selection against obtaining many more introns. This process of neutral selection for ever-building complexity is termed constructive neutral evolution and can act as a ratchet to increase the number of parts or steps in a process.

Although all eukaryotes contain introns, the intron density has decreased in some eukaryotes, such as the branch of fungi that includes *Saccharomyces cerevisiae*, in which less than 5% of genes contain introns (Narsai et al., 2007; Rogozin et al., 2012). This is a derived trait due to the loss of introns, as reflected by the intron density and position within conserved genes of organisms in the kingdoms on either side of fungi

within the eukaryotic family tree. There are other fungi groups with similar or even higher intron density than animals (Csuros et al., 2011). Also, fungi are closer relatives to animals than plants are, and yet both animals and plants have high intron density (80% of Arabidopsis genes and 93% of human genes contain at least one intron (Fedorova and Fedorov, 2005; Juneau et al., 2007)). Introns can be lost if an mRNA is mistakenly reverse-transcribed into cDNA and then used in homologous recombination with the genomic gene (Rogozin et al., 2012). However, the advantages that *S. cerevisiae* and its relatives experienced by losing introns are unclear. Yeast have also lost their U12 minor spliceosome altogether. The result is that plant and animal spliceosomes share more in common with each other than they do with well-studied yeast species. Yet, the core splicing mechanism is the same in all eukaryotes, and arguably the stripped-down spliceosome of *S. cerevisiae* has aided in investigating the basics of splicing.

Recent immigrants to the nuclear genome still show signs of their origins through intron content

The defining event in the formation of photosynthetic eukaryotes was the engulfment and endosymbiosis of a cyanobacterium in the common ancestor of Archaeplastida. Like the mitochondria that these cells already contained, the cyanobacteria had their own genomes, which did not contain introns in protein coding genes. Also, like mitochondria, there has been massive unidirectional movement of these genes into the nuclear genome: 18% of Arabidopsis genes are plastid-derived (Basu et al., 2008). When protein-coding genes first arrived in the nucleus, they would

have contained no introns, but over time most of them have obtained introns through mutation. Yet, on average, there are still slightly fewer introns in plastid-derived genes than the rest of the genome (Basu et al., 2008). The gain (and loss) of introns is an on-going process and the 'newer' plastid-derived genes have still not reached the same equilibrium as the rest of the genome. On average, plastid-derived genes are also shorter in length than genes in the rest of the genome, probably due to the generally smaller size of the ancestral prokaryotic genes. Fewer introns in plastid-derived genes contrasts with intron content in mitochondria-derived genes, which are the same as the rest of the nuclear genome (Ahmadinejad et al., 2010).

What defines an intron?

Eukaryotic introns are defined by common sequence features

In the course of the reactions that splice out an intron from an mRNA, an adenine in the interior of the intron (the branch point) attacks, severs, and then bonds to the 5' end of the intron, making a lasso or lariat shape. In a second step, the 3' end of the intron is severed, and the two exons are joined together (Figure 1- 1). The positions of the ends of the intron and the three-way branch point are partly determined by key sequence features of the intron. Since the eukaryotic common ancestor, the sequences that define introns have diverged slightly, but four core features have remained. 1) The 5' end of the intron (donor site) begins with GU. 2) The 3' end of the intron (acceptor site) concludes with AG. 3) There is a stretch of pyrimidine nucleotides (U and/or C) in the intron, usually 5-60nt upstream of the 3' end. 4) There is a branch point consensus

sequence in the intron 11-50 nt upstream from the 3' end of the intron that contains an adenine (A) that will serve as the 3-way intersection of the lariat structure.

These features are required of eukaryotic introns but are not sufficient. There are other base positions that show subtle consensus sequences. For example, the last nucleotide of the exon is usually a G, but it can be any base. Also, in some genes, there are sequences in the middle of introns that match the junction consensus sequence better than the actual junctions that the spliceosome uses (Senapathy et al., 1990). Additional sequence elements (exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers, and intronic splicing silencers) that inform the animal spliceosome have been found, and they are located amidst both introns and exons (Zhang et al., 2005). How these elements control splicing is being researched, and whether plants have similar features is yet to be determined.

There are differences between plant and animal introns

Despite the similarities in intron junction sequence discussed above, differences have accumulated between plant and animal introns. Animal introns have a polypyrimidine track located between the branch point and the 3' end of the intron. In plants, this stretch of nucleotides consists of nearly all uridines, and although it often is present in between the branch point and the acceptor site, it can also occur upstream of the branch point (Lorković et al., 2000; Reddy, 2007). There is also a generally higher uracil (U) and adenine (A) content in plant introns compared to animal introns. The branch point of introns is always an adenine, but the context around it is even more lax

in plants than in animals (Brown et al., 1996; Tolstrup et al., 1997). The 1-2 billion years of separation between the genes of plants and animals have resulted in plant introns not being very efficiently spliced out in animal cells, and animal introns barely being spliced in plant cells, although this varies from gene to gene (Van Santen and Spritz, 1987).

The Macromolecular Spliceosome

The major spliceosome is both a ribozyme and a protein enzyme

The spliceosome is a megadalton nuclear complex of both proteins and enzymatic RNAs. Small nuclear RNAs (snRNAs or UsnRNAs) are the RNA component and are required for catalysis of splicing (Valadkhan, 2007). The sequence and secondary structure of spliceosomal RNAs are conserved between plants and animals (Lorković et al., 2000). The major spliceosome (also called U2 spliceosome) of eukaryotes uses five RNAs: U1, U2, U4, U5, and U6. They are named for being **U**racil-rich. The minor U12 spliceosome also has five UsnRNAs (U11, U12, U4atac, U5, U6atac); four of them are different from the U2 major spliceosome. The UsnRNAs bind to a large network of proteins in order to recognize and splice mRNAs. Although most of the work on UsnRNAs has been done in yeast or mammalian cell culture, the core mechanism is likely the same for all eukaryotes.

The spliceosome core assembles in the cytoplasm

In animal cells, four out of five major UsnRNAs (U1, U2, U4, and U5) are exported to the cytoplasm where they fold and bind to core structural proteins, thus forming individual small nuclear RNA particles (snRNPs). UsnRNAs bind to some

shared proteins, such as the Sm family of proteins, although they also have their own unique binding partners. After these protein/RNA complexes have formed, they are imported back into the nucleus (Fischer et al., 1993). It is not clear what advantage there is in exporting the UsnRNAs since they need to be re-imported into the nucleus (Köhler and Hurt, 2007). Recent experiments have begun to investigate whether or not this cytoplasm-nuclear shuttling of UsnRNAs occurs in plants by looking for core proteins like Sm family member B (SmB) in cellular fractions, and indeed some can be found in cytoplasmic fractions of Arabidopsis protoplast cells (Lorković and Barta, 2008). The amount of spliceosomal proteins in the cytoplasm is very low and cannot be seen by fluorescent protein-tagged microscopy (Dino Rockel and Von Mikecz, 2002). Some of the proteins involved in UsnRNA export in animal cells are conserved in plants and yeast, such as multifunctional cap-binding complex/exportin1 (Izaurralde et al., 2002). However, other components, such as phosphorylated adapter RNA export (PHAX) do not have clear orthologs in plant (Ohno et al., 2000).

Smith proteins form a scaffold that assists UsnRNAs in adopting their functional conformations

The UsnRNAs bind with many proteins in the cytoplasm before re-entering the nucleus. Important among these are the Sm and Sm-like family of proteins. Sm proteins are named for a lupus patient, Stephanie Smith, who generated antibodies against a family of spliceosomal proteins, although their role in splicing was not understood at that time (Tan and Kunkel, 1966). Sm proteins bind the UsnRNAs through their numerous uracil residues, and like UsnRNAs, they are well conserved

between plants and animals. SmB, for example, shares 60% similarity between humans and Arabidopsis. The Sm proteins form a hetero heptamer consisting of SmB, SmD1, SmD2, SmD3, SmE, SmF, and SmG. The heptamer acts as a scaffold on which the UsnRNAs can form the 3D shapes required for catalysis. For all subsequent steps of splicing, each UsnRNA remains bound to these core proteins. Sm proteins are one of the few protein families that are found in both the major and minor spliceosomes. In animal cells, Survival of Motor Neurons and other Gemins (members of the components of gems family, named for their subnuclear localization) proteins mediate the interactions between UsnRNAs and Sm proteins (Liu et al., 1997). However, Arabidopsis does not have clear orthologs of these proteins.

Arginine- and serine-rich proteins are key in the spliceosome

Once back in the nucleus, the UsnRNAs and associated proteins bind to yet more players. Many of these spliceosomal proteins contain domains that are very rich in arginine (R) and serine (S) (RS domains). Proteins that have both an RS domain and an RNA recognition motif, which can bind RNA, are in a class of proteins called SR proteins (for serine/arginine). Please note that the serine/arginine-rich domain is called an RS domain, but the class of proteins containing this domain as well as fulfilling other criteria is called SR proteins. Some research groups apply more criteria for defining SR proteins including the order of the domains, binding to a specific monoclonal antibody, and their ability to rescue splicing factor-depleted HeLa cells (Koroleva et al., 2009; Reddy and Shad Ali, 2011). However, some of these criteria do not make sense for organisms distantly related to humans. Although a subset of Arabidopsis SR proteins

have successfully improved splicing in cytoplasmic extracts of HeLa cells that are deficient in exclusively nuclear spliceosome components (Krainer and Maniatis, 1985) (atRSp31: (Lopato et al., 1996) and RSZp22: (Lopato et al., 1999)), other Arabidopsis proteins (even those with high sequence similarity to human spliceosomal proteins) cannot function in HeLa cells (SR34: (Lazar et al., 1995)).

RS domains may aid in the many protein-protein interactions required in the massive spliceosome (Wu and Maniatis, 1993). Both human and plant genomes encode over a dozen SR proteins. However, the list of proteins that contain just an RS domain and lack a recognizable RNA-binding domain might be much longer, and these extended relatives may also function in splicing (Boucher et al., 2001). SR proteins are highly phosphorylated, and experimentally disrupting their phosphorylation state changes their localization within the nucleus of both plant and animal cells (Misteli et al., 1997; Ali et al., 2003). Spliceosome localization is discussed more in a later section.

There are many key SR proteins in the plant and animal spliceosome. For example, after returning from the cytoplasm, the U1 snRNA binds to SR protein U1-70K (U1 small nuclear ribonucleoprotein 70 kDa), which is unimpressively conserved between plants and animals (30% similarity) but maintains many of the same binding relationships with other spliceosomal proteins (Tanabe et al., 2009). The U2 snRNA also has specific binding partners in the SR family, such as the U2 auxiliary factor proteins (U2AF). U2AFs bind to both introns and the U2 spliceosomal RNA, thus acting as a match-maker between the spliceosome and target mRNA. They are only present

early in the splicing process as the complex is assembling. One of these proteins, U2-auxilliary-factor 35kda (U2AF35 or U2AF1) binds to the AG consensus sequence at the 3' end of the intron, and is very highly conserved between humans and Arabidopsis (70% similarity) (Merendino et al., 1999; Wu et al., 1999; Zorio and Blumenthal, 1999). Another U2AF protein, U2-auxilliary-factor 65kda (U2AF65 U2AF2), binds the polypyrimidine track in animal introns (Chusainow and Ajuh, 2005). Plants have a track of uracil instead of both cytosine and uracil, and its position is poorly defined. Perhaps it is not surprising that the Arabidopsis ortholog of U2AF65 is not as well conserved as U2AF35 (only 30% similarity) compared to animals.

The periphery of the spliceosome remains mysterious.

There are many critical spliceosomal components that are well-conserved between plants and animals whose specific roles in splicing remain enigmatic. In order to survey the full composition of the spliceosome, researchers have isolated spliceosomes using mRNAs with un-spliceable introns to 'trap' the spliceosome on the mRNA. These mRNAs also include a coliphage coat MS2 protein-binding sequence so that researchers can pull them out of animal cell extracts along with the spliceosome. The resulting proteins can be identified using mass spectrometry (Jurica et al., 2002; Bessonov et al., 2008). Hundreds of potentially spliceosome-associated proteins were identified using this technique in both human cells and flies, including proteins not known to be involved in splicing.

One of them is CACTIN, which is a conserved eukaryotic protein that may be involved in splicing. CACTIN may have a weak RS domain, and CACTIN has pulled down with spliceosomes and shows pairwise association with spliceosomal components in animal cells (Jurica et al., 2002; Giot et al., 2003; Ewing et al., 2007). CACTIN is likely to have an important role in the cell because it is essential in zebrafish, *C. elegans*, and Toxoplasma (Atzei et al., 2010b; Tannoury et al., 2010; Szatanek et al., 2012). CACTIN was originally identified as a player in the animal-specific Nf-κB inflammation pathway (named for CACTUS interactor) (Lin et al., 2000). However, CACTIN is more ancient than the Nf-κB pathway, and many organisms that lack that pathway have retained the *CACTIN* gene (Ruvkun and Hobert, 1998). Examining *CACTIN* in organisms that never had an Nf-κB pathway, such as plants, may reveal its original role, which may be in the spliceosome.

Another fascinating story about spliceosome-related proteins involves PININ, which has a dual role in animals. In the cytoplasm, it binds keratin and maintains cell-cell adhesion, and in the nucleus it helps with splicing. PININ was named for its role in pinning intermediate filaments (keratin) to the desmosome (an animal cell-cell adhesion structure) (Ouyang and Sugrue, 1996; Shi and Sugrue, 2000). Soon after, another group published that it was exclusively localized to the nucleus, which set off a particularly lively exchange of articles (Brandner et al., 1997, 1998; Ouyang, 1999). Through differential folding or possible alternative splicing, nuclear and cytosolic PININ are able to bind different, non-cross reactive monoclonal antibodies (Ouyang and Sugrue, 1996; Ouyang, 1999).

Animal PININ contains an RS domain and binds to another RS protein in the nucleus, RNPS1 (Li et al., 2003). Drosophila PININ binds the aforementioned CACTIN in yeast two hybrid assays (Giot et al., 2003). Plants do not have keratin proteins or desmosomes, although they do have a distant ortholog of PININ (AT1G15200). The N-terminal 98 amino acids of animal PININ are responsible for its interactions with keratin, and plant PININ lacks that domain (Riedmann and Schwentner, 2010). Most likely, the ancestral role of PININ was in the nucleus as a splicing-related factor, and in the animal lineage it developed a new role in cell-cell adhesion. In animal cells, PININ can be found bound to spliced mRNA in the regions where introns once were, suggesting that it plays a role in the exon junction complex (discussed more in a later section) (Li et al., 2003). However, over-expression of PININ in human cell culture shows aberrant 5' splice junctions, demonstrating that PININ also has a role in the active splicing process (Wang et al., 2002). Invertebrate and plant PININ have not been investigated yet, and *S. cerevisiae* has lost this gene.

Catalysis of splicing involves a lariat structure

Catalysis of splicing is multistep

Over 300 proteins are part of the spliceosome, and they participate in a multistep coordinated process to precisely excise introns (Jurica and Moore, 2003). Initially, all pre-mRNAs are bound by heterogeneous ribonucleoproteins regardless of whether they contain introns or not (Matlin and Moore, 2007). This state is referred to as the H complex or **h**eterogeneous ribonucleoprotein particle (hnRNP). If there are introns present in the mRNA, it then proceeds to the **e**arly splicing or E complex (Figure 1- 1).

During this phase, the U1 UsnRNA and its associated proteins (U1 snRNP) identify and bind to the 5' donor site at the beginning of the intron. As the spliceosome transitions to the A complex, auxiliary proteins help the U2 snRNA base pair to the branch point sequence of the intron. The base pairing is imperfect and leaves the critical adenine of the intron branch site unpaired and therefore bulging out. Although, it should be noted that the branch point consensus sequence (particularly the two bases flanking the adenine) is not strictly followed in animals, and is even looser in plants. The protein constituents of the spliceosome, rather than base pairing alone, might play a large part in positioning the bulging adenine. In transition to the B complex, the U1 snRNP bound to the 5' junction recruits, and is eventually replaced by, the U6 snRNP. The sequence of U6 is the most conserved across eukaryotes of all the UsnRNAs. In the catalytic C complex-stage, the spliceosome helps the bulging adenine nucleophilically attack the 5' end of the intron, thus initiating the first transesterification step in splicing (shown as a red arrow in Figure 1- 1). The U5 snRNA binds to both the 3' and 5' ends of the intron and may keep both exons in close proximity during the reaction so that they can be joined together in the final transesterification (Newman et al., 1995). The U4 splicing snRNA is needed for recycling of U6 so that it can be used in another round of splicing.

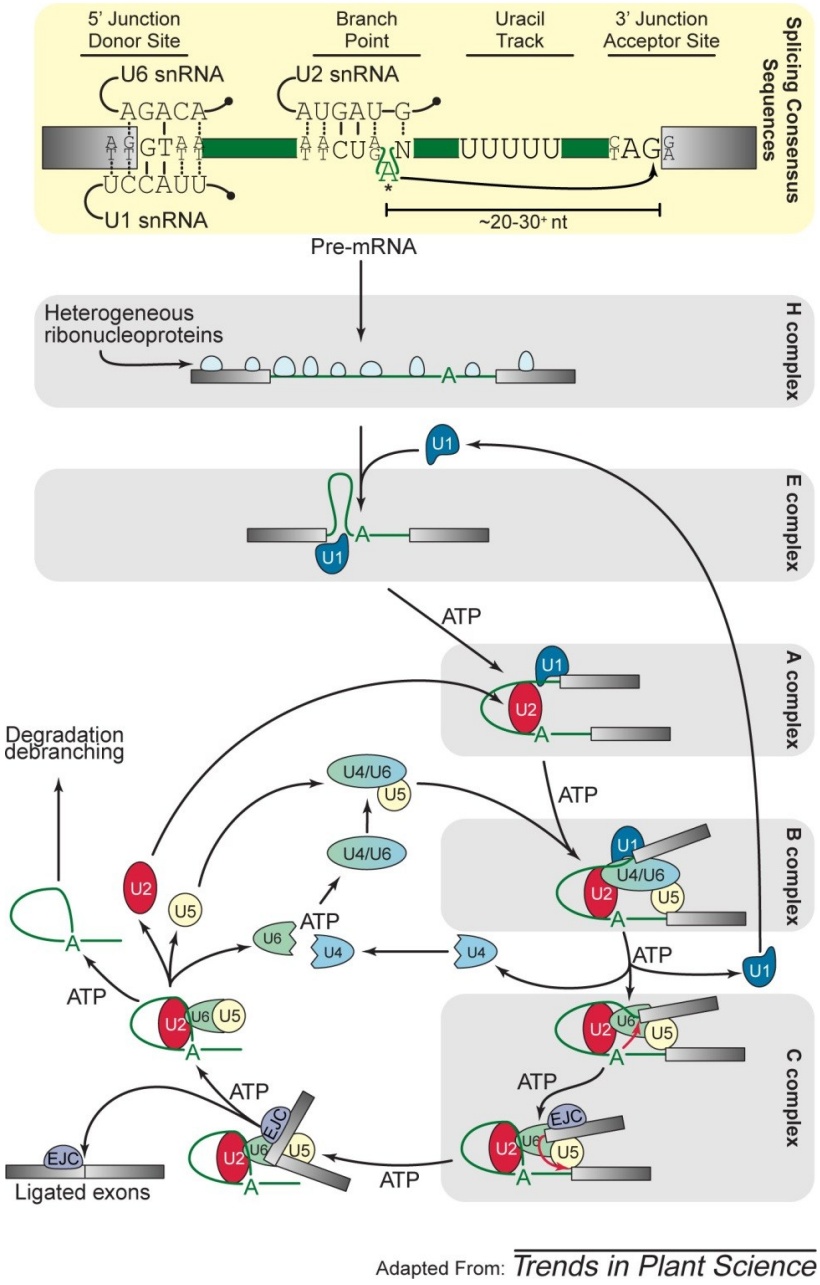

Figure 1- 1: mRNA splicing involves 5 UsnRNAs and their associated proteins.
Figure is adapted from (Lorković et al., 2000). Much of the research that was done to support this model was conducted in animals and yeast, but it seems very likely to be the same in plants. Base pairings of the U1 and U6 small nuclear RNAs (snRNAs) with the splice junctions and branch point are shown in the yellow box at the top. Intronic junction consensus sequences are from Arabidopsis (Reddy, 2007), as is the branch point sequence, which is a hybrid between two sequences given these articles (Brown et al., 1996; Tolstrup et al., 1997). The UsnRNAs are caped with 5' 7-methylguanylate caps (m7G) that are

depicted as filled black circles. Red arrows indicate the two transesterification reactions that occur during catalysis.

Localization of splicing factors within cells

In another example of the similarities between the plant and animal spliceosome, spliceosomal proteins show a combination of diffuse and speckled localizations within the nuclei of both plant and animal cells. Arabidopsis provides a great system for looking at spliceosomal protein localization in a multicellular organism. Its roots are optically clear, and seedlings are small enough to fit completely on a slide. Seedlings can also live under a microscope for many hours, allowing for live viewing while modifiers or chemicals are added. The root contains numerous well-defined cell types in predictable stages of differentiation. What's more, the nuclei of most of these cells are large enough to visualize subnuclear localization with light microscopes.

The cells within the meristem differentiate and divide frequently, and they are likely to have a high rate of transcription and mRNA splicing. In these cells, spliceosomal proteins are localized diffusely throughout the nucleus. In the older more shootward cells, spliceosomal proteins show a discrete speckling pattern within the nucleus (Ali et al., 2003; Docquier et al., 2004; Fang et al., 2004). Additional information suggests that spliceosomal protein localization correlates with the transcriptional activity of a cell. RNA polymerase inhibitors cause spliceosomal components to move to a tighter, more discrete speckle pattern in both animal and plant cells (Spector et al., 1983; Misteli et al., 1997; Docquier et al., 2004). Hypoxia treatment halts general translation and transcription, and also causes tighter speckle formation (Koroleva et al.,

2009). These observations are consistent with speckles being storage reservoirs for inactive spliceosomes (Spector and Lamond, 2011). When spliceosomes are actively splicing, they localize to more numerous tiny speckles or display more diffuse nucleoplasm localization.

In plant cells, there may be multiple pools of splicing factors in discrete speckles. Lorković et al., 2008 used GFP- and RFP-tagged Arabidopsis SR proteins expressed in tobacco cells to look at subnuclear localization. Although all the SR proteins that they tested did localize to speckles, the speckles did not colocalize for some pairwise comparisons (Lorković et al., 2008). This finding could mean that there are different spliceosomes within a nucleus, perhaps capable of causing different forms of alternative splicing on substrate mRNAs and then residing in different speckles when not actively splicing. However, these proteins were expressed in a heterologous system and no functional testing was done to verify that the tagged proteins were functional. It would be very interesting to repeat these experiments in stably transformed Arabidopsis.

Splicing is connected to other mRNA processes

Splicing occurs as a gene is being transcribed

In animals, electron microscopy and RT-PCR data suggest that mRNA splicing occurs simultaneously with transcription (Beyer and Osheim, 1988; Tennyson et al., 1995). These processes are linked and can affect each other. For example, a spliceosomal SR protein, SF2/ASF, is able to effect transcription of the genes that it is splicing because the two events are spatially near each other. SF2/ASF can inhibit the

DNA-relaxation-activity of topoisomerase I in animal cells (Andersen et al., 2002; Kowalska-Loth et al., 2002). SF2/ASF is 59% similar between humans and Arabidopsis, so it may have the same function in plants. Gene transcription causes negative supercoiling of the gene, which is usually remedied by topoisomerase I. When a gene is being cotranscriptionally spliced, SF2/ASF inhibits topoisomerase I, and the gene becomes negatively supercoiled. This may provide easier access for RNA polymerase II to transcribe the next mRNA from this gene, thus increasing gene expression (Niu and Yang, 2011).

There is also evidence that transcription can encourage splicing through the C-terminal domain of RNA polymerase II. This domain increases the efficiency of in-vitro splicing, and increasing the activity of RNA polymerase increases the recruitment of spliceosomes to nascent transcripts unless the C terminal domain is removed (Hirose et al., 1999; Misteli and Spector, 1999).

Additional evidence for the tight link between transcription and splicing in animal cells comes from promoter studies which show that genomic sequence upstream of the transcriptional start site can affect which introns are spliced, and this depends on certain SR proteins (Cramer et al., 1999). The connection between transcription and splicing has not been as well studied in plants, but they are likely to reciprocally affect each other.

Splicing affects downstream processes via the exon junction complex

Not only is splicing connected to upstream processes, but it is also linked to downstream ones. Again, these connections have been better studied in animal systems, but even in plant cells, the presence of an intron was sufficient to cause 30-times higher translation of a reporter mRNA (Rose and Last, 1997). In animal cells, introns and the spliceosome are able to affect mRNAs even after splicing is done by depositing other proteins onto the spliced mRNA, called the exon junction complex (EJC).

RNaseH-protection experiments in mammalian cells showed that the proteins of the exon junction complex are found 20-24 nt upstream of the site where introns were removed (Le Hir et al., 2000). The presence of the EJC on a transcript is important for helping it export from the nucleus efficiently (Zhou et al., 2000). In mammalian cells, the EJC has a special role in nonsense-mediated decay. This conserved eukaryotic process detects and degrades transcripts with early stop codons (Le Hir et al., 2001). Mammalian ribosomes determine that stop codons are 'early' if they precede EJCs on a transcript by 55nt or more (Nagy and Maquat, 1998). In a typical protein-coding mRNA, the stop codon is in the last exon so there are no EJCs after the stop codon. The result is that introns are required for nonsense mediated decay in mammals. In contrast, Drosophila don't use EJCs to identify transcripts with premature stop codons (Gatfield et al., 2003). This is also likely to be the case for Arabidopsis because a higher percentage of genes don't contain introns compared to mammals, and nonsense mediated decay has been observed for genes without introns in plants, which doesn't occur in animals (Van Hoof and Green, 2006). Also, *S. cerevisiae* have lost the exon

junction complex, but are still able to conduct nonsense mediated decay. These observations may suggest that the ancestral mode of identification of transcripts with premature stop codons did not require the exon junction complex and that this connection is a derived trait in mammals. The mode of identification for nonsense mediated decay in plants is still unknown, although it may require that stop codons be near sequence signatures in 3' UTRs (Van Hoof and Green, 2006). In plants and non-mammalian animals, the EJC likely has it's ancestral role of facilitating smooth delivery of mature mRNAs to the site of translation.

The mammalian EJC contains at least 14 proteins, and Arabidopsis has orthologs of all of these proteins except for one (Pendle et al., 2005). At least some of these proteins localize to the same regions and behave in the same way as in animal cells (Koroleva et al., 2009). The spliceosome and the EJC contain an overlapping list of proteins that have roles in both processes such as RNPS1 and the previously discussed PININ (Li et al., 2003). RNPS1 was named for RNA binding protein S1, and it binds to the aforementioned CACTIN as well as PININ in animal cells (Giot et al., 2003; Ewing et al., 2007; Murachelli et al., 2012). RNPS1 is one of the critical proteins for nonsense-mediated decay in the cytoplasm, but it is also critical for constitutive and alternative splicing in the nucleus (Mayeda et al., 1999; Viegas et al., 2007). In plant cells, the ortholog of RNPS1 is called SR45 for serine arginine protein 45, and it is alternatively spliced, as we will discuss in the next section.

Alternative splicing allows for increased complexity and regulation

Whole transcriptome sequencing has revealed that a large percentage of Arabidopsis genes are expressed as more than one spliceoform. In plants, most of the alternate forms retain an intron, which usually results in premature stop codons that can cause the mRNA to be degraded or translated into truncated proteins (Marquez et al., 2012). In animals, exon skipping is the most common type of alternative splicing and can result in proteins with fewer domains and/or different localization (Modrek et al., 2001; Sakabe and de Souza, 2007; Mastrangelo et al., 2012). Both intron retention and exon skipping occur in plants and animals, along with other forms of differential splicing including altered 5' junction, altered 3' junction, and combinations of all of these. Current estimates are that 60% of Arabidopsis intron-containing genes are present in multiple spliceoforms, whereas the estimates for humans are over 90% (Pan et al., 2008; Marquez et al., 2012). Sequencing of RNAs extracted from more tissues and/of from plants subjected to more environmental conditions may yield additional spliceoforms. However, it remains to be seen what proportions of these are meaningful spliceoforms versus merely rare splicing 'mistakes'. For some genes, the preferred spliceoform has been shown to change under conditions such as stress, temperature, or hormone treatment (Palusa et al., 2007; Tanabe et al., 2007; Duque, 2011). Many of the alternatively spliced transcripts that have been studied encode parts of the spliceosome, mainly SR proteins. This may be as a form of feedback regulation so that the spliceosome can regulate itself.

One example of alternative splicing in Arabidopsis with functional consequences is RS domain-containing SR45 (Zhang and Mount, 2009). Plants with loss-of-function mutations in this gene are small, have narrow flower petals, are late flowering, and show altered spliceoform preference of some genes when tested by RT-PCR (Ali et al., 2007). In Arabidopsis, SR45 has two isoforms that differ by the exclusion or inclusion of eight amino acids due to altered 3' splice site (Zhang and Mount, 2009). Despite this seemingly subtle difference between these two proteins, they have separate functions. One spliceoform can rescue the root phenotype of the SR45 knockout mutant, and the other spliceoform can rescue only the flower petal morphology. Both isoforms are expressed throughout the plant and show the same type of localization with the nuclei of cells. How these eight amino acids change the protein function is still being studied.

Another example of functional alternative splicing is the JASMONATE-ZIM DOMAIN (JAZ) repressor proteins. Jasmonic acid is a key plant hormone involved in many processes including stress response. In the absence of jasmonic acid, the JAZ proteins bind to and repress the MYC2 (named for its similarity to an animal virus oncogene, 'myelocytomatosis viral oncogene') transcription factor, preventing it from regulating the transcription of target genes. When the plant experiences stress, it produces jasmonic acid in order to activate response pathways. As jasmonic acid enters a cell, it binds to the COI1 (Coronatine-insensitive protein 1) receptor which stimulates the ubiquitination and degradation of the JAZ repressors. Once the repressors are gone, the MYC2 transcription factor is free to regulate expression of jasmonic acid-responsive genes (Pauwels and Goossens, 2011). However, alternative

splicing of JAZ receptors, particularly JAZ10, results in a truncated protein missing the domain that COI1 regulates (Chung et al., 2010). Thus, the truncated JAZ10 is immune to jasmonic acid-mediated degradation and is able to repress MYC2 constitutively. The plant likely uses these truncated proteins as a way to temper the stress response.

Another emerging story about the functional uses of alternative splicing in Arabidopsis comes from ZIFL1 (Remy et al., 2013). ZIFL1 stands for ZINC-INDUCED FACILITATOR-LIKE 1 and belongs to a family of diverse transporters. One spliceoform of this gene is found only in guard cells and localizes to the plasma membrane. Another spliceoform, which includes two additional transmembrane domains, is found in numerous plant tissues, but localizes to the vacuole membrane (tonoplast). In plants, *zifl1* mutants have classic auxin-related phenotypes such as defective gravitropism and reduced lateral root elongation; however they are also drought-sensitive and have more open stomata. When cDNA constructs were used to rescue the phenotypes of the *zifl1* mutant, only the guard-cell specific spliceoform rescued the drought and water loss phenotype, and only the ubiquitous spliceoform rescued the root growth phenotypes.

The physiologically relevant substrate for the ZIFL1 transporters is still unclear. ZIFL1 does not contain ATPase domains, and likely utilizes existing proton gradients to power its transport of target molecules. Consistent with an auxin-related function, expression of both isoforms of ZIFL1 in yeast resulted in increased resistance to auxin (which slows yeast growth), suggesting that ZIFL1 may be able to move auxin out of cells. However, yeast experiments also suggested that ZIFL1 can move potassium

ions, which could be an indirect cause of both the plant and yeast auxin related observations. Many transporters are powered by ionic gradients, and ZIFL1 may use its ability to convert a proton gradient into a potassium gradient to simulate other transporters to pump auxin. Consistent with this hypothesis, guard cell closure is dependent on potassium fluxes.

Both isoforms of this gene behaved similarly in yeast cells, so the differential expression and localization in plants is likely the result of organ-specific splicing and isoform-specific subcellular localization. The two isoforms may transport the same substances, but their different subcellular localizations could dramatically change their function. The molecular mechanism of how the guard-cell spliceoform is favored in those cells is an intriguing mystery. It will also be interesting to determine what substrates each isoform pumps out of cells or into the vacuole.

The plant spliceosome continues to shroud mysteries

The prevalence of alternative splicing in plants is a recent discovery and the functions of most of these spliceoforms are yet to be examined. Undoubtedly, future studies will yield more information about alternative splicing as a gene regulatory system in plants. Also, the spliceosome itself contains critical yet still enigmatic components. We still do not know the specific roles of these peripheral splicing proteins. New technologies, like whole transcriptome sequencing and spliceoform analysis will facilitate examination of mutants and help unravel these mysteries.

The goal of this thesis is to present new findings regarding one small corner of the spliceosome that was unknown previously. In chapter 2, we use Arabidopsis to uncover evidence supporting the existence of a new protein component of the spliceosome, CACTIN. We did not set out to study the spliceosome, but rather to find the function of this conserved eukaryotic gene. CACTIN protein levels, as determined by two dimensional electrophoresis, increased 1.8-fold (standard deviation 0.2, 7 trials) in the root tips of gravistimulated Arabidopsis seedlings (Murthy et al., unpublished data.). Our experiments led us to nuclear speckles and eventually to the spliceosome. It is conceivable that increased CACTIN levels could affect the alternative splicing of transcripts and contribute to the gravitropism response. In chapter 3, we present genetic experiments that led us to attribute the phenotypes initially associated with a second allele of *cactin* to another gene mutation. In appendix 1, we show a series of experiments aimed at silencing Arabidopsis *CACTIN*. Finally, a review on root gravitropism is presented in appendix 2.

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Chapter 2: CACTIN is an Essential Nuclear Protein in Arabidopsis and may be Associated with the Eukaryotic Spliceosome[1]

Katherine L. Baldwin, Elizabeth M. Dinh, Brian M. Hart, Patrick H. Masson

Abstract

CACTIN is a conserved eukaryotic protein without known functional domains. Previous research revealed that CACTIN is essential in animals and protists and that it may function in inflammation pathways in animals; however, these pathways are not as broadly conserved as CACTIN. Therefore, the ancestral molecular function of CACTIN remains unknown. Our studies using Arabidopsis show that CACTIN is required for embryogenesis. Fluorescently tagged CACTIN localizes to the nucleus and shows nuclear speckles in many cell types of the seedling. CACTIN colocalizes with known splicing proteins SR45 and RSP31. Sodium azide treatment is known to change the localization of spliceosomal proteins from being more diffusely nuclear to discrete speckles, and we demonstrate the same phenomenon for CACTIN. In yeast-two-hybrid studies, we found that CACTIN binds to a putative component of the spliceosome. These findings support a possible role for CACTIN in splicing.

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[1] This chapter is a modified version of the publication: Baldwin KL, Dinh EM, Hart BM, Masson PH. (2013) CACTIN is an Essential Nuclear Protein in Arabidopsis and may be Associated with the Eukaryotic Spliceosome. FEBS Letters 587, 873-9. Elizabeth Dinh and Brian Hart are undergraduate students who worked with Katherine Baldwin on this project. Brian Hart worked on paralogous genes (AT5G66310 & AT3G51150) that also came up in a yeast-two-hybrid screen for binding to CACTIN. They do not appear in this manuscript because we were unable to confirm or refute this interaction. Elizabeth Dinh worked on RINTIN, including crossing *rintin* to *sintin* as well as other mutants. She also helped clone many of the genes tested by pairwise yeast-two-hybrid.

Introduction

CACTIN (*CTN*) is highly conserved throughout eukaryotes (Lin et al., 2000) (Figure 2- 1A), but its function is largely unknown. CACTIN in most organisms contains a nuclear localization signal, coiled-coil domain/s, and a region rich in arginine and serine residues predicted to have low complexity (Wootton and Federhen, 1996) (Figure 2- 1B). The *CACTIN* gene appears to be single copy in most organisms, including Arabidopsis (TAIR: AT1G03910), although the latter also contains a related pseudogene (TAIR: AT2G36815) that contains many premature stop codons. *CACTIN* is found in plants, animals, protists, and fungi; however, it has been lost from the subphylum Saccharomycotina, which contains *S. cerevisiae*.

CACTIN was originally identified in Drosophila as a yeast-two-hybrid interactor of CACTUS, an IκB component of the NF-κB transcriptional signaling cascade that controls developmental cell polarity and inflammation (Lin et al., 2000). However, several components of this pathway are only found in animals, suggesting that it may have evolved in the metazoan common ancestor. Also, the NF-κB pathway has been lost in the *C. elegans* lineage (Ruvkun and Hobert, 1998). This contrasts with the *CACTIN* gene, which is broadly found throughout eukaryotes, including *C. elegans*. If animal and non-animal CACTIN proteins share a function, as their sequence similarities suggest, then CACTIN plays a role distinct from the NF-κB pathway. In human cells, CACTIN does not bind IκB as determined by coimmunoprecipitation (Atzei et al.,

2010a), suggesting that the role of CACTIN in the NF-κB pathway may not be conserved even within animals.

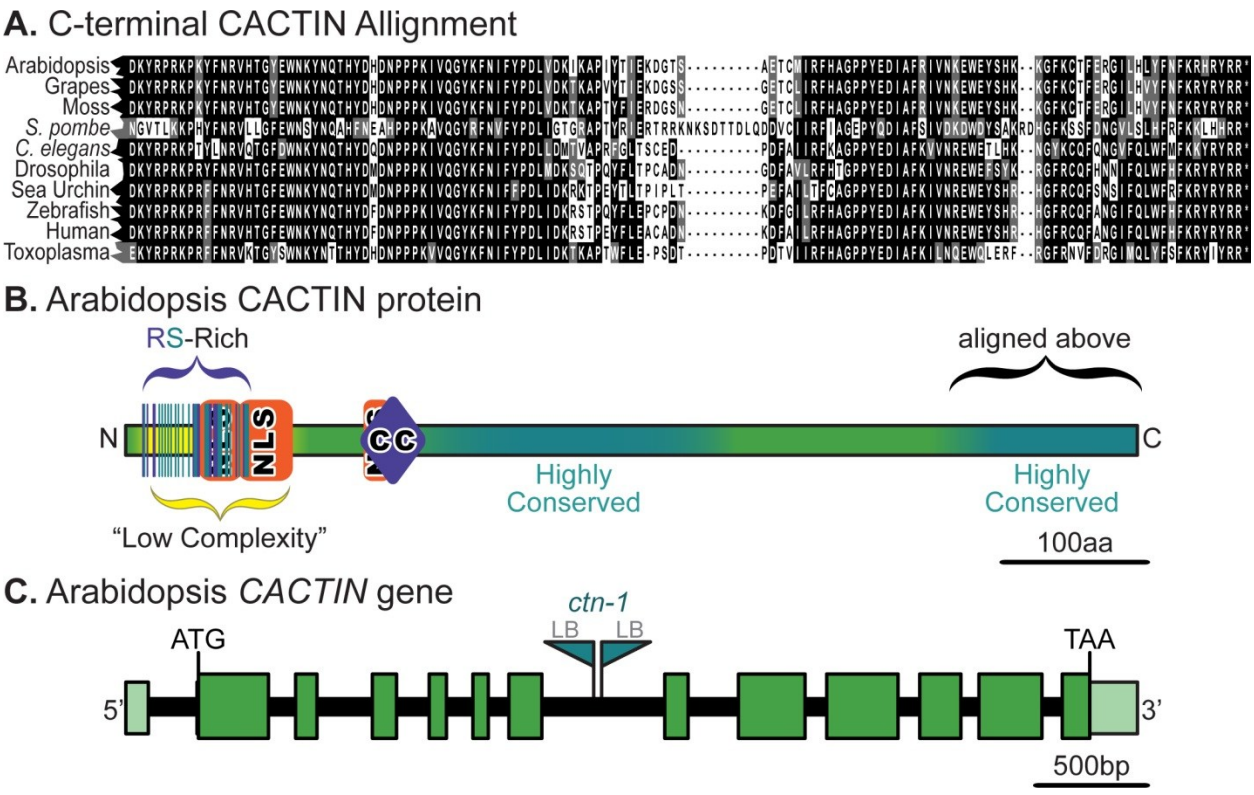


Figure 2- 1 CACTIN is a conserved eukaryotic protein.
A: Alignment of the C-terminal domain of CACTIN from various eukaryotes. The region of the protein that is aligned is indicated by the bracket below. Black boxes indicate conserved amino acids; grey boxes indicate amino acids that are similar bases on a 50% threshold and the BLOSUM62 similarity matrix. Sequence source information is given in the methods section. B: The domain structure of Arabidopsis CACTIN. In the RS-rich region, arginine and serine residues are shown by blue lines. NLS indicates predicted nuclear localization signals and CC indicates a predicted coiled-coil domain. C: Gene structure of *CACTIN* with the T-DNA insertional site of *ctn-1* shown. Introns are represented by black rectangles, exons are dark green boxes, and UTRs are light green boxes (UTRs inferred from RNA seq reads(Gulledge et al., 2012)). The translational start and stop codons are also shown. "LB" indicates the left border of the T-DNA insert.

In all the model organisms thus tested, *CACTIN* is required for viability. *cactin* strong-allele mutants arrest in the larval stage in *C. elegans* (Tannoury et al., 2010), and morpholino silencing of *CACTIN* in zebrafish results in developmental abnormalities and embryo death (Atzei et al., 2010b). A temperature-sensitive allele of *CACTIN* in Toxoplasma results in cell cycle arrest when incubated at the non-permissive temperature (Szatanek et al., 2012). These experiments suggest *CACTIN* is required for eukaryotic cell viability.

The function of CACTIN is still unknown despite analyses in several organisms. CACTIN-fluorescent protein fusions show nuclear localization in *C. elegans*, human cells, and toxoplasma (Atzei et al., 2010a; Tannoury et al., 2010; Szatanek et al., 2012). *CACTIN* knockdown in human cells results in transcriptional changes of many of the genes tested by qRT-PCR, including multiple immune-related genes (Atzei et al., 2010a). *CACTIN* silencing in *C. elegans* results in germline development abnormalities including distal tip cell migration problems (Tannoury et al., 2010). CACTIN is also important for cell cycle progression in Toxoplasma where mutants show misexpression of many genes as demonstrated by microarray experiments (Szatanek et al., 2012). Many of these experiments suggest CACTIN modulates gene expression. Our studies on Arabidopsis CACTIN discussed below are consistent with this hypothesis, and we propose that CACTIN affects the transcriptome through connections to the spliceosome.

The spliceosome is a large macromolecular complex consisting of both catalytic RNAs and over 300 different proteins (Jurica and Moore, 2003). Many of the core

splicing components from animals are conserved in plants, suggesting that plants and animals share similar splicing processes. In contrast, yeast have a reduced spliceosome that contains far fewer proteins (Fabrizio et al., 2009).

One group of spliceosomal proteins that is particularly prevalent in plant genomes is the SR family (Reddy, 2007). SR proteins are named for their arginine and serine-rich RS domains of low predicted complexity [3-5], similar to the arginine and serine-rich region of CACTIN, and usually have an RNA binding domain. SR proteins are important for not only core transcript splicing, but also for spliceoform selection of alternatively spliced genes (Ali et al., 2007). In both plant and animal cells, SR proteins localize to the nucleus, and in many cells they form nuclear speckles. Although the function of the speckle structure is not completely clear, they may be storage clusters of inactive spliceosomes, as reviewed (Spector and Lamond, 2011). Splicing proteins have dynamic localization between the speckles and the general nucleoplasm, which can be regulated with various treatments and compounds in plant and animal cells (Misteli et al., 1997; Ali et al., 2003). In plants, meristematic cells show a more diffuse nuclear pattern of splicing factors compared to mature tissues, which generally show more punctate speckles (Ali et al., 2003; Fang et al., 2004). This may indicate a correlation between transcriptional activity and speckle formation of splicing factors.

In this study, we aimed to determine the function of CACTIN and propose that CACTIN may be involved in mRNA splicing.

Methods

Plant materials and genotyping

Mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH) (Table 2- 1). Seeds were surface sterilized with 95% ethanol and sown onto half-strength Linsmaier & Skoog media containing 1.5% sucrose (Caisson Laboratories LSP04-1LT, Logan, UT) and supplemented with 1.5% agar type E (Sigma, A4675-1KG, St Louis, MO). The plates were stratified at 4°C in the dark for 2 days before being placed in a growth chamber (16 hours light (50-70 μmol $m^{-2}s^{-1}$) and 8 hours dark, 22°C, 70% relative humidity).

Plants were moved to soil after approximately 10 days of growth on petri plates. Genotyping was performed using standard PCR methods and the primers in Table 2- 1.

Pollen Staining

Alexander staining was used to identify dead pollen as described (Alexander, 1969).

Table 2- 1: Mutant stock numbers and genotyping primers.

Mutant			Genomic Wild-Type Primers		T-DNA Primer
ctn-1	TAIR: AT1G03910	SALK_094586	GCTCAGTGAACCGTACATGG	GTCCACTCTCGATCTCAAAAGC	AAAAACGTCCGCAATGTGTT
			GCTCAGTGAACCGTACATGG[1]	GCCACTTTCGTCTTCTCCAG[1]	
			TGGTCTTTTAGGGACTCTCTTTG[1]	GTCCACTCTCGATCTCAAAAGC[1]	
RINTIN-1	TAIR: AT5G63440	SALK_070923C	CGTGCTGAAGAGAATCTGGA	TTCCCATGAATTCCAGAAGC	AAAAACGTCCGCAATGTGTT
RINTIN-2	TAIR: AT5G63440	SALK_075173C	CGTGCTGAAGAGAATCTGGA	TTCCCATGAATTCCAGAAGC	AAAAACGTCCGCAATGTGTT
SINTIN-1	TAIR: AT1G49170	SALK_016988	TGCCATCTTCTCGTCTTTGA	AGCTGCGTTAGCTTCACCAT	AAAAACGTCCGCAATGTGTT

[1]These pairs of primers were used for genotyping plants transformed with CTN-RFP to avoid detection of the transgene.

The genomic wild-type reaction uses two primers aligning to the gene that flank the insertional site. Because the inserts are quite large, these primers only result in a ~400bp product on templates that contain wild-type copies of the gene. The T-DNA border reaction uses one gene primer and one primer that anneals to the T-DNA insert. Because all of these alleles contain left-boarder (LB) T-DNA sequence on both sides of the insert, the T-DNA primer can be used with either genomic WT reaction primer to produce an amplicon indicative of the mutation. This reaction only results in amplification if there is an insertional mutant copy of the gene in the template. All primer sequences are listed in the 5' to 3' direction.

Table 2- 2: Junction sequences of mutant T-DNA lines.

Mutant		Junction Sequence 5' end of insert		Junction Sequence 3' end of insert	
		20bp of Gene Seq	20bp of T-DNA Seq	20bp of T-DNA Seq	20bp of Gene Seq
ctn-1	SALK_094586	TGCTGGGATAGCATCTATTC	CA[1]CAGGATATATTGTGGTGTAA	GTTTACACCACAATATATCC	TGAA[2]TATATGTAGAAAAAAAA
RINTIN-1	SALK_070923C	GTATTATGGCACAAAATCTA	TTGACGCTTAGACAACTTAA	AAACGTCCGCAATGTGTTATT[3]GTTATTTG	TTTTCTTACTCACTTGACAT
RINTIN-2	SALK_075173C	AATCTTTTCAATTCCAAGAT	TATATTGTGG[4]AAACAAATTG	TTTACACCACAATATATCCT[5]TCAGTTGTGTTATTAAGTTGTCCGCAATGTGTTATTAAGCAATGTGTTATGTG	GATCACTAATCTGTTGTGGC
SINTIN-1	SALK_016988	GAAGAAAGGAAAGAAGACCA	GACAACTTAATAACACATTG	CAATGTGTTATTAAGTTGTC	CGACGTGTTTGCGTCTCCTT

[1] the previous 2 bases are an additional insertion
[2]the following 2 bases are swapped from normal *CACTIN* sequence
[3]the following is an additional insertion
[4]3bp deletion relative to T-DNA sequence
[5]the following is an additional insertion of unknown origin

20 base pairs on either side of each T-DNA junction are shown. Each junction sequence is separated into two columns to denote the portion of the sequence derived from the gene and from the T-DNA. As with many T-DNA mutations, all four of these alleles have left borders on both sides of the insert. The side of the insert that is registered in the Arabidopsis Information Resource database is the 3' junction for all four of these alleles.

mRNA analysis of *RINTIN-1 and SINTIN-1* mutants

RNA was isolated from seedlings using the RNeasy Plant Mini Kit (Qiagen, 74904, Hilden, Germany). DNA was eliminated using RQ1 DNase (Promega M6101, Madison WI). cDNA was made from total RNA using iScript (170-8891,Bio-Rad, Hercules, CA). Primers for RT-PCR are listed in Table 2- 3.

Table 2- 3: Primers used for testing mutants for mRNA expression using Reverse Transcription PCR.

Gene	Forward Primer	Reverse Primer	Amplicon position relative to insert
TAIR: *AT1G13320*	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT	Reference gene
RINTIN reaction 1	TGGACCCGACTCTGATCTCT	CTGTCGGTCTTCCTTTTTGG	Upstream
RINTIN reaction 2	CACAACGTTCAGCAATCACA	CAGAAGCTCGTTGTTAGCTTCC	Spans insert
RINTIN reaction 3	CGTGTGACTGTTGCTGCAC	TGGCTCAACCTTAAACCAAGA	Immediately downstream
RINTIN reaction 4	TCCTCGTGGTCGAGGATTTA	TCAGCTAATGTCGTTCACCAA	Downstream
SINTIN reaction 1	TCCGACGAAGAAAGGAAAGA	CGGAGGAAGGTGTAAGGAGA	Spans insert
SINTIN reaction 2	GACGTGTTTGCGTCTCCTTAC	TTGAACGCCTACCGCTTC	Immediately downstream
SINTIN reaction 3	GCTCTGTATTGGGGGTGAAA	GCTTGGGACAAAGCTTGAAA	Downstream

All primer sequences are listed in the 5' to 3' direction. The results of these reactions are shown in Figure 2- 14, and diagrammed in Figure 2- 12.

Arrested embryo counting

Seeds were sown in soil and grown to flowering stage at which time we harvested elongated green siliques and preserved them in 50% acetic acid and 50% ethanol. Siliques were dissected on a stereo microscope. The seeds within each silique were counted and tallied as either full size, or underdeveloped.

Microscopic analysis of embryo development

Siliques at 3-4 days post-anthesis were collected and fixed in Histochoice (Amresco H112, Solon, OH) on ice for 15 minutes, then dissected on a slide to remove the seeds from the valves. A drop of Hoyers Solution (0.21g/ml gum arabic, 14% glycerine, 2.9g/ml chloral hydrate) was applied, and a cover slip was added. The slide was sealed with nail polish and allowed to sit at room temperature overnight. We then

photographed the slides on a phase contrast Leica DM LB2 microscope using a 100X objective. The contrast of embryo images was increased using Adobe Photoshop.

Transgenic constructs

CACTIN-RFP

We amplified *CACTIN* from genomic Arabidopsis DNA using primers on the start codon and just before the stop codon (caccATGGGTTCTCATGGTAAGGGTAAGAG and TCGCCTGTACCTATGTCGTTTA). The PCR product was cloned into pENTR-D-Topo, which required the addition of "cacc" to the forward primer (Invitrogen K2400-20, Carlsbad, CA). This construct was then recombined into pH7RWG2 (Karimi et al., 2005), which contains the cauliflower mosaic virus strong constitutive 35S promoter and a C-terminal RFP tag. The gateway sites in this vector result in a short peptide linker sequence in-between CACTIN and RFP.

Native Promoter and Native UTR CACTIN Construct with RFP

The genomic region around CACTIN was amplified using these primers: caccTTTATTTTACCGATTGCTTCAAA and GAACGAACCTGAGTTCATCAAA. This 5518bp amplicon encompasses all of the sequence after the stop codon of the gene before *CACTIN* (*AT1G03905*) and the sequence up until the start codon of gene after *CACTIN* (*AT1G03920*). This results in 330bp upstream of the apparent transcriptional start, 640bp upstream of the start codon, 874bp downstream of the presumptive transcriptional end site, and 1078bp downstream of the stop codon. The transcription start and stop sites were inferred from RNA seq data (Gulledge et al., 2012). The genomic amplicon was cloned in pENTR-D-Topo. We inserted the RFP tag just before

the stop codon of CACTIN along with the same linker that is present in the gateway-based CACTIN-RFP construct discussed above. We used a site-directed-mutagenesis-like technique to insert RFP and the linker into the larger genomic construct in pENTR. To start, we amplified a segment genomic *CACTIN* in pH7RWG2 (discussed above) which includes the last 51bp of coding *CACTIN*, the 48bp gateway linker, and the RFP gene from pH7RWG2. We used these primers: TCCATGCTGGTCCACCTTAT and AGCCAAACTCCGATAAGGTTAGGCGCCGGTGGAGT. The reverse primer includes a wing that is complementary to the stop codon and 3'UTR of *CACTIN* (AGCCAAACTCCGATAAGGTTA). This PCR product was mixed with *CACTIN* from the gene before to the gene after in pENTR-D-topo in a PCR reaction with no additional primers. The PCR product acts as a primer on the plasmid and creates a new plasmid that includes RFP inserted just before the stop codon and full 3'UTR of CACTIN. After thermocycling, this reaction was digested with DpnI to in order to eliminate the initial template plasmid, which contains no RFP. This mixture was transformed into *E. coli* and coloies were screened and sequenced. This construct was then recombined into pMDC99, which encodes for hygromycin resistance in plants (Curtis and Grossniklaus, 2003).

GFP-SR45 and GFP-RSP31

Plasmids containing the coding domains of *SR45* (stock #G68687) and *RSP31* (stock #G14975) in pENTR223 (Invitrogen, Carlsbad, CA) were obtained from the ABRC. Stock #G68687 contains the coding domain of splice variant SR45.1 (TAIR: AT1G16610.1) and a conservative change in base pair 617 (C to T, which causes an

alanine-to-valine change). Stock #G14975 contains the coding domain for TAIR: AT3G61860.1. These plasmids were recombined into pEarleygate104, which contains the 35S promoter and an N-terminal GFP tag (Earley et al., 2006).

GFP-RINTIN

RINTIN coding domains were amplified from the plasmids obtained from our yeast-two-hybrid screen using the primers caccATGCCGAAGAGAACGACAC and TTACGGTACAACGGCCTCG. The PCR product was cloned into pENTR-D-Topo (Invitrogen K2400-20, Carlsbad, CA) and recombined into pMDC43 (Curtis and Grossniklaus, 2003), which contains a 35S promoter and an N-terminal GFP tag (Xu and Li, 2008).

Agrobacterium and plant transformation

All entry vectors were recombined into destination vectors using Invitrogen LR clonase II (Invitrogen 11791-100 Carlsbad, CA). All constructs were confirmed by sequencing. Plants were transformed using standard methods (Clough and Bent, 1998), and transformants were screened on antibiotic plates (Harrison et al., 2006).

Fluorescence Microscopy

Fluorescence microscopy was performed on seedlings that were grown on agar plates. The seedlings were placed on a slide in a drop of water and enclosed with a cover slip. We imaged the slides on a Leica DM LB2 equipped with a mercury bulb, a Leica GFP fluorescence cube, a Chroma 41002C fluorescence cube and a 100X objective. Photographs were captured using an Olympus DP72 camera. Because the

expression levels of the RFP and GFP fusion constructs varied, the camera exposure time was chosen to best capture the range of brightness and pattern within each nucleus. Colocalization was quantified using the Pearson coefficient of correlation (also known as Pearson's R value) for colocalization, which measures the linear dependence between the intensity of a pixel on one color channel compared to another channel (Bolte and Cordelières, 2006; Adler and Parmryd, 2010). The Pearson coefficients of correlation (R-values) for colocalization were calculated using the "Coloc2" analysis tool within the FIJI plugin for image J using the nucleus and the region of interest (Schindelin et al., 2012). No threshold assumptions were made. Completely unmodified images were used for all colocalization quantification. The images chosen for publication were cropped in Adobe Photoshop and the brightness was increased. No gamma adjustments were used. For the 'merge' pictures, Adobe Photoshop CS4's "linear dodge add" blending mode was used.

Sodium Azide Treatment Microscopy

Three day old CTN-RFP Seedlings were mounted on a slide with 20ul of water and a cover slip. Many florescent nuclei were photographed in a systematic manner beginning at the root tip and taking note of the region of the root they were located in. Then, 20ul of 100mM sodium azide (dissolved in water) was pipetted under the cover slip while the slide was still under the microscope using a thin gel loading tip. Again, many nuclei were photographed. Photographs of the same nuclei from before and after sodium addition were matched up with each other. The same process was used on PhyB-YFP seedlings given to us by Robert Stankey and Richard Vierstra (Laboratory of

Genetics, University of Wisconsin-Madison). The seedlings were grown in the dark and mounted on slides in low light and hypocotyl nuclei were photographed.

PHYTOCHOME B Localization

Seed carrying 35S::PHYTOCHROME B-YFP (phyB-YFP) was obtained from the laboratory of Richard Vierstra (Laboratory of Genetics, University of Wisconsin-Madison). These plants were crossed to plants carrying CACTIN-RFP, and the F1 seedlings were imaged. The seedlings were grown in darkness and then put in the light for two hours prior to imaging because PHYTOCHROME B does not form nuclear speckles under all lighting conditions (Christians et al., 2012).

Quantifying CACTIN-RFP overexpression

Quantitative Real Time PCR (qRT-PCR) was used to quantify the level of expression of the *CACTIN-RFP* transgene. Rosette leaves from 35S::CACTIN-RFP plants and Columbia wild-type plants were harvested and RNA isolated using RNeasy Plant Mini Kit (Qiagen 74904, Hilden Germany). DNA was eliminated using RQ1 DNase (Promega M6101, Madison WI) and cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen 18080-044, Grand Island NY). Reactions were conducted in the Roche 480 light cycler using SYBR Green I (Roche 4707516001, Basel, Switzerland). CACTIN transcript was quantified using primers: GAAGAAGAAATGGCATTGTTAGC and TGCTTTGATCGAAATGGAACT (average primer efficiency: 1.86). We used two reference genes (Czechowski et al., 2005): 1) *AT1G58050*, amplified with primers: CCATTCTACTTTTTGGCGGCT and

TCAATGGTAACTGATCCACTCTGATG (average primer efficiency: 1.83); and 2) *AT1G13320*, amplified with primers: TAACGTGGCCAAAATGATGC and GTTCTCCACAACCGCTTGGT (average primer efficiency: 1.89). Primer efficiencies and transcript abundance were calculated using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). The standard deviation derives from the quantification of three independently run technical replicates of the chosen line.

Identification of CACTIN-interacting proteins

RINTIN was identified through a yeast-two-hybrid screen using the coding domains of *CACTIN* as bait and an Arabidopsis seedling-derived cDNA library as prey. We amplified *CACTIN* cDNA using the primers: ATGGGTTCTCATGGTAAGGGTA and TTATCGCCTGTACCTATGTCGTT. In order to keep the plasmid at lower copy number to alleviate toxicity problems, we used CopyCutter EPI400 *E. coli* (Epicentre Biotechnologies EPI400, Madison WI). This allowed us to clone the Arabidopsis *CACTIN* coding domains into pENTR-D-Topo (Invitrogen K2400-20, Carlsbad, CA). This clone was sequenced and was found to contain a silent mutation in base pair number 1,725 from the start codon in the coding domains (T→G). We continued with this construct for use in yeast. The *CACTIN* coding domain was recombined into pDEST32, which contains the ADH1 yeast promoter and an N-terminal Gal4 DNA binding domain. This plasmid was sequenced and mailed to the Indiana University Yeast-Two-Hybrid Facility (http://sites.bio.indiana.edu/~michaelslab/yeast_two_hybrid_facility.html; Scott Michaels Indiana University, Bloomington, IN) where it was used to probe an Arabidopsis leaf and meristem mixed cDNA library in pEX-AD502, which contains a

fusion N-terminal Gal4 activation domain. A total of 10^7 clones were screened for their ability to grow on media lacking histidine plus 25mM 3AT.

For pairwise confirmation of the CACTIN and RINTIN interaction, we cloned the coding domains of *RINTIN* into pDEST22 and cotransformed it and pDEST32-CACTIN into Mav203 yeast as well as the following controls: ATG7 (TAIR: AT5G45900) and ATG12a (TAIR: AT1G54210), which are known interaction partners from the autophagy pathway (Damodaran and Vierstra, 2007) and were given to us in pDEST32 and pDEST22 by the Richard Vierstra laboratory. We used these plasmids together as a positive control and separately with CACTIN and RINTIN as negative controls. Colonies were mixed with water and optical densities at 600nm were measured; cultures were diluted to a standard concentration of yeast (OD600: 0.5). The cultures were then spotted on petri dishes containing –leu –trp media and –leu –trp –his + 25mM 3AT, as shown in Figure 2- 10.

We also tested a number of other candidate genes for pairwise interaction with CACTIN and RINTIN. SmB (AT4G20440), PININ (AT1G15200), and the ankyrin repeat coiled coil candidate (At4G19150) were amplified from Arabidopsis Columbia cDNA using these primers: caccATGTCGATGTCGAAGAGTTCA and TCACTGCTGCTGATTATGTGGT, caccATGGGAGACACCGCCTT and TTAGAGAACCTCATGTTTAATATCTTCC, caccATGGCAGGCAGAAGCG and CTCTTCTTCTTGATCAGCTTCTGTG. These PCR products were gel-extracted and cloned into pENTR-D-Topo. After sequencing they were recombined into pDEST32 and

pDEST22. It is important to note that the At4G19150 amplicon does not include a stop codon. The pDEST32 and pDEST22 vectors are designed for N-terminal fusions and thus this clone results in a C-terminal tag of unplanned amino acids (pDEST32: FLVQSGLMAAK*) (pDEST22: KGGRADPAFLYKVV*). This unplanned tag, or the planned N-terminal DNA-binding-domain tag or transcriptional-activation-domain tag could disrupt the function of any of these proteins.

Phylogenetic analysis

For the CACTIN alignment shown in Figure S1A, the protein sequences were aligned using ClustalW, and colored using BioEdit (Hall, 1999). The GenBank accession number for the protein sequences used were as follows: Arabidopsis: GenBank: NP_171887.1; Grapes: GenBank: CAN74141.1; Moss: GenBank: XP_001780481.1; *S. pombe*: GenBank: NP_595704.1; *C. elegans*: GenBank: NP_001254422.1; Drosophila: GenBank: NP_523422.3; Sea Urchin: GenBank: XP_783684.3; Zebrafish: GenBank: F1Q8W0.1. Human: GenBank: NP_001074012.1; Toxoplasma: GenBank: EEE29532.1.

For the phylogenetic tree shown in Figure S4, protein sequences were aligned in MEGA using ClustalW and were then used to build a neighbor joining tree with pairwise deletion and Poisson modeling with 1000 bootstrap replicates (Tamura et al., 2007). The protein sequences used for the RINTIN homologs were: Arabidopsis: TAIR: AT5G63440.2; Soybean: GenBank: ACU15411; Corn: GenBank: ACF78397; Moss: GenBank: XP_001770071; Human: GenBank: EAW63367; Chicken: GenBank: XP_426277; Drosophila: GenBank: CG16865; *C. elegans*: GenBank: Y66D12A. The protein sequences used for the AT1G49170

homologs were: Arabidopsis: TAIR: AT1G49170.1; Soybean: GenBank: ACU16361; Corn: GenBank: LOC100284194; Moss: GenBank: XP_001768912; Human: GenBank: Q8WUR7; Chicken: GenBank: XP_413838; Drosophila: GenBank: NP_647784; *C. elegans*: GenBank: W01A8; The following prokaryote sequences were also used: Gallionella: GenBank: ZP_04831751; Laribacter: GenBank: YP_002797168; Heliobacterium: GenBank: YP_001680663; *E. coli*: GenBank: ZP_06655036; Nitrosospira: GenBank: YP_411279; Legionella: GenBank: ZP_05110179; and Sideroxydans: GenBank: YP_003525367.

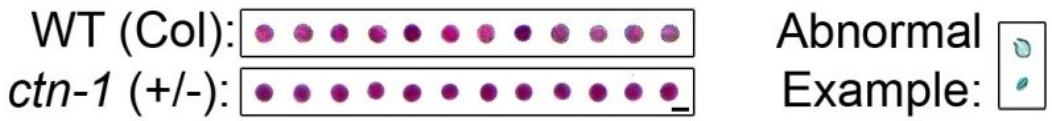


Figure 2- 2: The pollen of ctn-1(+/-) heterozygous plants stain normally with Alexander staining. Alexander staining marks the cytoplasm of pollen magenta and the cell wall teal. Pollen without cytoplasm appear teal, as in the panel showing abnormal pollen. The scale bar is 20μM.

Results

CACTIN is necessary for embryogenesis

To characterize the function of plant *CACTIN*, we identified a T-DNA insertion mutant allele (*ctn-1*) and isolated *ctn-1* (+/-) plants. This T-DNA insertion is located after the 6th exon of *CACTIN* (Figure 2- 1C). The insertion is limited to this position, and flanking sequences along the T-DNA junctions are given in Table 2- 2. Self-crossing of c*tn-1(+/-)* plants resulted in only c*tn-1(+/-)* and *CTN* wild-type plants at approximately a 2:1 ratio. We found 89 heterozygote plants and 49 wild-type plants, which is not statistically different from a 2:1 ratio (X^2=0.29, df=1,p=0.59). The mutant allele of *ctn-1* can be transmitted through the pollen or the ovule, as demonstrated by reciprocal crosses confirmed through genotyping. Although chromosomal abnormalities are

common in Arabidopsis T-DNA lines (Clark and Krysan, 2010), the *ctn-1* allele does not show a common hallmark of this phenomenon because the heterozygous plants have viable pollen as determined by Alexander staining (Figure 2- 2). Therefore, this is a suitable line to use for *CACTIN* analysis.

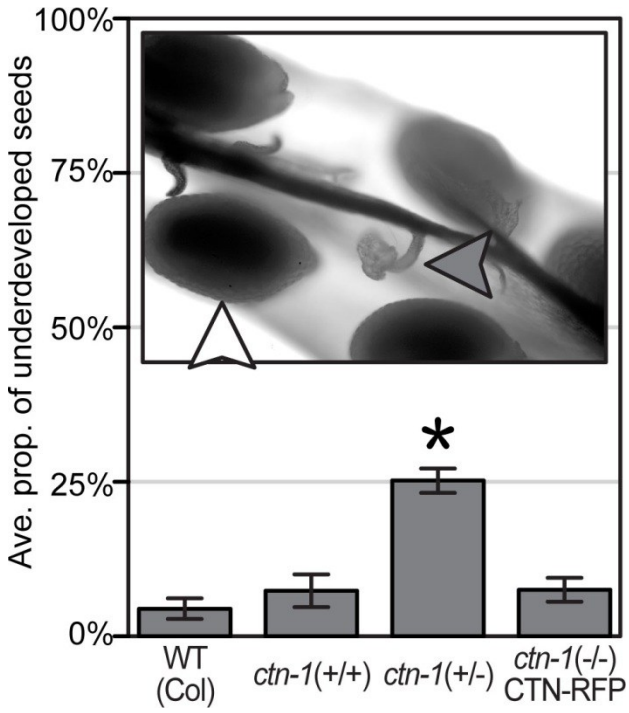


Figure 2- 3: Approximately one fourth of the seeds in the siliques from *ctn-1* heterozygous plants are small and underdeveloped. The height of the bars corresponds to the average proportion of seeds that were underdeveloped in the siliques of the indicated genotype. Examples of underdeveloped and regular seeds are shown in the inset photomicrograph and denoted with grey and white arrowheads respectively. The *ctn-1*(+/+) bar refers to the WT siblings of *ctn-1*(+/-) plants. The asterisk indicates that the average proportion of underdeveloped seeds is significantly different between the *ctn-1*(-/+) plants and all the other plants on the graph (Student's T-Test, $p<10\text{-}5$). The rescued homozygous mutant (*ctn-1*(-/-) 35S::CTN-RFP) does not show different ratios of developmentally arrested seed compared to wild-type plants. Error bars show standard error of the mean. The seeds in 12-37 elongated green siliques were counted for each genotype; the siliques contained an average of 43 seeds. A total of 4091 seeds were counted.

The siliques of the ctn-1(+/-) plants contained 74.8% regular large seeds and 25.2% underdeveloped seeds, whereas wild-type siliques contained 95.6% large seeds and 4.4% underdeveloped seeds (Figure 2- 3). The 25.2% underdeveloped seeds observed in the siliques of the ctn-1(+/-) plants is not significantly different from 25%, which is the expected ratio of homozygous progeny from a segregating population

(X^2=0.02, df=1, p=0.89). We observed this proportion of underdeveloped seeds in the siliques of c*tn-1(+/-)* heterozygous plants through multiple generations after back crossing. The c*tn-1(+/-)* heterozygous plants show an otherwise normal phenotype. These results suggest that the *ctn-1* mutation is embryonic lethal.

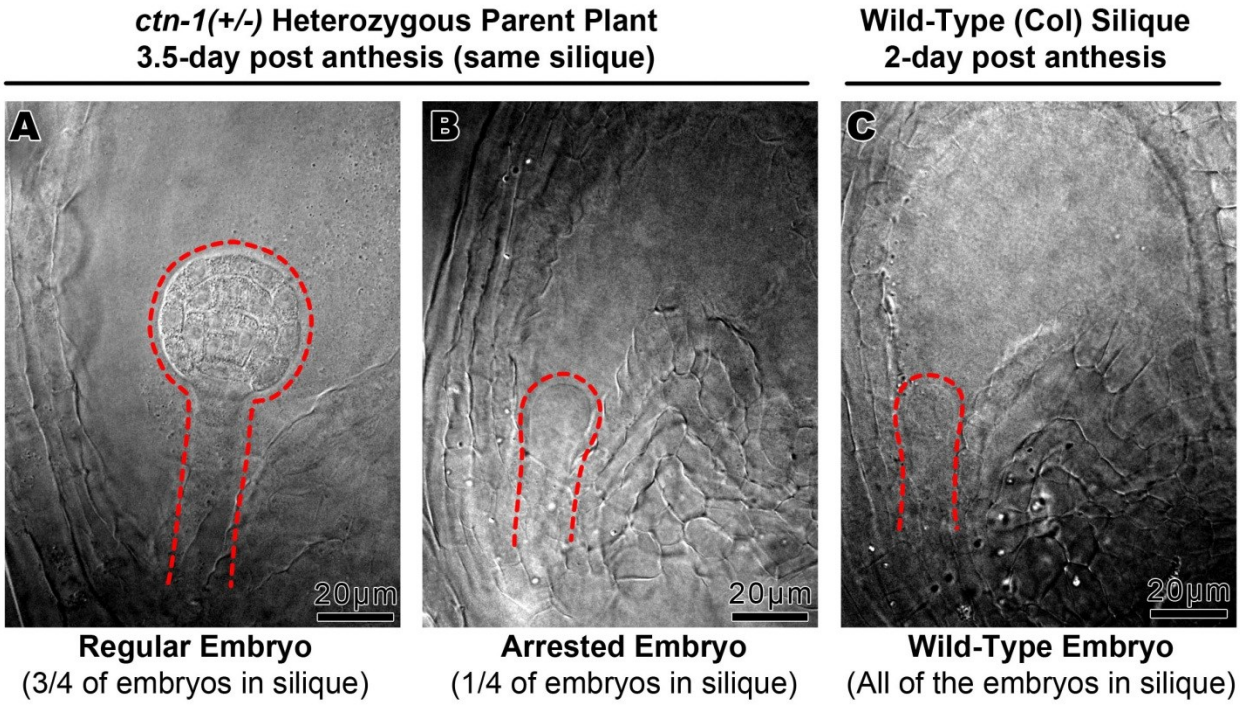


Figure 2- 4: *ctn-1* embryos arrest early in development.
A and B. Photographs of cleared embryos from 3 ½ days post-anthesis self-crossed *ctn-1(+/-)* siliques. Approximately three quarters of the embryos are regularly sized for this stage (A), and one quarter of the embryos arrest at a smaller size (B) that resembles the embryos in a younger silique (C). **C.** An embryo from a two-day post-anthesis wild-type silique.

The *cactin* mutant shows early embryo arrest

Microscopy of the seeds inside the siliques of c*tn-1(+/-)* plants showed approximately ¾ regular embryos and approximately ¼ embryos arresting in the one- to three-cell stage (Figure 2- 4A-B). These 3.5-day post-anthesis arrested embryos looked like two-day post-anthesis wild-type embryos, suggesting that the small embryos

had failed to progress (Figure 2- 4C). These findings suggest that *CACTIN* is required for early embryo development. We observed a similar stage of arrest in the underdeveloped seeds in all of the c*tn-1(+/-)* siliques we examined (3 siliques from each of 11 plants).

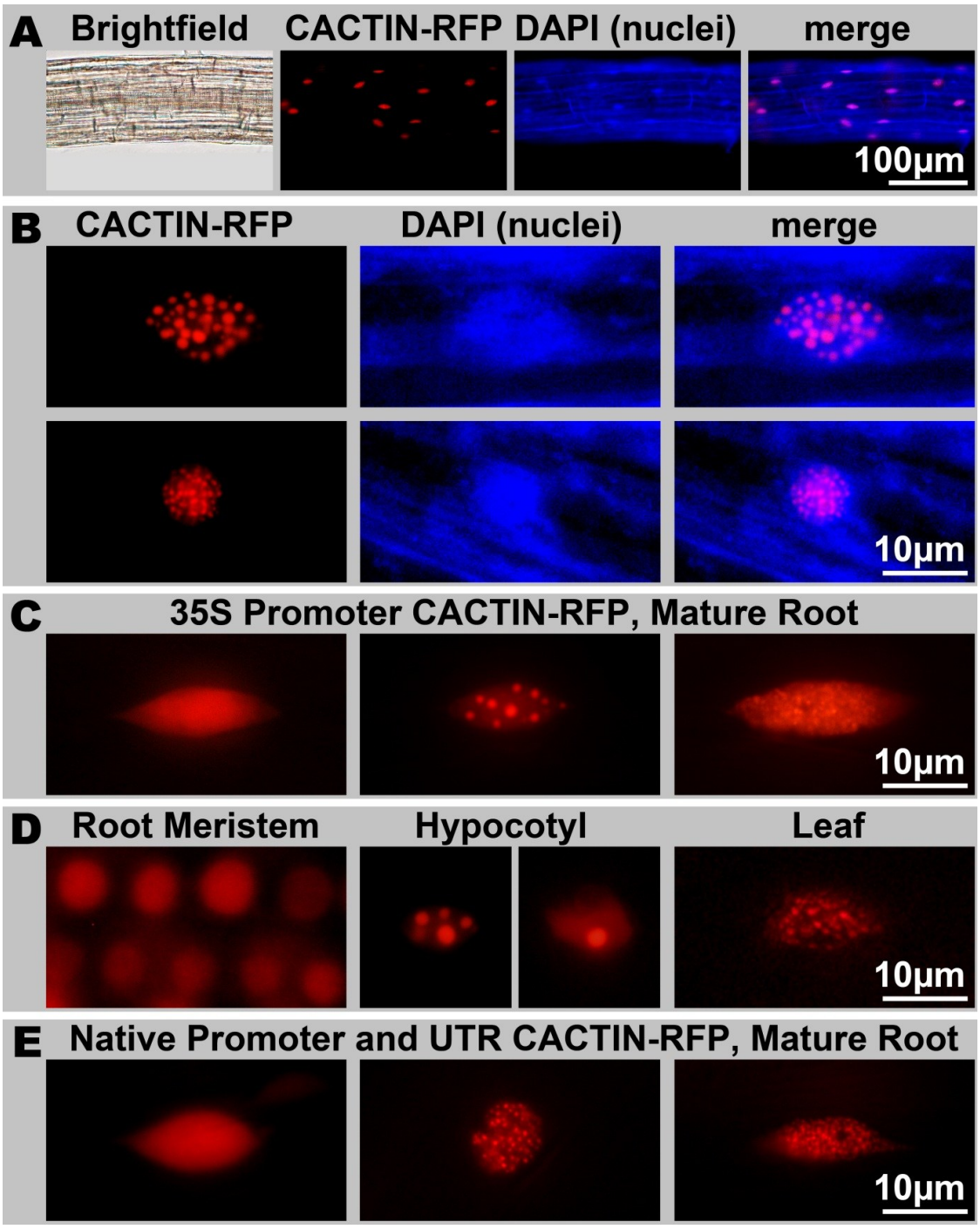
CACTIN-RFP rescues *ctn-1* embryonic lethality and localizes to the nucleus

To verify that the embryonic lethality identified in this mutant is truly due to the *ctn-1* mutation, we generated a CACTIN-RFP construct and introduced it into heterozygous c*tn-1(+/-)* plants under the control of the 35S promoter. Transformed progeny were genotyped and *ctn-1* (-/-) plants were identified, signifying that CACTIN-RFP rescues the *ctn-1* lethality. Backcrossing these plants and allowing the F1 progeny to self-cross allowed us to regenerate homozygous *ctn-1* mutants, all of which contained the CACTIN-RFP transgene, further supporting that CACTIN-RFP rescues the lethal *ctn-1* mutant. The CACTIN-RFP transgene also rescues the underdeveloped seed phenotype (Figure 2- 3). Homozygous *ctn-1* plants with the CACTIN-RFP transgene showed no obvious phenotypic differences from wild-type plants, despite overexpressing *CACTIN* mRNA at 30.5-fold higher levels than wild-type (standard deviation=0.61), as determined by qRT-PCR.

Epifluorescence microscopy shows nuclear localization of 35S promoter driven CACTIN-RFP (Figure 2- 5A). Many cells show a diffuse nuclear localization, especially in younger seedlings (Figure 2- 9A). In the mature root, however, many cells show discrete speckles within the nucleus (Figure 2- 5B). We observed that different cell

types have different sizes and patterns of speckles and that this changes with the age of the seedling (Figure 2- 5C, D). We found the same pattern of localization for a native promoter and native UTR construct (Figure 2- 5E). This speckle phenomenon has been observed for splicing-related proteins (Ali et al., 2003; Docquier et al., 2004; Fang et al., 2004). Fluorescent protein fusions with human CACTIN also show a nuclear speckle localization in cell culture (Atzei et al., 2010a), suggesting that the localization of CACTIN is conserved.

Figure 2- 5 CACTIN-RFP is nuclear and shows a nuclear-speckle pattern in some cells. **(Figure Next Page) A.** Epifluorescence photomicrographs of mature root epidermal cells. **B.** Close-up on nuclei from seedlings grown on agar plates. Diamidino-2-phenylindole (DAPI) was used as a nuclear stain, although it also stains cell walls. **C.** Additional images of nuclei from mature root epidermal cells. Some cells show a diffuse pattern, and some are more speckled. **D.** Examples of nuclei from other tissues of the plant. **E.** A different construct which contains the upstream and downstream regions around the CACTIN gene shows the same localization as the 35S promoter construct (CACTIN-RFP).



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CACTIN-RFP localization becomes more speckled when oxidative phosphorylation is inhibited

Because nuclear speckles are thought to be storage location for inactive splicing components, we tested CACTIN localization under conditions that would reduce splicing. Sodium azide is a potent inhibitor of cytochrome oxidase in the electron transport chain. Although inhibiting respiration has broad effects in cells, translation and transcription become generally repressed meaning that there are fewer pre-mRNAs that require splicing. We found that nuclei with a diffuse CACTIN-RFP localization became speckled within less than a minute after addition of 100mM sodium azide. This same phenomenon has been observed for splicing factors in both plant and animal cells after treatments that would be expected to inhibit splicing (Spector et al., 1983; Misteli et al., 1997; Docquier et al., 2004; Koroleva et al., 2009).

As a negative control for speckle-localization after Sodium azide treatment, we used 35S::PHYTOCHROME B-YFP seedlings. PhyB is a nuclear protein that forms speckles under some conditions but has not been implicated in splicing. PhyB is diffusely nuclear in dark grown seedlings (Christians et al., 2012). Sodium azide treatment did not cause the PhyB signal to change to speckles (Figure 2- 7).

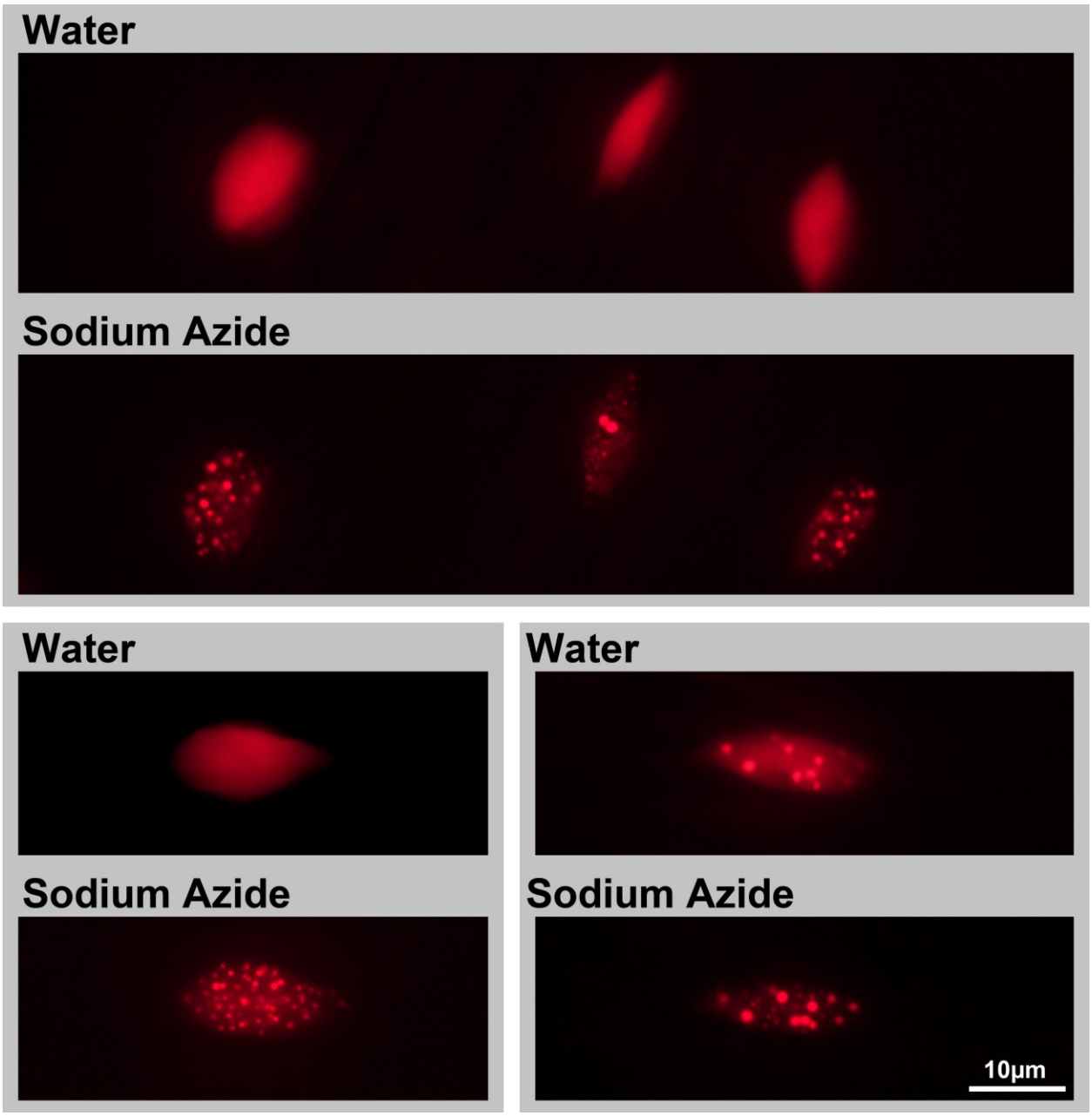

Figure 2- 6: Sodium Azide treatment changes the sub-nuclear localization of CACTIN-RFP causing it to become more tightly speckled.
Each photomicrograph is shown as pair. The top photo is from before addition of sodium azide to the slide and the lower photo is the same nuclei after 100mM sodium azide was added to the slide. Nuclei that started with a diffuse CACTIN-RFP localization changed to speckles within one minute. Nuclei that already showed nuclear speckles changed to having tighter speckles with less diffuse background. Nuclei are from epidermal cells of the mature root of a 4-day old seedling.

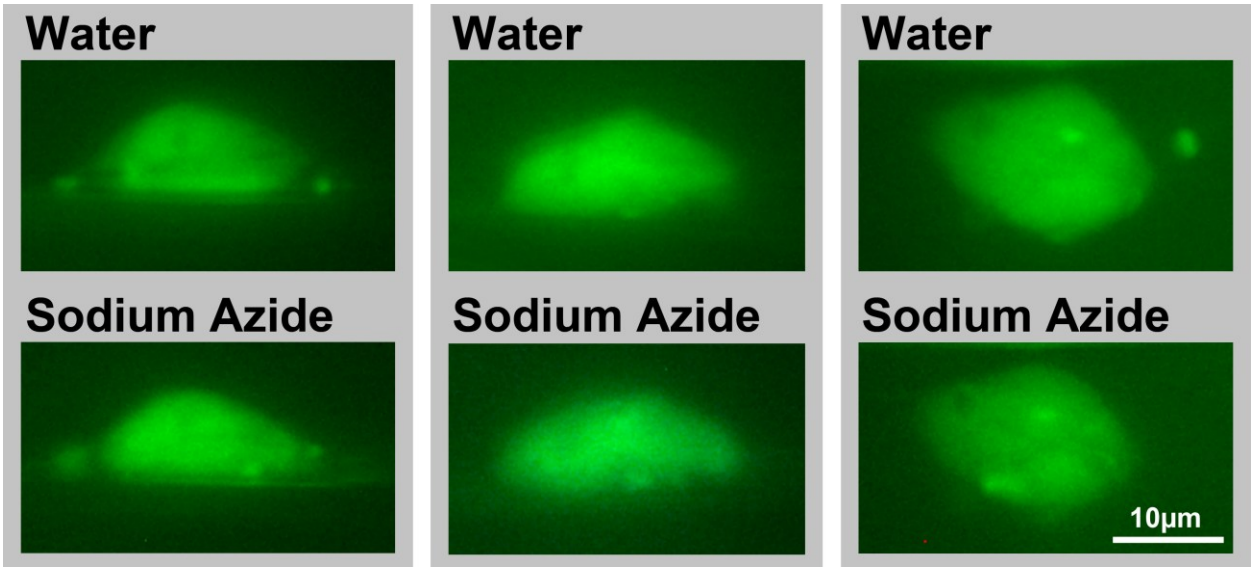

Figure 2- 7: Sodium Azide treatment does not change the localization of PhyB-YFP.
Each photomicrograph is shown as pair. The top photo is from before addition of sodium azide to the slide and the lower photo is the same nucleus after 100mM sodium azide was added to the slide. Nuclei are from epidermal cells of the mature root

CACTIN-RFP colocalizes with known spliceosome components.

Like CACTIN, many spliceosome proteins appear as speckles in the nucleus (Lorković et al., 2008). SR45 is a component of the spliceosome that is thought to function in 5' and 3' site selection (Day et al., 2012). GFP-SR45 colocalizes with CACTIN-RFP. They are both diffusely nuclear in the cells near the root tip and localize to the same discrete speckles in many cells elsewhere in the root (Figure 2- 9A-B). Also, we noticed that GFP-SR45 is more excluded than CACTIN-RFP from a single spot within the nucleus that is likely the nucleolus, although the implications of this are unclear (Figure 2- 8A). Quantification of the colocalization between CACTIN-RFP and GFP-SR45 within root nuclei resulted in an average Pearson coefficient of correlation of 0.96, suggesting high colocalization (Figure 2- 9).

Arabidopsis RSP31 is another SR protein that forms nuclear speckles and is known to be involved with the spliceosome (Docquier et al., 2004). The nuclear speckle pattern of over-expressed fluorescent protein fusions to RSP31 has been confirmed as similar to that obtained by immunolocalization of endogenous RSP31, making it an appropriate splicing speckle marker (Docquier et al., 2004). GFP-RSP31 and CACTIN-RFP colocalize with an average Pearson coefficient of correlation of 0.97, again indicating high colocalization (Figure 2- 8C and Figure 2- 9).

As a negative control for nuclear speckle colocalization, we crossed plants carrying CACTIN-RFP with those carrying 35S::PHYTOCHROME B-YFP. PhyB forms nuclear speckles when plants are moved from the dark to the light, but this protein has not been implicated in splicing (Christians et al., 2012). We saw no correlation between the speckle patterns of CACTIN and PhyB (Figure 2- 8), and the average Pearson coefficient of correlation was 0.58, suggesting low colocalization (Figure 2- 9). The correlation coefficient of colocalization of CACTIN-RFP with GFP-SR45 and GFP-RSP31 is statistically different from that of colocalization with PhyB-YFP (Student's T-test, $p<10^{-4}$). This supports the hypothesis that CACTIN specifically colocalizes with splicing-related protein speckles, and not with any sub-nuclear compartments.

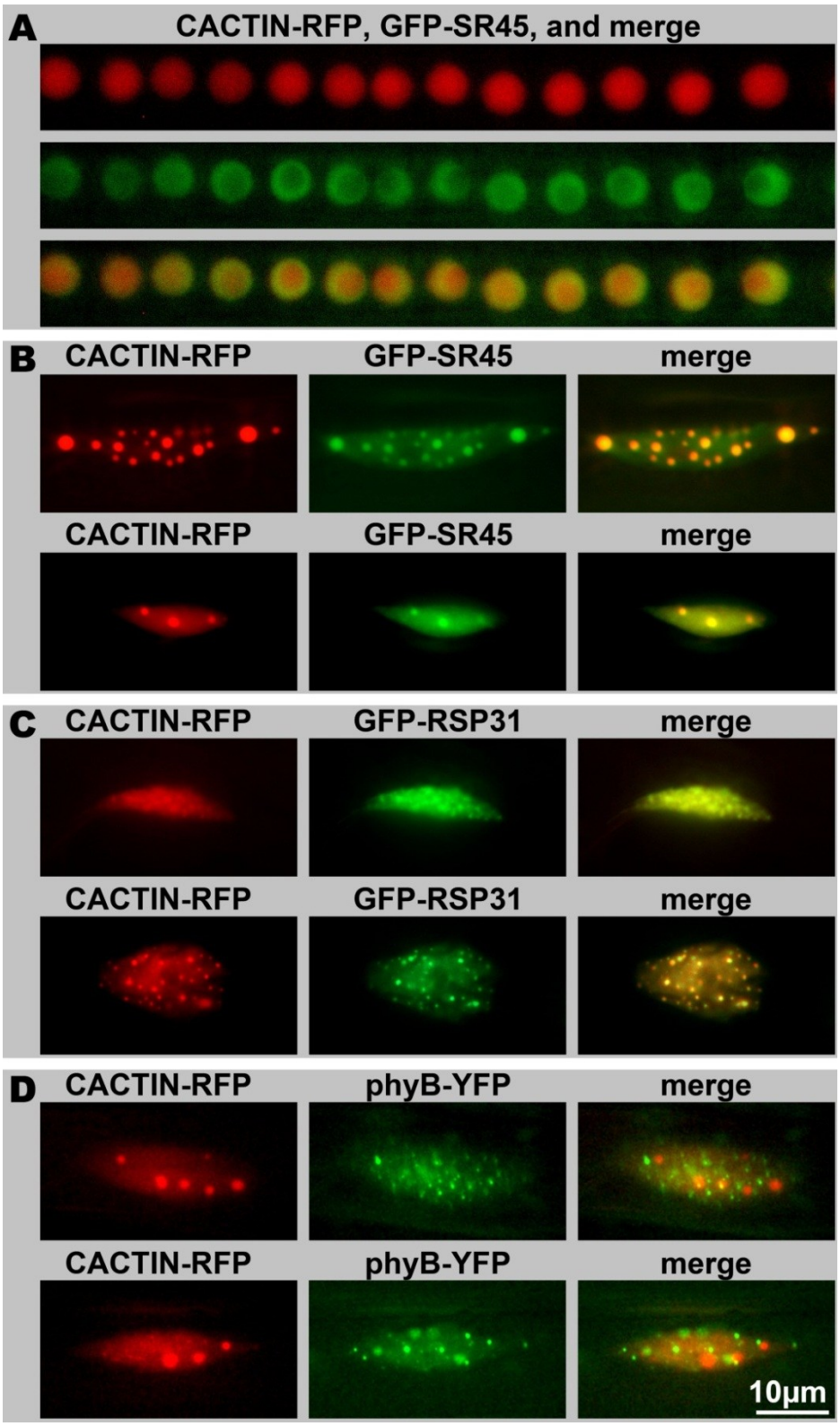

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Figure 2- 8: CACTIN-RFP colocalizes with two spliceosomal proteins.
A. Both CACTIN-RFP and GFP-SR45 show diffuse nuclear localization in many cells of four-day-old seedlings. Imaged from cells in a single cell file in the root meristem, these nuclei show a diffuse pattern without speckles. **B-D**. Mature root nuclei from seven-day-old seedlings grown on plates. These seedlings were 1.5-1.75cm long, and these nuclei are from the root within 0.5cm of the root/shoot juncture. The degree of speckles of these fusions varies between the cells of root, and we show some of the diversity here. These images are representative of dozens of images, and multiple transformed lines. **B:** CACTIN-RFP colocalizes with GFP-SR45. **C:** CACTIN-RFP colocalizes with GFP-RSP31. **D:** CACTIN-RFP does not colocalize with PhyB.

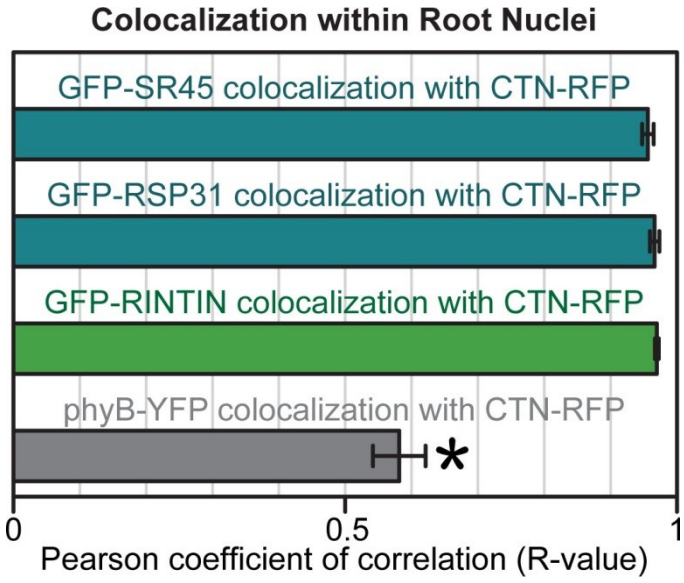

Figure 2- 9: CACTIN colocalizes with spliceosomal proteins and GFP-RINTIN. Quantification of colocalization using "Coloc2" analysis within the FIJI plugin for image J. Zero indicates no colocalization and one indicates absolute correlation. Representative images of those used for quantification are shown in Figure 2- 8 and Figure 2- 15. phyB was used as a negative control. The difference in colocalization between the negative control and the other samples is statistically significant (T-Test $p<10^{-4}$). Error bars show standard error of the mean. The number of nuclei measured for each comparison from top to bottom of the graph were 17, 13, 19, & 8.

CACTIN interacts with the conserved gene *RINTIN*

Arabidopsis CACTIN interacts by yeast-two-hybrid with the conserved protein, AT5G63440, which we have named RINTIN (RIT) for **R**elationship with CACTIN (Figure 2- 10). In Drosophila, the orthologs of RINTIN and CACTIN also bind each other in high-throughput yeast-two-hybrid studies (Giot et al., 2003). Because the same orthologous gene came up in CACTIN-binding screens conducted in such distantly related organisms, we believe RINTIN is a true interacting partner with CACTIN.

Like *CACTIN*, *RINTIN* is found in plants and animals and is missing from *S. cerevisiae*. However, unlike *CACTIN*, *RINTIN* may have been lost in a larger group within the fungi kingdom, not just subphylum Saccharomycotina. RINTIN has no recognizable informative functional domains, and its function is unknown. *RINTIN* has a **S**horter distant paralog, *SINTIN* (*SIT*, *AT1G49170)*, which is present in eukaryotes, and there is also a relative in bacteria (*E. coli*: YGGU). RINTIN, SINTIN, and YGGU share a Domain of Unknown Function (DUF) #167. Two prokaryote DUF167 proteins have been structurally analyzed by NMR, but their functions have not been explored (Yee et al., 2002; Aramini et al., 2003). In phylogenetic trees, the longer DUF167 proteins (*RINTIN* orthologs) of eukaryotes all cluster together separately from the shorter DUF167 proteins (*SINTIN* orthologs), suggesting that they diverged in the eukaryotic common ancestor and have been maintained separately ever since then (Figure 2- 11).

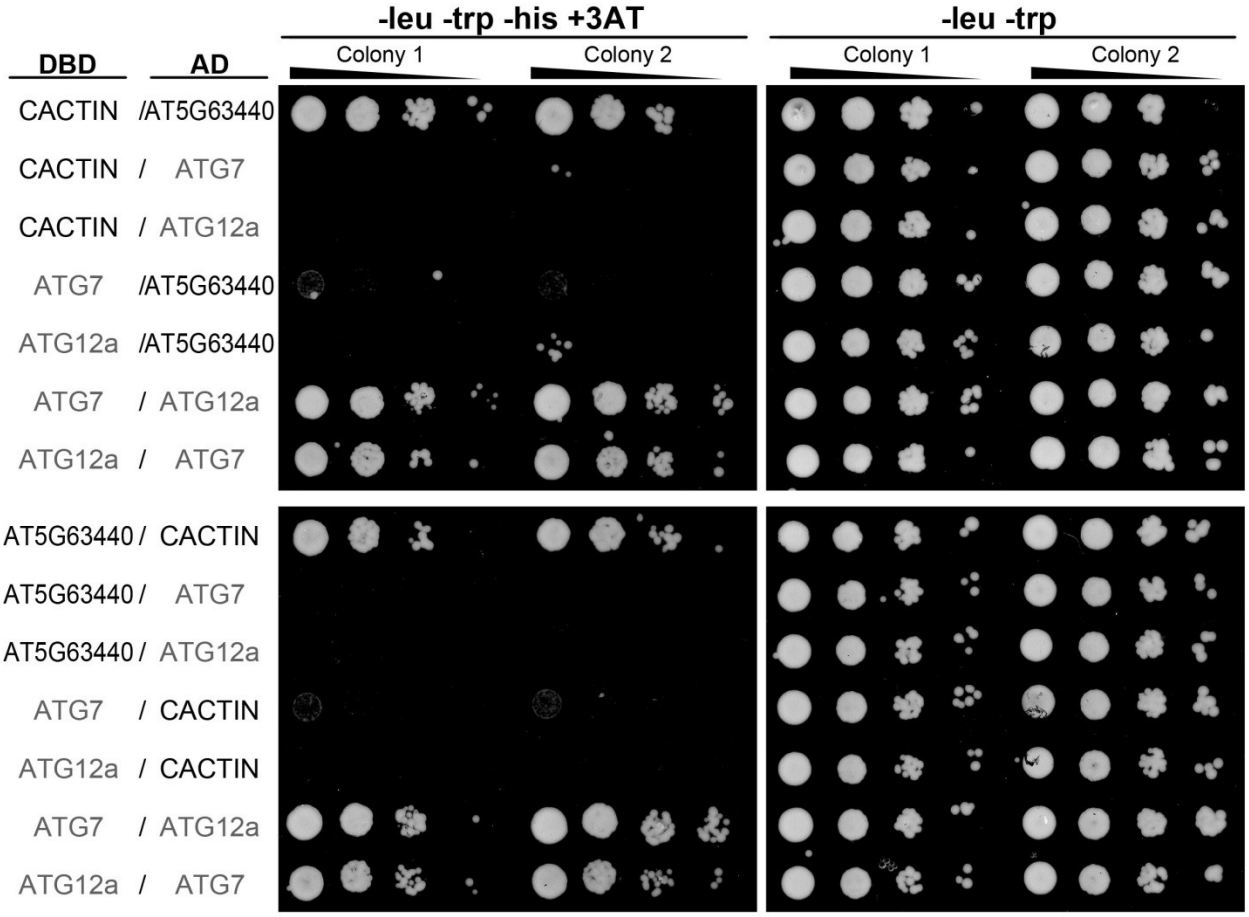

Figure 2- 10: CACTIN binds RINTIN in a yeast two-hybrid assay.
The left panel shows yeast growth on media that select for yeast cells displaying protein-protein interactions (-leu –trp –his +3AT) whereas the right panel illustrates growth on media that select for yeast cells carrying both the bait and prey vectors (-leu -trp), but do not select for cells displaying protein-protein interaction. The first protein listed corresponds to the gene in the bait vector pDEST32 (which makes a protein fusion with the GAL4 DNA binding domain (DBD)), and the second one corresponds to the gene in the prey vector pDEST22 (which makes a protein fusion with the GAL4 transcriptional activation domain (AD)). The genes ATG7 and ATG12a were used as positive controls with each other and negative controls with CACTIN and RINTIN (Damodaran and Vierstra, 2007). Two different colonies are shown for each protein combination. The plates were spotted with 5µl of four sequential serial 10-fold dilutions of cultures.

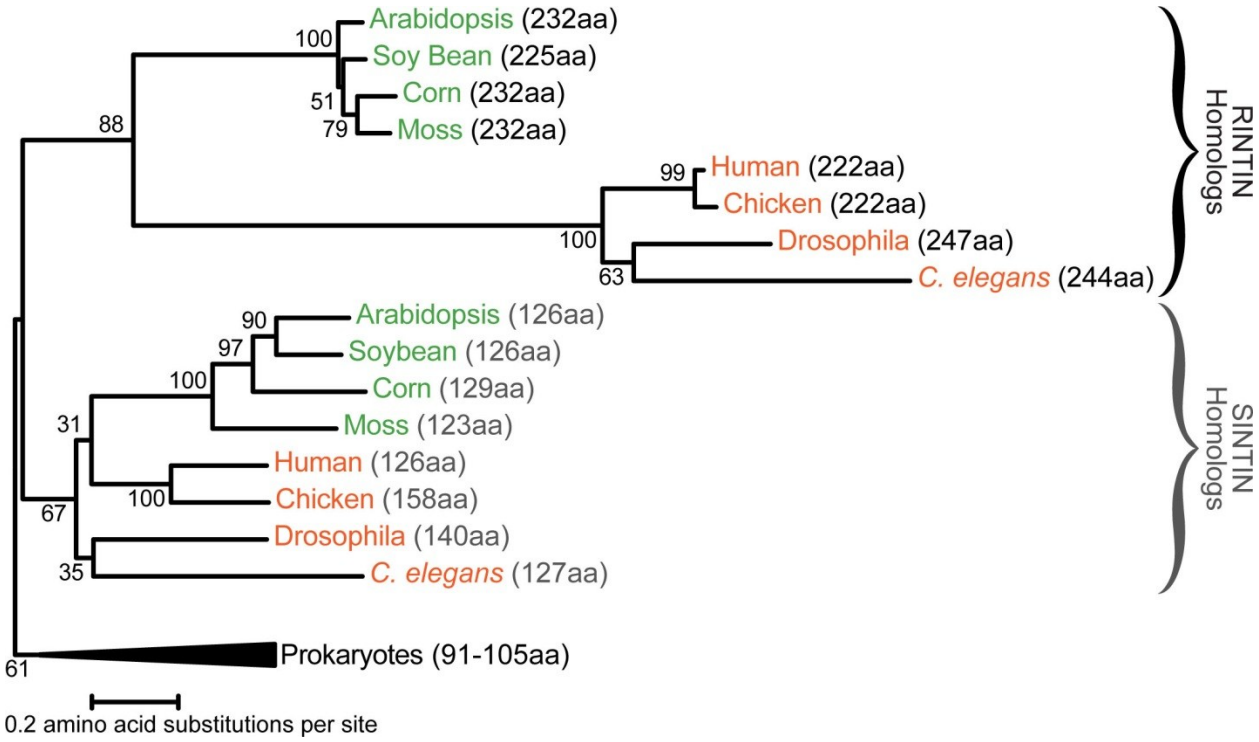

Figure 2- 11 The RINTIN orthology group and the SINTIN orthology group have been maintained in eukaryotes since the common ancestor.
Neighbor joining phylogenetic tree of RINTIN and SINTIN proteins. Sequences from plants and animals are shown in green and orange, respectively. Bootstrap numbers are given at branch points. The length of the protein in amino acids is indicated. Sequence accession and algorithm details are described in the materials and methods section.

We examined two Arabidopsis *RINTIN* mutants (*rit-1* and *rit-2*) and found no abnormal phenotypes (Figure 2- 12 and Figure 2- 13). We also made double mutants between *rit-1* and a mutant in its distant paralog, *sit-1,* which also have a normal phenotype under standard growth conditions (Figure 2- 13). The mutational inserts of *rit-1* and *sit-1* disrupt the expression of their respective full-length mRNAs (Figure 2- 14).

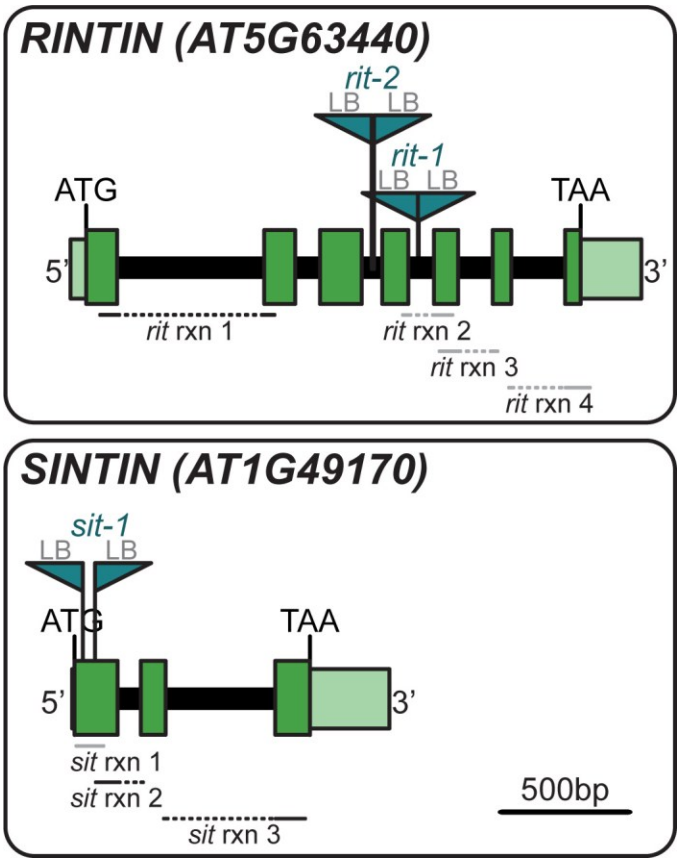

Figure 2- 12 Gene diagram of RINTIN and SINTIN.
Introns are represented by black rectangles, the exons as dark green boxes, and the UTRs as light green boxes. The translational start and stop codons are also shown. The positions of the T-DNA insertions for *rit-1*, *rit-2*, and *sit-1* are shown as triangles. All of these alleles have left-borders on both sides of the insert. The lines under the gene diagrams represent the cDNA amplicons used to assay expression from *rit-1* and *sit-1*. Dotted lines show where the amplicon spanned an intron. Black lines indicate that amplicons were obtained for the mutant, and grey indicates that they were not. The gels for these reactions are shown in Figure S7, and primer sequences are shown in Table 2- 3.

Although the function of RINTIN and its homologs is unknown, it associates with the spliceosome in Drosophila and human cells (Bessonov et al., 2008; Herold et al., 2009). We made *GFP-RINTIN* constructs, transformed them into Arabidopsis, and crossed these plants with those carrying CACTIN-RFP. Like CACTIN, RINTIN has a diffuse nuclear localization in many cells but also shows a nuclear speckle pattern in some cells. GFP-RINTIN colocalizes with CACTIN-RFP, and we used cells showing a speckle pattern to measure an average Pearson coefficient of correlation of 0.97, which is significantly different from the negative control (Figure 2- 15 and Figure 2- 9).

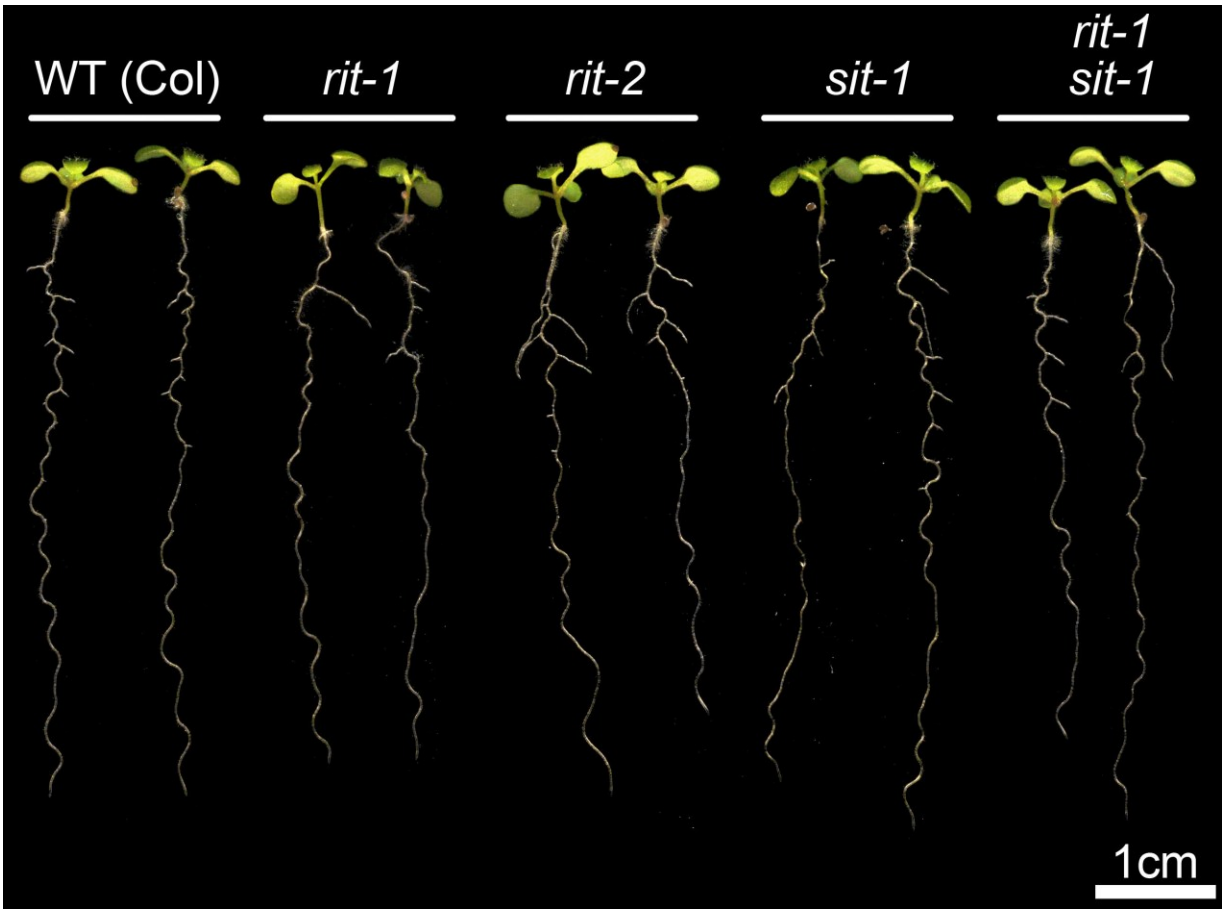

Figure 2- 13: *rit-1*, *rit-2*, *sit-1*, and *rit-1 sit-1* mutants have the same phenotypes as wild-type plants under standard growth conditions.
Mutant allele information is given in Figure 2- 12 and Table 2- 2. Primers used for genotyping these lines are given in Table 2- 1, and gels showing the extent of expression of the mutated genes in these lines are given in Figure 2- 14.

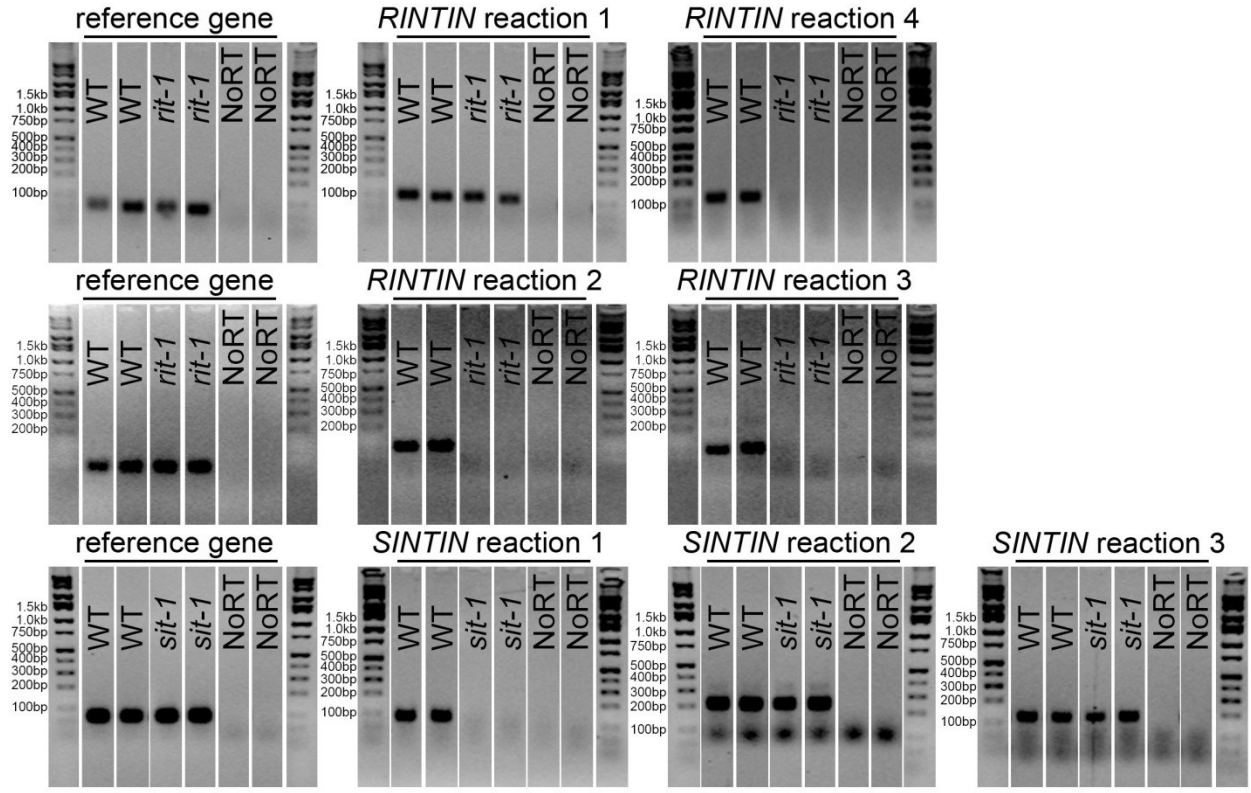

Figure 2- 14 The *rit-1* and *sit-1* mutations result in altered transcripts.
Gels showing products from Reverse Transcription-PCR using various primer pairs across the *RINTIN* and *SINTIN* transcripts. To confirm that all samples contained cDNA, we used the reference gene TAIR: *AT1G13320*. The primer sequences and positions relative to the *rit-1* and *sit-1* insertional mutations are shown in Table 2- 1. Each row corresponds to reactions (rxn) done at the same time and ran on one gel. ColWT= control Columbia wild-type plant cDNA sample. NoRT= Negative control where no reverse transcriptase was added to a plant RNA sample. On each gel, one NoRT sample is the same RNA as one of the WT samples on the same gel, and one of the mutant samples. Molecular weight markers (Minnesota Molecular Hi-Lo Marker, Minneapolis, MN) are shown on the sides of each gel and the size of the bands is shown on the left of each gel. ColWT and NoRT samples were scattered within the samples as a better control for cross contamination. The lanes were re-ordered in Adobe Photoshop for the ease of the reader.

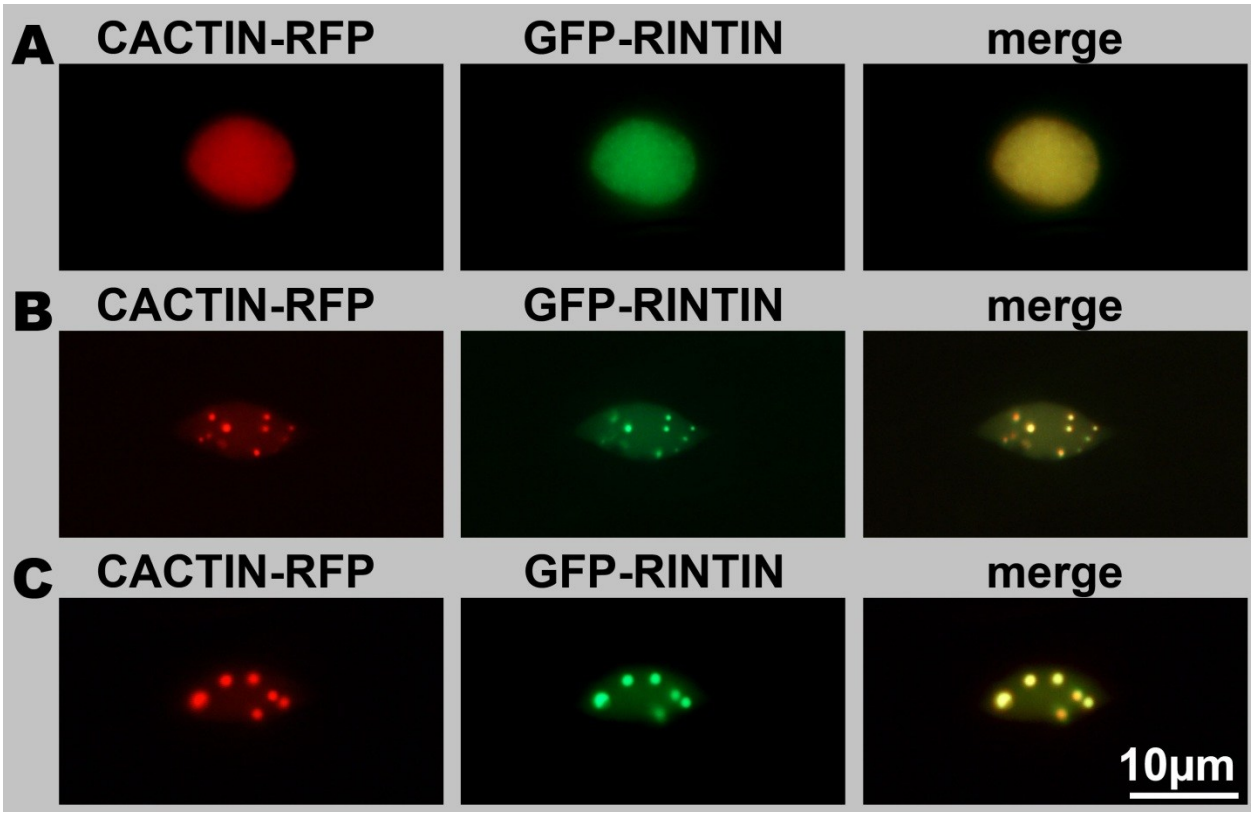

Figure 2- 15 CACTIN-RFP and GFP-RINTIN colocalize.
Mature root nuclei from eight-day-old seedlings grown on plates. These seedlings were 1.5-1.75cm long, and these nuclei are from the root within 0.5cm of the root/shoot junction. These images are representative of dozens of images. A. A nucleus with a diffuse pattern of both GFP-RINTIN and CACTIN-RFP. B and C. Colocalized speckles.

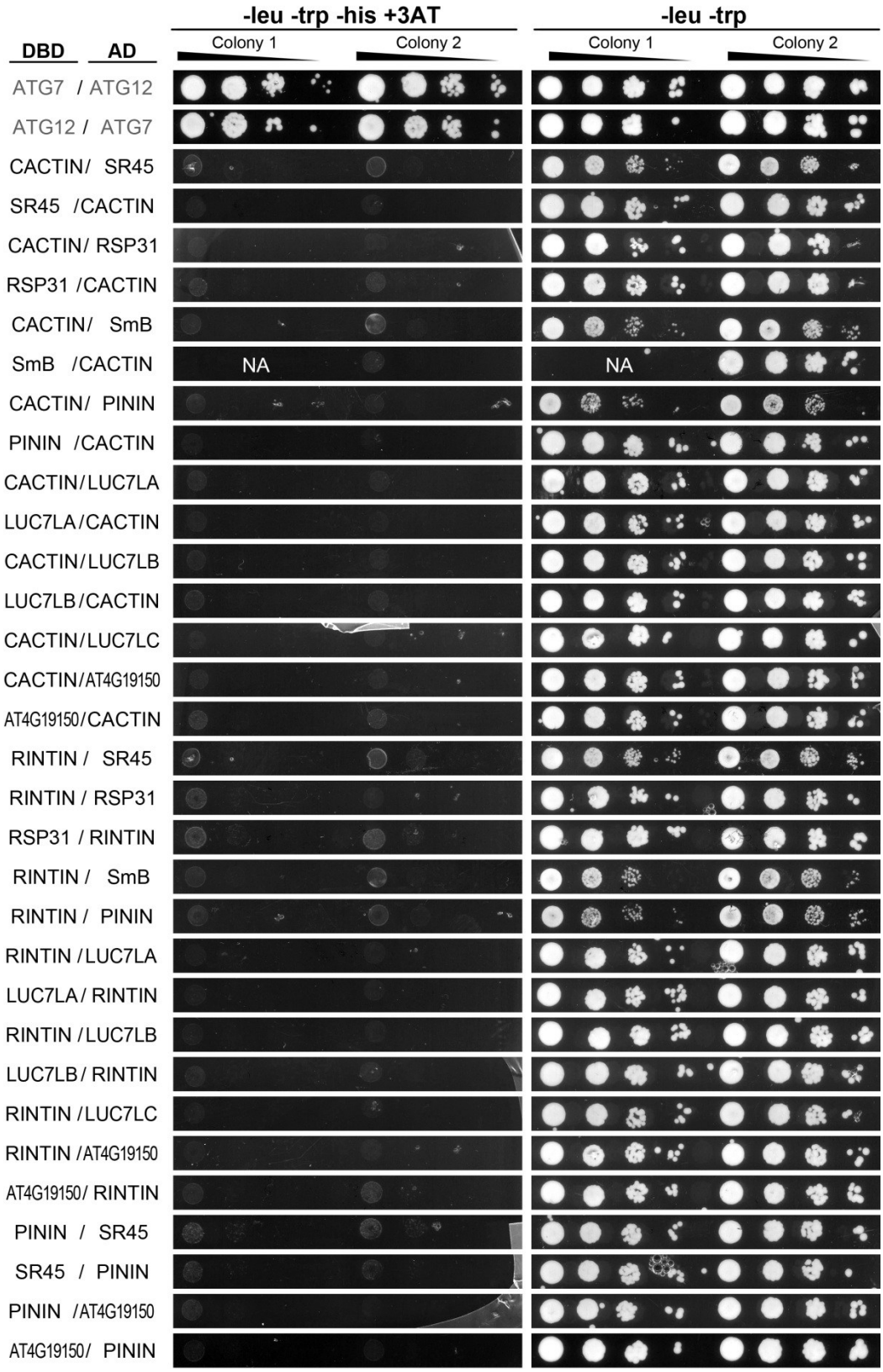

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Figure 2- 16: CACTIN & RINTIN don't bind to some spliceosome components in a pair-wise fashion.
(Figure is on previous page) The left panel shows yeast growth on media that select for yeast cells displaying protein-protein interactions (-leu –trp –his +3AT) whereas the right panel illustrates growth on media that select for yeast cells carrying both the bait and prey vectors (-leu -trp), but do not select for cells displaying protein-protein interaction. The first protein listed corresponds to the gene in the bait vector pDEST32 (which makes a protein fusion with the GAL4 DNA binding domain (DBD)), and the second one corresponds to the gene in the prey vector pDEST22 (which makes a protein fusion with the GAL4 transcriptional activation domain (AD)). We found no-pairwise interactions between CACTIN or RINTIN with SR45, RSP31, SmB, PININ, Luc7LA, Luc7LB, Luc7LC, and AT4G19150.

Discussion

Our results show that CACTIN is an important plant protein required for embryogenesis. CACTIN-RFP is nuclear and shows nuclear-speckle localization in some cells. We found a physical interaction between CACTIN and another conserved protein, RINTIN, and the corresponding fluorescent protein fusions colocalize in Arabidopsis seedlings.

Multiple findings have led us to propose that CACTIN may be involved with the spliceosome. We noticed that the N-terminal region of Arabidopsis CACTIN is rich in arginine and serine. Many spliceosomal proteins contain arginine and serine-rich RS domains, which are thought to aid in protein-protein interactions within the spliceosome. The CACTIN protein from humans also has a possible RS domain as identified by bioinformatics (Boucher et al., 2001). Additionally, orthologs of CACTIN and its yeast-two-hybrid binding partner, RINTIN, have both pulled down with the spliceosome in animal cells (Jurica et al., 2002; Bessonov et al., 2008; Herold et al., 2009). Like many spliceosome components, CACTIN and RINTIN show nuclear-speckle localization, and CACTIN also colocalizes with known spliceosome proteins, SR45 and RSP31. CACTIN

is also like splicing components in that treatments that would be expected to reduce splicing, such as sodium azide treatment, cause CACTIN-RFP to localize to tighter speckles.

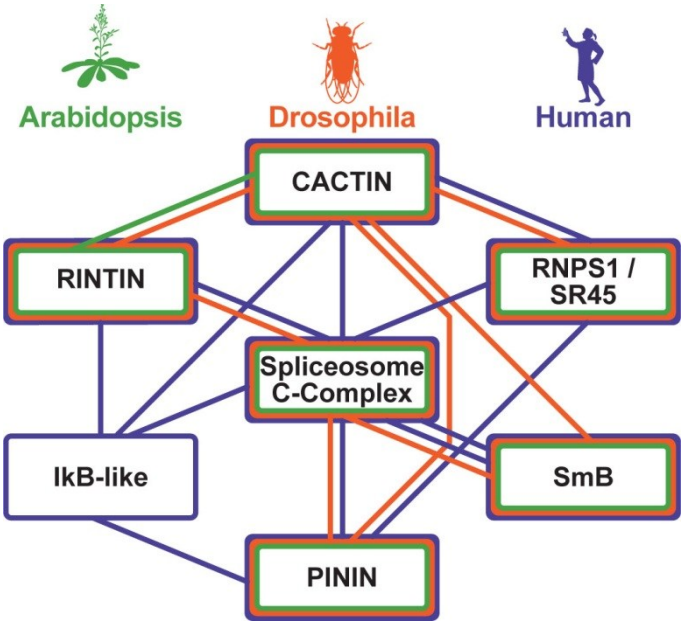

Figure 2- 17 CACTIN may interact with components of the spliceosome. Each box represents a protein group of orthologs. The colors of the lines around the boxes indicate which organisms contain gene encoding that protein. The lines that connect the boxes indicate physical interactions identified from the literature, and the color represents the organism in which the experiment was conducted. Each interaction and reference is detailed in the supplemental **Table 2- 4**.

Data mining from high-throughput interaction databases and from the literature also suggests a connection between CACTIN and the spliceosome. Yeast-two-hybrid and tagged-immunoprecipitation mass spectrometry experiments in Drosophila and humans show physical interaction between CACTIN and other proteins associated with the spliceosome, such as RNPS1, whose closest sequence match in Arabidopsis is SR45 (Giot et al., 2003; Ewing et al., 2007). Drosophila CACTIN also physically interacts with Small nuclear ribonucleoprotein-associated protein B (SmB), a core component of the spliceosome (Saltzman et al., 2011). Figure 2- 17 shows a summary of protein-protein interactions involving CACTIN and RINTIN. We have tested Arabidopsis CACTIN for pair-wise interaction in Yeast-2-Hybrid with Arabidopsis SR45

and obtained negative results (Figure 2- 16). Although this could be due to any number of technical problems with Yeast-2-Hybrid, it could also be due to Arabidopsis CACTIN needing other proteins in order to bind with SR45 and that a pairwise situation is not sufficient for binding.

Both CACTIN and RINTIN physically interact with human IκB-like (Ewing et al., 2007; Atzei et al., 2010a). IκB-like was named because it has ankyrin repeats like IκB proteins (Albertella MR, 1994). However, ankyrin repeats are now known to be one of the most common structural domains in all of nature (Li et al., 2006). There is little evidence that IκB-like shares functions with IκB, and these two proteins have different subcellular localizations (Atzei et al., 2010a). Also, only IκB-like has shown physical association with the spliceosome (Bessonov et al., 2008). Orthologs of IκB-like are difficult to find in invertebrates or plants because the prominent ankyrin repeats make traditional BLAST searches unproductive. Instead, we used a domain-feature approach to find orthologs. Other than ankyrin repeats, IκB-like only contains one additional recognizable feature, a coiled-coil domain, which is an extremely common protein-protein interaction domain. Arabidopsis contains approximately 100 Ankyrin repeat proteins (Li et al., 2006). Of these, only one has only ankyrin repeats and coiled coil domains (AT4G19150). Not very much is known about this protein, although it does show a nuclear localization in the root meristem, like CACTIN (Figure 2- 5D) (Cutler et al., 2000). We tested Arabidopsis CACTIN and AT4G19150 for pairwise interaction in yeast-two-hybrid, and observed negative results. Very little can be interpreted from negative yeast-two-hybrid results.

Table 2- 4 List of CACTIN interacting proteins corresponding to Figure 2- 17. legend: next page.

Binding Partners from Literature				Experiment, Citation		
Name	Ortholog IDs	Name	Ortholog IDs	Material Source	Technique	Reference
CACTIN	C19orf29, FBgn0031114, AT1G03910	RINTIN	CXorf56, FBgn0035415, RINTIN	Arabidopsis Seedlings	Y2H Screen	This Work
				Pooled Fly Stages	Y2H Screen	(Giot et al., 2003)
CACTIN	C19orf29, FBgn0031114, AT1G03910	RNPS1/ SR45	RNPS1, FBgn0037707, AT1G16610	Pooled Fly Stages	Y2H Screen	(Giot et al., 2003)
				Human emb. kid. cells	tagIP-HTMS	(Ewing et al., 2007)
CACTIN	C19orf29, FBgn0031114, AT1G03910	PININ	PNN, FBgn0037737, AT1G15200	Pooled Fly Stages	Y2H Screen	(Giot et al., 2003)
CACTIN	C19orf29, FBgn0031114, AT1G03910	Spliceosome (Catalytic C-Complex)		HeLa cell nuc. extract	RNA-MS	(Jurica et al., 2002)
CACTIN	C19orf29, FBgn0031114, AT1G03910	IkB-like	IKBL, No Clear Fly or Plant Ortholog	Human emb. kid. cells	tagPairwise IP	(Atzei et al., 2010a)
CACTIN	C19orf29, FBgn0031114, AT1G03910	CACTIN	C19orf29, FBgn0031114, AT1G03910	Toxoplasma Cactin cDNA	Pairwise Y2H	(Szatanek et al., 2012)
CACTIN	C19orf29, FBgn0031114, AT1G03910	SmB	SmB, FBgn0262601, AT4G20440	Pooled Fly Stages	Y2H Screen	(Giot et al., 2003)
RINTIN	CXorf56, FBgn0035415, RINTIN	IkB-like	IKBL, No Clear Fly or Plant Ortholog	Human emb. kid. cells	tagIP-HTMS	(Ewing et al., 2007)
RINTIN	CXorf56, FBgn0035415, RINTIN	Spliceosome (Catalytic C-Complex)		Fly Kc Cells	RNA-MS	(Herold et al., 2009)
				HeLa cell nuc. extract	RNA-MS	(Bessonov et al., 2008)
RNPS1/ SR45	RNPS1, FBgn0037707, AT1G16610	PININ	PNN, FBgn0037737, AT1G15200	Human emb. kid. cells	tagIP-HTMS	(Ewing et al., 2007)
RNPS1/ SR45	RNPS1, FBgn0037707, AT1G16610	Spliceosome (Catalytic C-Complex)		HeLa cell nuc. extract	RNA-MS	(Bessonov et al., 2008)
IkB-like	IKBL, No Clear Fly or Plt Ortholog	Spliceosome (Catalytic C-Complex)		HeLa cell nuc. extract	RNA-MS	(Bessonov et al., 2008)
IkB-like	IKBL, No Clear Fly or Plt Ortholog	PININ	PNN, FBgn0037737, AT1G15200	Human emb. kid. cells	tagIP-HTMS	(Ewing et al., 2007)
PININ	PNN, FBgn0037737, AT1G15200	Spliceosome (Catalytic C-Complex)		Fly Kc Cells	RNA-MS	(Herold et al., 2009)
				HeLa cell nuc. extract	RNA-MS	(Jurica et al., 2002)
PININ	PNN, FBgn0037737, AT1G15200	Spliceosome (Catalytic C-Complex)		HeLa cell nuc. extract	RNA-MS	(Bessonov et al., 2008)
SmB	SmB, FBgn0262601, AT4G20440	Spliceosome (Catalytic C-Complex)		HeLa cell nuc. extract	RNA-MS	(Bessonov et al., 2008)
				HeLa cell nuc. extract	RNA-MS	(Jurica et al., 2002)
				Fly Kc Cells	RNA-MS	(Herold et al., 2009)

Table 2- 4: The colors of the text represent the organism the gene or experiment was conducted in (see Figure 2- 5). **Y2H Screen =** Yeast-two-hybrid screen. **tagIP-HTMS =** Proteins were epitope tagged and immunoprecipitated, and binding partners were identified through high-throughput mass spectrometry. **RNA-MS =** The spliceosome was pulled down using an affinity tagged pre-mRNA with a splice-site mutation, and binding partners were identified through mass spectrometry. **tagPairwiseIP =** Two candidate genes were epitope-tagged and tested for interaction using immunoprecipitation and western blots. **Pairwise Y2H =** Two candidate genes were tested for interaction using a yeast-two-hybrid.

Drosophila CACTIN, human RNPS1, and human IκB-like have been found physically associated with another spliceosome-related protein called PININ (Giot et al., 2003; Ewing et al., 2007). Animal PININ has a role in cell-cell adhesion as well as physical and functional connections to splicing (Jurica et al., 2002; Bessonov et al., 2008; Herold et al., 2009; Leu et al., 2012). PININ also has an ortholog in plants (TAIR: AT1g15200); however its function has not been investigated. PININ and CACTIN may together contribute to splicing in eukaryotes including Arabidopsis. We tested CACTIN and PININ for interaction in Yeast and obtained negative results (Figure 2- 16).

The data from Figure 2- 17 is largely from high-throughput screens that have not been tested using alternate methods. However, the number of CACTIN and spliceosome connections is striking, and combined with our findings suggest that CACTIN's role in splicing deserves further investigation. Most of these literature findings relied on immunoprecipitation techniques that allow for clusters of proteins binding each other. We hypothesize that Arabidopsis CACTIN's interaction with the spliceosome requires multiple proteins due to our negative results using pairwise yeast-two-hybrid, although there are also other possible explanations for these observations. It is also interesting to note that the loss of CACTIN and RINTIN within parts of the fungi lineage could correlate with the reduced complexity of the spliceosome in that phylum.

Although the molecular function of CACTIN remains unclear, our research suggests that CACTIN may have a role in the spliceosome. *cactin* mutants or plants with reduced CACTIN may have different splicing efficiency or changed alternative splicing choices, which could result in broad changes in gene expression and expansive problems for cell function. However, it remains possible that CACTIN modulates gene expression through a different mechanism, perhaps as a transcription factor or by linking transcription and splicing.

Chapter 2 Acknowledgements

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Chapter 3: Transgenic rescue experiments and genetic complementation tests suggest the phenotypes of *cactin-2* are not due to loss of *CACTIN*.

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Abstract

CACTIN is a conserved eukaryotic gene that likely functions in splicing. In Arabidopsis, the homozygous *ctn-1* allele causes very early embryo arrest, which makes further analysis of the mutant challenging. Recently, a new viable allele of CACTIN became available. In this chapter, we present the results of the characterization of *cactin-2* (*ctn-2*) plants. These plants have a T-DNA insertion near the end of *CACTIN* that results in a stop codon that truncates the last three amino acids of CACTIN, which are highly conserved. We found that the *ctn-2* plants are smaller and paler than wild-type plants. These abnormal phenotypes co-segregate with the mutation, and are recessive. Unexpectedly, a complementation test with *ctn-1* resulted in F1s with an intermediate phenotype, and transgenic wild-type CACTIN failed to rescue the phenotype. These findings have led us to propose that the phenotypes associated with *cnt-2* are likely caused by disruption of another linked gene.

[2] Elizabeth Dinh is an undergraduate student who worked with Katherine Baldwin. She sequenced the junctions of the insertional *ctn-2* allele, and helped with plant-care.

Introduction

Plants with homozygous mutations in *ctn-1* arrest at the one- to three-cell stage of embryogenesis (Baldwin et al., 2013; chapter 2 of this thesis). These plants carry a large T-DNA insert in the middle of CACTIN (Figure 3- 2); *ctn-1* might be a functional knock out. The experiments described in chapter 2 led us to propose that CACTIN functions in splicing. One way to test this hypothesis is to look for mis-splicing in a loss-of-function mutant. However, the only allele available resulted in lethality, and the artificial micro RNAs we used to target *CACTIN* resulted in only mild silencing (see appendix 1). An alternative arose when we obtained a new allele of *CACTIN* that was found in a forward screen in another lab due to its pleiotropic phenotypes. In this chapter we describe our attempts to test the validity of this allele as a tool for studying CACTIN.

Methods

Plant materials and genotyping

The *ctn-1* mutant line was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). The *ctn-2* allele was generously sent to us by Linda Savage and Professor Robert Last at Michigan State University. Seeds were surface-sterilized with 95% ethanol and sown onto half-strength Linsmaier & Skoog media containing 1.5% sucrose (Caisson Laboratories LSP04-1LT, Logan, UT) and supplemented with 1.5% agar type E (Sigma, A4675-1KG, St Louis, MO). The plates were stratified at 4°C in the dark for 2 days before being placed in a growth

chamber (16 hours light (50-70 µmol $m^{-2}s^{-1}$) and 8 hours dark, 22°C, 70% relative humidity).

Plants were moved to soil after approximately 10 days of growth on petri plates. Genotyping was performed using standard PCR methods and the primers in Table 3- 1.

Table 3- 1: Allele numbers and genotyping primer information for *ctn-1* and *ctn-2*.

Allele		Gene	Rxn Purpose	Primer seq 5'--->3'	Amplicon Size
ctn-1	SALK_ 094586	*AT1G03910*	WT Rxn	GCTCAGTGAACCGTACATGG	418bp
				GTCCACTCTCGATCTCAAAAGC	
			ctn-1 Rxn1	GCTCAGTGAACCGTACATGG	322bp
				AAAAACGTCCGCAATGTGTT	
			ctn-1 Rxn2	GTCCACTCTCGATCTCAAAAGC	194bp
				AAAAACGTCCGCAATGTGTT	
ctn-2	WISCDSLOX363	*AT1G03910*	WT Rxn	TCCATGCTGGTCCACCTTAT	491bp
				GGTTCTGTTTGATGATCAGCTTC	
			ctn-2 Rxn1	TCCATGCTGGTCCACCTTAT	270bp
				AAAAACGTCCGCAATGTGTT	
			ctn-2 Rxn2	GGTTCTGTTTGATGATCAGCTTC	247bp
				AAAAACGTCCGCAATGTGTT	

Pollen Staining

Alexander staining was used to identify dead pollen as described (Alexander, 1969).

qPCR analysis of *CACTIN* expression

Seedlings were harvested one week after sowing. They were dissected at the root/shoot junction, and the roots were collected separately from the hypocotyls and cotyledons. The tissue was harvested in liquid nitrogen and RNA isolated using

RNeasy Plant Mini Kit (Qiagen 74904). DNA was eliminated using RQ1 DNase (Promega M6101, Madison WI), and cDNA was synthesized using iScript Reverse Transcriptase (BioRad 170-8891 Hercules, California). Reactions were conducted in a Roche 480 Light Cycler using SYBR Green I (Roche 4707516001, Basel, Switzerland). *CACTIN* transcript was quantified using primers: GAAGAAGAAATGGCATTGTTAGC and TGCTTTGATCGAAATGGAACT (average primer efficiency: 1.86). We used two reference genes (Czechowski et al., 2005): 1) *AT1G58050*, amplified with primers: CCATTCTACTTTTTGGCGGCT and TCAATGGTAACTGATCCACTCTGATG (average primer efficiency: 1.83); and 2) *AT1G13320*, amplified with primers: TAACGTGGCCAAAATGATGC and GTTCTCCACAACCGCTTGGT (average primer efficiency: 1.89). Primer efficiencies and transcript abundance were calculated using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). More information about LinRegPCR is given in the qPCR methods section of appendix 1 on page 113.

Phenotypic analysis

Agar plates with seedlings were photographed from above using a standard digital camera (Olympus SP-350). These images were loaded in the NeuronJ plugin for ImageJ and root length was measured (Meijering et al., 2004).

The paleness of cotyledons was quantified in Adobe Photoshop CS4 by selecting the pixels of the cotyledons using the wand and lasso tools. The median green channel pixel intensity from the histogram palette was recorded from the selected region. These values are between 0 and 255 with an inverted scale (paler greens have a higher

value). In order to make it more intuitive, we inverted the numbers by taking 255-median value. In order to correct for subtle lighting differences between the photos, each value was divided by the average of the values on that plate. (Each genotype was evenly represented on each plate.) The values were then averaged for all the seedlings within a genotype group. Columbia wild type was set equal to one.

CTN-RFP

The construction and transformation details for this transgene are given in Chapter 2 and in (Baldwin et al., 2013). The construct includes genomic Arabidopsis *CACTIN* from the start codon to just before the stop codon under the strong ubiquitous CaMV 35S promoter, and it also has a C-terminal RFP tag. This transgene was transformed into *CTN*/*ctn-1* heterozygous plants and is able to rescue the embryo lethality of *ctn-1*. It was introduced into *ctn-2* by crossing.

Linkage Analysis

In order to calculate the linkage between *CTN-RFP* and *ctn-1*, we used a maximum likelihood approach (Hutchinson, 1929). Equations were written describing the probability of obtaining each of the genotypes relative to the probability of crossing over, designated “p”. Because some genotypes are indistinguishable by our PCR methods (for example, hemizygous verses homozygous for *CTN-RFP*), the probability of obtaining a plant with the ambiguous molecular phenotype was estimated by adding the probabilities of occurrence of each genotype as a function of “p”. Then, the observed number of plants actually obtained for each genotypic group was multiplied by

the base-10 logarithm of the corresponding expectancy equation. The sum of these corresponds to the likelihood function of p (L(p)), and is graphed with Y=L(p) and X=p (top of Figure 3-1). The resulting graph is a non-symmetric rainbow-shape function within the recombination range of 0 to 0.5 (Figure 3- 1). The X value for the maximum point of the graph (Maximum Likelihood (ML)) is the best estimate of the recombination frequency p, between *CTN-RFP* and *ctn-1* (Liu, 1997). To find the 95% confidence interval, we subtracted one from the maximum Y value of the graph, and determined the two X values associated with Y-1. We then used the Kosambi function to convert the estimated recombination frequency with its confidence interval into genetic distance in centi-Morgans (Kosambi, 1944).

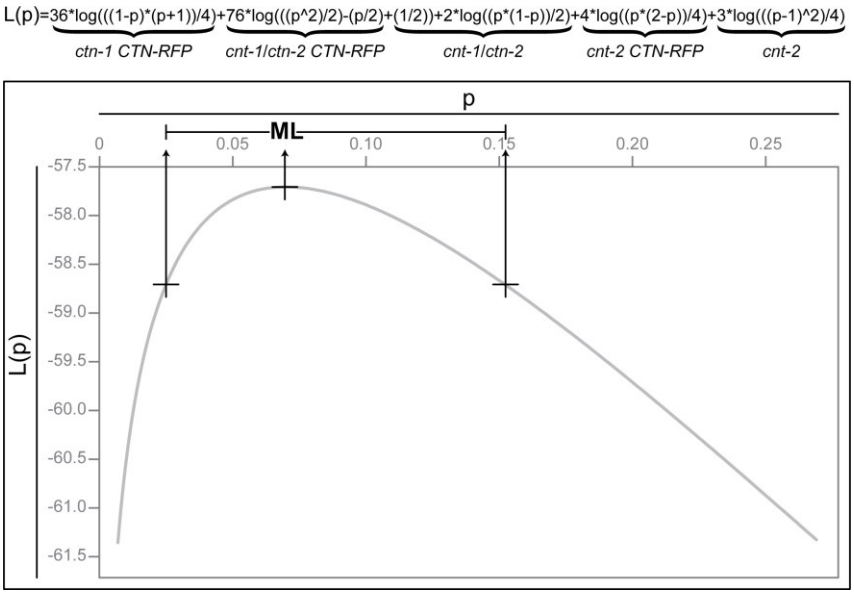

Figure 3- 1: Maximum likelihood calculation graph example. The equation is listed above and graphed below. The maximum of the graph is indicated and X-value is the best estimate for the linkage (ML). The 95% confidence interval is shown as well and was found by subtracting one from the Y-value of the maximum.

Results

ctn-2 mutational insertion is located near the stop codon

We amplified both sides of the *ctn-2* insertion and sequenced the junctions using the primers listed in Table 3- 1. Like many T-DNA insertional mutants, *ctn-2* has left borders on both sides of the insert (Figure 3- 2A). The junction sequences that we obtained are given in Table 3- 2. The result of this insertion is a stop codon seven base pairs upstream of the normal stop codon. At the protein level, this mutation would truncate the last three amino acids (Figure 3- 2B) and the mRNA made from *ctn-2* also has an abnormal 3'UTR. The *CACTIN* mRNA is expressed at a normal level in *ctn-2* seedlings as measured by qPCR in both hypocotyls and roots (Figure 3- 3).

Previous experiments have shown that the native UTRs of *CACTIN* are not required to rescue *ctn-1* (Baldwin et al., 2013), but having T-DNA sequence as a UTR could have detrimental effects. Although only three amino acids are missing from these coding domains, they are among the most conserved within CACTIN across eukaryotes (Figure 3- 2).

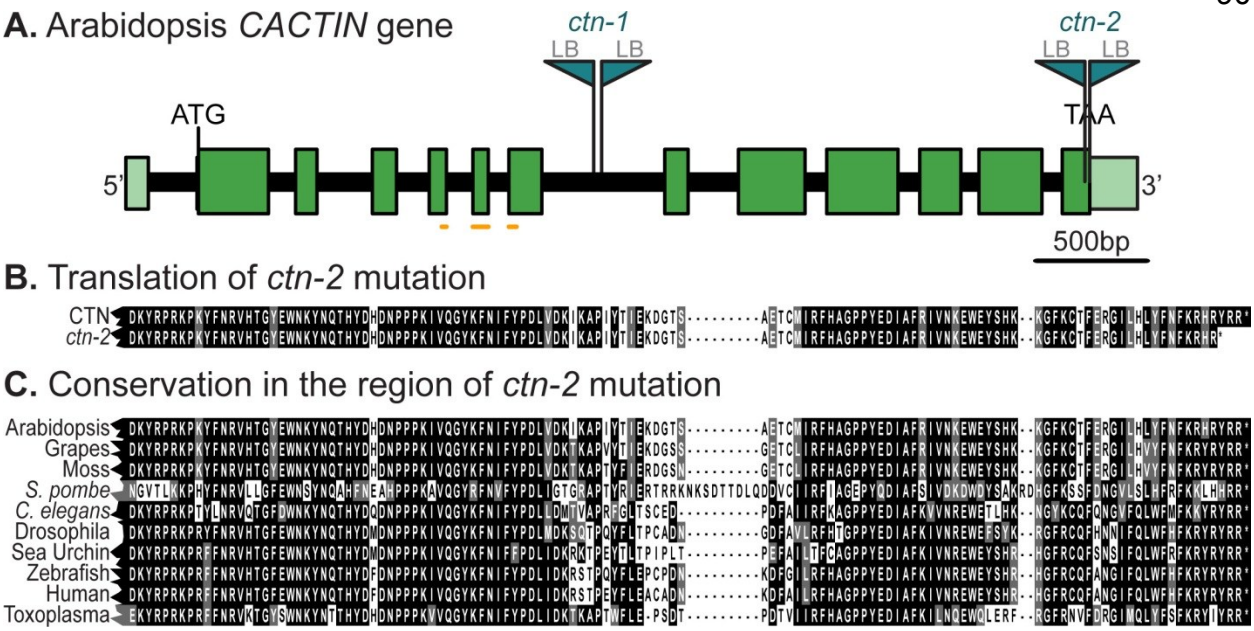

Figure 3- 2: *ctn-2* is an insertional mutant with the T-DNA at the end of the coding domain.
A. *ctn-2* is inserted downstream of ctn-1. The orange line shows the qPCR amplicon made from the cDNA of this gene in Figure 3- 3. **B**. The insert is nine nucleotides before the stop codon, and the first bases of the insert happen to make a stop codon. The protein made from these transcripts would likely have a three amino acid truncation. **C**. The final 3 amino acids of CACTIN are particularly well conserved through eukaryotes and may therefore have an important function.

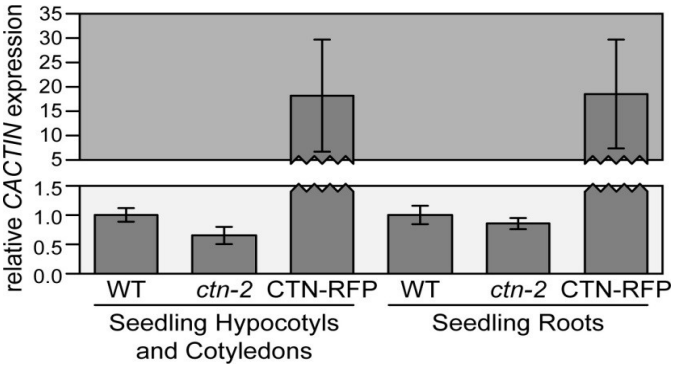

Figure 3- 3: *CACTIN* is expressed at a comparable level in ctn-2 and wild type plants.
Please note that the scale changes in the upper 'broken' part of the graph. CTN-RFP plants are homozygous for *ctn-1* and have a rescuing *35S::CTN-RFP* transgene. Tissue was collected from seedlings roots and tops separately to facilitate comparisons because the roots of *ctn-2* seedlings are much shorter than wild type whereas the hypocotyls and cotyledons are more similar in size (Figure 3- 4). Transcript levels are shown relative to reference gene *AT1G13320*, although similar results were obtained using *AT1G58050*. Error bars show the standard error of two biological repeats. The *CACTIN* transcript levels in *ctn-2* are not statistically different from wild type. The amplicon location relative to the insertional mutation is shown as an orange line in Figure 3- 2.

Table 3- 2 Junction sequences of mutant T-DNA lines.

Mutant	Junction Sequence 5' end of insert		Junction Sequence 3' end of insert	
	20bp of Gene Seq	20bp of T-DNA Seq	20bp of T-DNA Seq	20bp of Gene Seq
ctn-1	TGCTGGGATAGCATCT ATTC	CA[1]CAGGATATATTGTGGT GTAA	GTTTACACCACAATATATCC	TGAA[2]TATATGTAGAAAAAAA
ctn-2	AAC TTT AAA CGA CAT AGG **TA**	**A** CAA ATT GAC GCT TAG ACA A	TTGTTTACACCACAATATAT	CCTTATCGGAGTTTGGCTG G

[1] The previous 2 bases were improperly inserted

[2] The following 2 bases are swapped from normal *CACTIN* sequence

20 base pairs on either side of each T-DNA junction are shown. Each junction sequence is separated into two columns to denote the portion of the sequence derived from the gene and from the T-DNA. As with many T-DNA mutations, both of these alleles have left borders on both sides of the insert. The 5' end of *ctn-2* is inserted within the coding region of CACTIN, and there are spaces separating the codons. The slightly premature stop codon that is created by this insertion is bolded and underlined.

ctn-2 seedlings have a short root and are pale.

In a segregating population, we found a perfect correlation between the homozygous *ctn-2* genotype and a short root and pale cotyledons (Figure 3- 4). As these plants grow, they continue to be slightly developmentally delayed and a little pale, but they do make flowers and viable seed. In the seed collected from a selfed heterozygous plant that we were sent from Michigan, the *ctn-2* allele segregates at the expected 25%:50%:25% ratios (WT: 9 plants; *CTN*/*ctn-2*: 15 plants; *ctn-2*: 8 plants (χ^2=0.19, df=2, p-value=0.91)) as determined by PCR genotyping. The short root and pale phenotype is present only in the *ctn-2* homozygous plants and the *CTN*/*ctn-2* plants are phenotypically wild type.

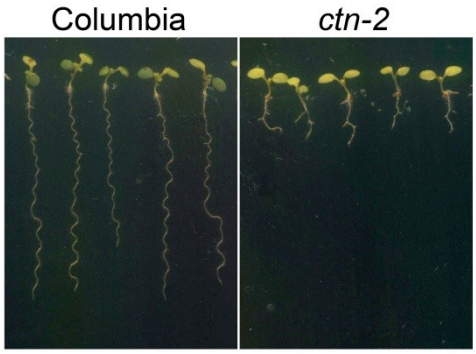

Figure 3- 4: *ctn-2* seedlings have shorter roots and pale cotyledons. Seeds were grown at a 30° tilt backwards.

The *ctn-1*/*ctn-2* F1 plants appear nearly wild type

To test if the phenotype of *ctn-2* was due to the mutation in *CACTIN*, we crossed it to heterozygous *ctn-1* plants for a complementation test. The seedlings that have the *ctn-1* allele on one chromosome and *ctn-2* on the other chromosome were expected to have the short root and pale phenotype of *ctn-2*, or perhaps a more severe phenotype. Much to our surprise, the F1 seedlings looked nearly normal. The roots of the *ctn-1*/*ctn-2* seedlings are significantly longer than the *ctn-2* seedlings, even though the *ctn-1* allele is a more severe mutation (Figure 3- 2 and Figure 3- 5). However, they are also not as long as wild type seedling roots. When we quantified the color of the cotyledons of these seedlings, we found that the F1 seedlings were not significantly paler than *CTN*/*ctn-1*, but both of these genotypes were significantly paler than wild type.

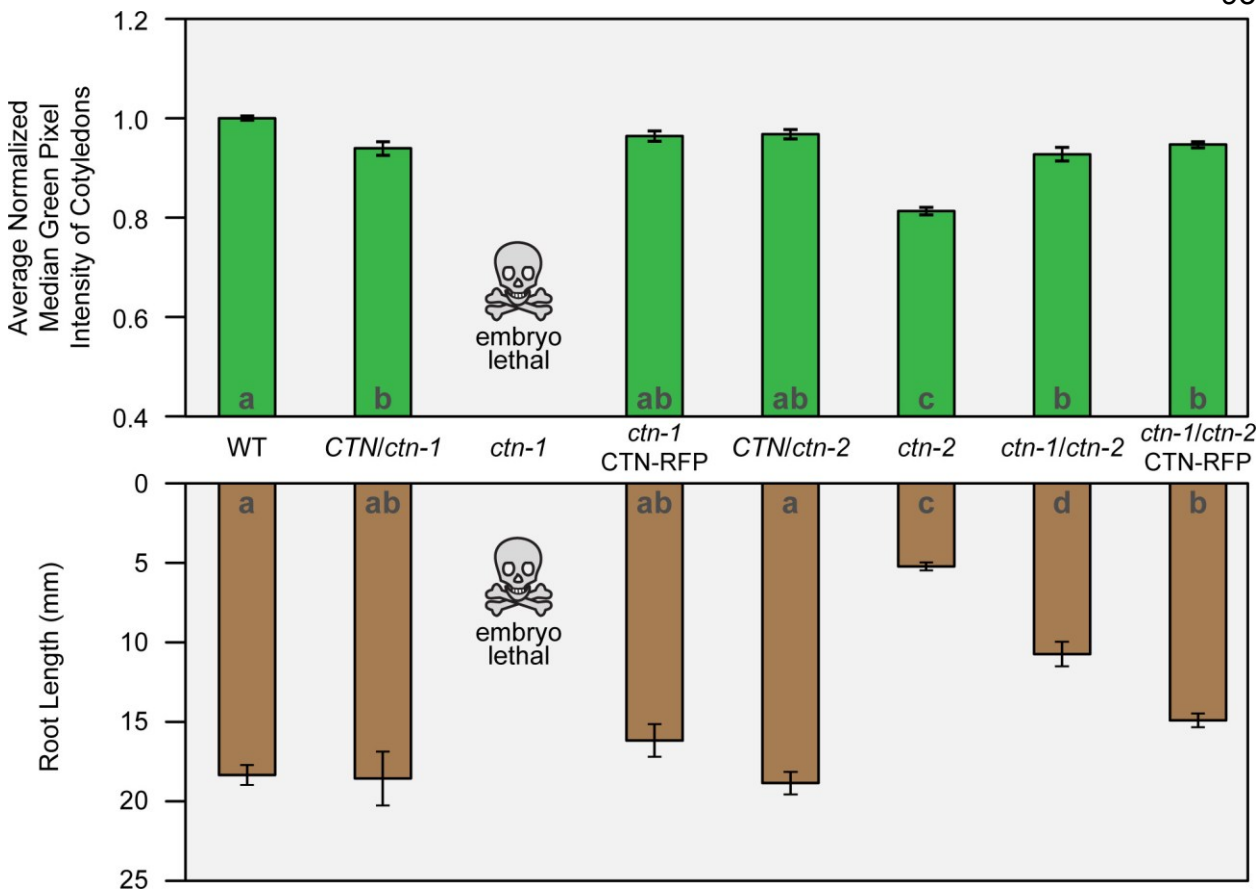

Figure 3- 5: The *ctn-2* allele partially complements *ctn-1*.
The *ctn-1*/*ctn-2* plants look more like wild type than *ctn-2*. The upper green graph shows the relative paleness of the cotyledons of the indicated genotypes (paler seedlings have a smaller bar). The lower graph shows the root length for the same seedling groups. Grey letters on each bar show the statistical groups. Because we were making 21 pairwise comparisons, we used a Bonferroni corrected p-value of 0.05, which resulted in a threshold of $p<0.002$. Bars that don't have the same letter are statistically different from each other. CTN-RFP refers to a 35S driven transgene that is able to rescue *ctn-1*.

CTN-RFP does not rescue *ctn-2*

The RFP-tagged rescuing transgene in the *ctn-1* background was introduced into *ctn-2* by crossing. There is likely only one insertion site of CTN-RFP in the line that we used because when this particular line is back crossed to wild type, only 63% of the segregating F2 plants have RFP signal by microscopy (n=59 seedlings). Also, the

segregation ratios of *ctn-1* and CTN-RFP are consistent with linkage between the transgene and the *CACTIN* locus corresponding to a genetic distance of 3cM, with a 95% confidence interval of 0.5-10cM. T-DNA transgenes are thought to insert randomly in the genome, and it appears that the T-DNA in this line inserted somewhat near the *CACTIN* gene on the first arm of chromosome one.

We found seven F2 seedlings that were homozygous for *ctn-2*. Four of the seedlings strongly expressed CTN-RFP throughout the plant, and the other three did not show RFP signal. Surprisingly, all seven seedlings had short roots and pale cotyledons (Figure 3- 7). Therefore, the same transgene that is able to rescue the lethality of *ctn-1* in this line cannot rescue the phenotype of *ctn-2*.

Another unexpected finding from this experiment was the low percentage of F2 homozygous *ctn-2* seedlings that resulted from the self-crossing of *ctn-1*/*ctn-2* CTN-RFP. We were expecting 25% of the offspring to be *ctn-2*, but we instead found only 6% (N=121 seedlings). This is a statistically significant under representation of this genotype (χ^2= 29.3, df=4, p-value=6.8X10^{-6}). Previous experiments had suggested that the *ctn-2* seeds germinate with the same frequency as other genotypes. However, they were greatly underrepresented in this population as well as from two other segregating populations that were plated out from other crosses. The rest of the genotypes in this F2 population were present at the expected ratios (χ^2=

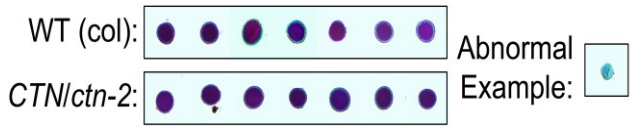

Figure 3- 6: The pollen from heterozygous *CTN*/*ctn-2* plants is normal.

2.5, df=4, p-value= 0.65), and showed similar linkage distances as past experiments (*ctn-1* and CTN-RFP were calculated as being 7cM apart with a 95% confidence interval of 2.6-16cM). We tested the pollen quality of heterozygous *ctn-2* plants because it can be an indicator of chromosomal abnormalities (Clark and Krysan, 2010), but we found normal pollen (Figure 3- 6). One possible explanation for the missing *ctn-2* seedlings is that the seeds for these experiments were harvested from green plants from our growth chamber. Our previous segregating population seed was sent from the Last Lab where the plants may have been grown under different conditions. It is possible that another facet of the *ctn-2* phenotype is bad germination rates when the plants are grown under our conditions or when the seed is not dried for a long time. One way to test if seed drying is important is to plate out this same seed after drying it thoroughly to see if the proportion of seedlings homozygous for *ctn-2* increases.

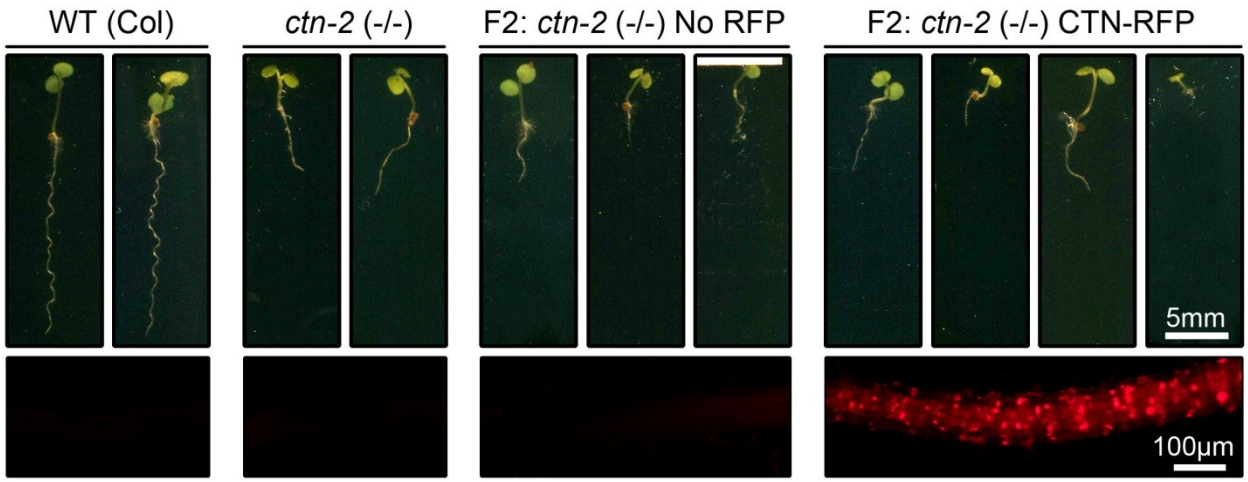

Figure 3- 7: CTN-RFP does not rescue the abnormal phenotypes of *ctn-2*.
Representative seedlings of labeled genotypes are shown in the upper panel. The lower panel shows representative photomicrographs of RFP in the roots of seedlings of the indicated genotypes. The same exposure settings were used for all images. Seedlings were grown for 1 week on ½ LS 1.5% agar media at a 30° tilt backwards.

Discussion

Our experiments suggest that the phenotype of *ctn-2* seedlings is not due to the *CACTIN* gene, but rather recessive alterations to another linked gene. *ctn-2* complemented *ctn-1*, and a transgene known to rescue *ctn-1* was unable to rescue *ctn-2*. The cause of the phenotype may be a separate mutation in a gene near *CACTIN*. We have not observed a crossing over event between these theoretical mutations in any of our experiments because there has always been a perfect correlation between the *ctn-2* mutation and the phenotype. Another explanation is that the mutation at the end of *CACTIN* affects another gene. Based on our molecular data, the *ctn-2* T-DNA is contained within the CACTIN gene, but perhaps it disrupts the expression of the downstream gene or a gene located elsewhere.

An interesting caveat to this explanation is that the *ctn-1*/*ctn-2* seedlings in the complementation test had slightly shorter roots than wild type. Although this could be explained by partial allelic complementation, we do not favor this hypothesis in light of the failed rescue experiment. Another possibility is that *ctn-2* mutation does have a subtle effect on CACTIN activity that is only seen in a sensitized background that has no other functional copies of the *CACTIN* gene. A third explanation is that there is a mutation in another gene, and when it is in the heterozygous configuration it slightly enhances the *CTN*/*ctn-1* phenotype. This could simply be the result of both mutations making the plants slightly sick or stressed even when heterozygous.

We also tested the *ctn-2* mutants for spliceoform abundance differences in candidate genes in order to test our hypothesis that *CACTIN* is involved in mRNA splicing. We did not find any reproducible differences between *ctn-2* seedlings and wild type (data not shown), but in light of our recent findings with this allele, it seems likely the *CACTIN* gene within *ctn-2* is functional and we do not expect splicing defects.

These experiments highlight the importance of testing mutants to be certain that phenotypes are truly due to defects in the genes of interest. Although this allele is not currently useful for probing the function of CACTIN, perhaps backcrossing could separate the *CACTIN* mutation from the causative mutation and allow the relevant mutation to be mapped and identified.

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Appendix 1: amiRNAs targeted against *CACTIN*

Katherine L. Baldwin & Patrick H. Masson

Abstract

The phenotypes of loss-of-function mutants are one of the best ways to probe gene function. However, for *CACTIN*, the only available Arabidopsis mutant allele caused very early embryo arrest (as described in chapter 2), thus precluding further analysis. As an alternative strategy, we adopted a silencing technique in order to investigate the consequences of reduced CACTIN. We had hoped that reducing the expression of *CACTIN* would allow us to see informative phenotypes, while still maintaining enough expression to keep the plant alive. In this chapter, we will discuss these attempts and ultimately how we failed to obtain lines with strong silencing that correlated with any abnormal phenotypes. It is our hope that this chapter will serve as a guide for future researchers using similar techniques and as documentation of our results with *CACTIN* amiRNAs.

Introduction

Eukaryotes use RNAi to reduce expression of target genes

RNAi-based gene silencing can be used as a tool to reduce target gene expression (Wesley et al., 2001). This technique takes advantage of the natural RNA interference pathway (RNAi), found in most eukaryotes. Briefly, the RNAi pathway detects double stranded RNA and reduces the expression of genes with similar sequence to the double stranded RNA by either degrading/stopping translation of the

mRNA, and/or by directing chromatin modification near the target gene's location within the nuclear genome. Eukaryotic cells use this system to restrain expression of transposable elements, repress viral expression, and as a form of endogenous gene regulation (Buchon and Vaury, 2006). Experimentally, this pathway can be utilized for target-gene silencing by expressing double stranded RNA with sequence similarity to the target gene.

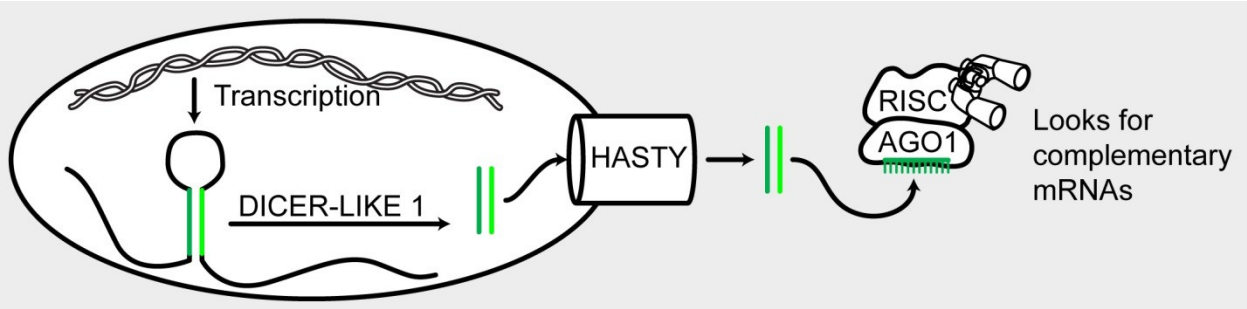

Figure A1- 1: The processing of miRNAs in the Cell.
miRNAs are transcribed from the plant genome and chopped by DICER-LIKE1. The double stranded RNA is exported to the cytoplasm by HASTY. One strand of the RNA is loaded into AGO1, which is part of RISC.

Endogenous MicroRNAs are processed to regulate target genes

Micro RNAs (miRNAs) are genome-encoded RNAs that regulate the expression of other target genes using cellular pathways related to RNAi. Endogenous microRNAs are small, 21 bp RNAs derived from precursor RNAs encoded by chromosomal genes and transcribed by RNA polymerase II. Pre-MicroRNAs have an imperfect self-complementary secondary structure that results in a hairpin. In plants, the double stranded hair pin structure is recognized by DICER-LIKE1 in the nucleus, and the hairpin structure is clipped off from the rest of the transcript in a multi-step process,

finally resulting in just 21 bp of double stranded RNA (Figure A1- 1) (Kurihara and Watanabe, 2004). This processed miRNA is then methylated by HUA-ENHANCER 1, and exported from the nucleus by an EXPORTIN 5 homolog, HASTY (Park et al., 2005; Ji and Chen, 2012). Once in the cytoplasm, the mature miRNA is recognized by the RNA-Induced Silencing Complex (RISC), which preferentially loads the miRNA strand with the lower thermodynamic stability at its 5′ end (Khvorova et al., 2003). A family of proteins called ARGONAUTEs constitute the catalytic activity of the RISC. Although most of the RNAi pathway genes have been best characterized in *C. elegans* (Riedmann and Schwentner, 2010), the ARGONAUTEs are named for the phenotype of *ago1* in Arabidopsis, which has pleiotropic defects including abnormal seedling and flower morphology. The first researchers to describe this mutant reported that the malformed young plants looked like small squids, and thus they named it after a rare pelagic octopus called *Argonauta argo* (Bohmert et al., 1998), although the resemblance is questionable (Figure A1- 2). The severe pleiotropic phenotypes of this mutant show the broad importance of the RNAi pathway for normal plant development.

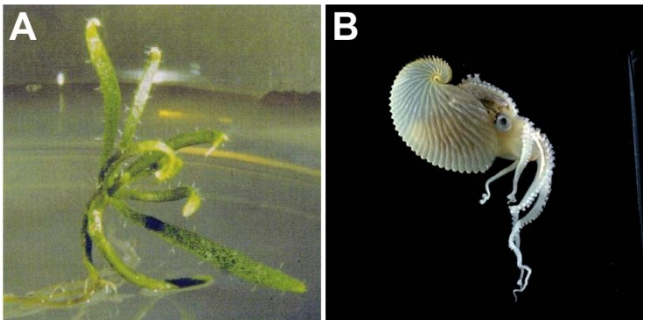

Figure A1- 2: *ago-1* Arabidopsis seedlings resemble octopuses.
A. 35-day old *ago-1* seedling photo from (Bohmert et al., 1998). **B.** Female Argonaut octopus from news.softpedia.com. This figure is included as a “fun fact”.

Once the RISC is loaded with one strand of the miRNA, it binds to mRNAs that are complementary to it. The sequence matching does not have to be perfect, though. Of the 21 bases, zero to five bases may be mismatches as long as they do not correspond to key positions within the sequence (Llave et al., 2002a). In plants, the matching mRNA is generally cleaved at the site corresponding to the middle of the bound miRNA by the Argonaut endonucleases (in between the 10^{th} and 11^{th} nucleotides of the miRNA) (Llave et al., 2002b). This cleavage results in the degradation of the mRNA. There is at least one example of plant miRNAs reducing the target protein levels without cleaving or effecting the mRNA levels, presumably by inhibiting translation of the target mRNA (Chen, 2004), however this seems to be uncommon (Schwab et al., 2006).

miRNAs can silence more than just one target gene

Because miRNAs do not need to have a perfect match to their target, and because they are only 21nt long, there inevitably are multiple target genes. In animals, more mismatches between miRNA and target are tolerated than in plants. This results in many more targets for any one miRNA in animals than in plants. Both plants and animals use the polyspecific nature of miRNAs to target networks of genes simultaneously with one miRNA.

The cell treats miRNAs differently from other double-stranded RNA

miRNAs have some important differences from other forms of RNAi in the cell. Double stranded RNA in the cytoplasm, like those from viral infections, are chopped by

cytoplasmic DICER-LIKE2 and DICER-LIKE4. DICER-LIKE 2 cuts double stranded RNA into 22nt-long siRNAs (Mlotshwa et al., 2008). The 22nt siRNAs are loaded on the RISC using the same AGO1 protein that 21nt miRNAs use. For both siRNAs and miRNAs, RISC finds complementary mRNA and silences it. However, the length-difference, in part, controls whether or not this signal is amplified (Schwab and Voinnet, 2010). For 22nt siRNAs, the target RNA - siRNA duplex serves as template for amplification using RNA-dependent-RNA-polymerases. The amplified double-stranded RNA is chopped up by cytoplasmic DICERs, resulting in more siRNAs complimentary to the same gene. This feeds forward the silencing signal. In contrast, 21nt miRNAs rarely produce secondary (transitive) siRNAs (Schwab and Voinnet, 2010). There are exceptions which are due to bulges in the hairpin that make DICER-LIKE1 cut the miRNA into 22nt segments, or due to too-few mismatches between miRNA and target (Schwab et al., 2006; Schwab and Voinnet, 2010).

Amplification of the silencing signal has important implications. Secondary siRNAs are more likely to spread from cell-to-cell, thus causing silencing of a viral gene in more than just the original infected cell (Schwab et al., 2006). Because miRNAs, are not typically amplified, they usually do not spread from the cell they are expressed in (Schwab et al., 2006). Therefore, it is possible to silence a defined gene target in a specific cell type by simply introducing a miRNA construct designed to target this gene and driving its expression in transgenic plants with the appropriate cell-type specific promoters. Another implication of siRNA amplification is that the new siRNAs are derived from all parts of the target mRNAs. The original siRNA may have targeted a

unique part of the gene, but secondary siRNAs can be produced from common-domain regions of the gene which can go on to also silence many other genes as well.

Utilizing the miRNA pathway to silence genes of interest

Artificial micro RNAs (amiRNAs) are lab-made constructs that aim to mimic miRNAs. Upon transformation into the plant genome, these artificially made genes will silence the targeted endogenous gene by either promoting the degradation of its mRNA or by interfering with its translation.

We used amiRNAs targeted against Arabidopsis *CACTIN* in an attempt to reduce its expression and reveal an informative phenotype. We used three different types of promoters to express two different amiRNAs. We used qPCR to quantify the level of silencing in some of these lines and tested them for phenotypes. In this chapter we present the results of this approach.

Methods

Plant growth conditions

Seeds were surface-sterilized with 95% ethanol and sown onto half-strength Linsmaier & Skoog media containing 1.5% sucrose (Caisson Laboratories LSP04-1LT, Logan, UT) and supplemented with 1.5% agar type E (Sigma, A4675-1KG, St Louis, MO). The plates were stratified at 4°C in the dark for 2 days before being placed in a growth chamber (16 hours light (50-70 μmol $m^{-2}s^{-1}$) and 8 hours dark, 22°C, 70% relative humidity). Plants were moved to soil after approximately 10 days of growth on petri plates. For antibiotic selection of transformed plants, we followed standard protocols (Harrison et al., 2006). For estrogen induction of expression, we used 1μM β-estradiol mixed in with the agar medium after autoclaving. The stock concentration of β-estradiol was 5mM in 95% ethanol. 100μl of this stock was added to 500ml of 1/2LS 1.5% agar, and vehicle control plates were made by adding 100μl of just 95% ethanol to the same type of media.

Gravitropism Hair-Pin Assay

Seedlings were grown on agar plates tilted backwards 30° for approximately 4 days. The plates were then rotated 180° upside-down and put back in the other-wise same position. Root growth was analyzed after at least 24 hours by scanning the plates on an Epson flatbed scanner and using Adobe Photoshop to measure the diameter of the "U" in the roots.

amiRNA Design and Construction

This section includes some additional information and advice for other researchers planning to use this technique, but which is not necessary to understand our results.

amiRNA sequence is planned to target the gene of interest

In order to silence *CACTIN* in Arabidopsis, we designed miRNA constructs and transformed them into Arabidopsis. The Weigel lab has created a web interface that uses criteria found in naturally occurring miRNAs to design an amiRNA against any plant gene of interest (http://wmd3.weigelworld.org/) (Schwab et al., 2006). The algorithm uses the following criteria: 1) the amiRNA should have a uridine in the first position, and an adenine in position 10; 2) the 5' end of the amiRNA should have almost no mismatches from the target mRNA; 3) the 3' end of the amiRNA should have one to three mismatches because too-good of a match is correlated with amplification of the miRNA into secondary siRNAs; 4) there should be no mismatches at the presumptive mRNA cleavage site (positions 10 and 11 of the miRNA); 5) and the side of the hairpin that is antisense to the target mRNA should have higher 5' instability relative to the other stand of the hair pin so that the preferred strand is loaded into the RISC. We chose the top two distinct amiRNAs that the Weigel lab website recommended for Arabidopsis *CACTIN* (Figure A1- 4). The sequence of the strand of the amiRNA that will be loaded into RISC is listed in Figure A1- 4. It is very important to use more than one amiRNA against a target gene because like endogenous miRNAs, the short length and permitted mismatches result in not enough specificity to target only one gene. Although *CACTIN* has no paralogs, by random chance the amiRNA will partially match

unrelated genes. Some Arabidopsis amiRNAs designed with the goal of silencing a single gene have been shown to have as many as 10 off-target genes that are also silenced (Schwab et al., 2005). However, two distinct amiRNAs would not be expected to have any of the same unrelated-gene off-targets. Indeed, there are no common genes within the lists of predicted possible off-targets for amiR-ctn-1 and amiR-ctn-2 (Figure A1-4, and data not shown). Also, not every amiRNA is effective at silencing its target, so it is best to try multiple. As we will discuss in the next section, construction of the amiRNAs usually works very well, but some sequences seem difficult to assemble using the standard protocol. This is another important reason to initially start with multiple amiRNA designs for each target.

amiRNAs are constructed using a variation on PCR-fusion

Dr. Rebecca Schwab, in the Weigel lab at that time, developed a streamlined protocol for cloning and constructing amiRNAs which is available as a PDF on the Weigel lab website. Starting with a plasmid that contains a natural miRNA (pRS300), and through a variation on site-directed-mutagenesis and PCR fusion, the sequence of the hairpin is changed to a sequence complementary to your target gene (Figure A1- 3). In addition to designing the sequence of the amiRNA, the Weigel lab website prepares the sequences of the primers needed to construct the amiRNA from pRS300. We had success with these instructions except that we had to use much lower annealing temperatures for the initial PCR reactions A, B, & C (36°C-46°C) (Figure A1- 3). We recommend doing a PCR annealing temperature gradient for these reactions to find the best conditions.

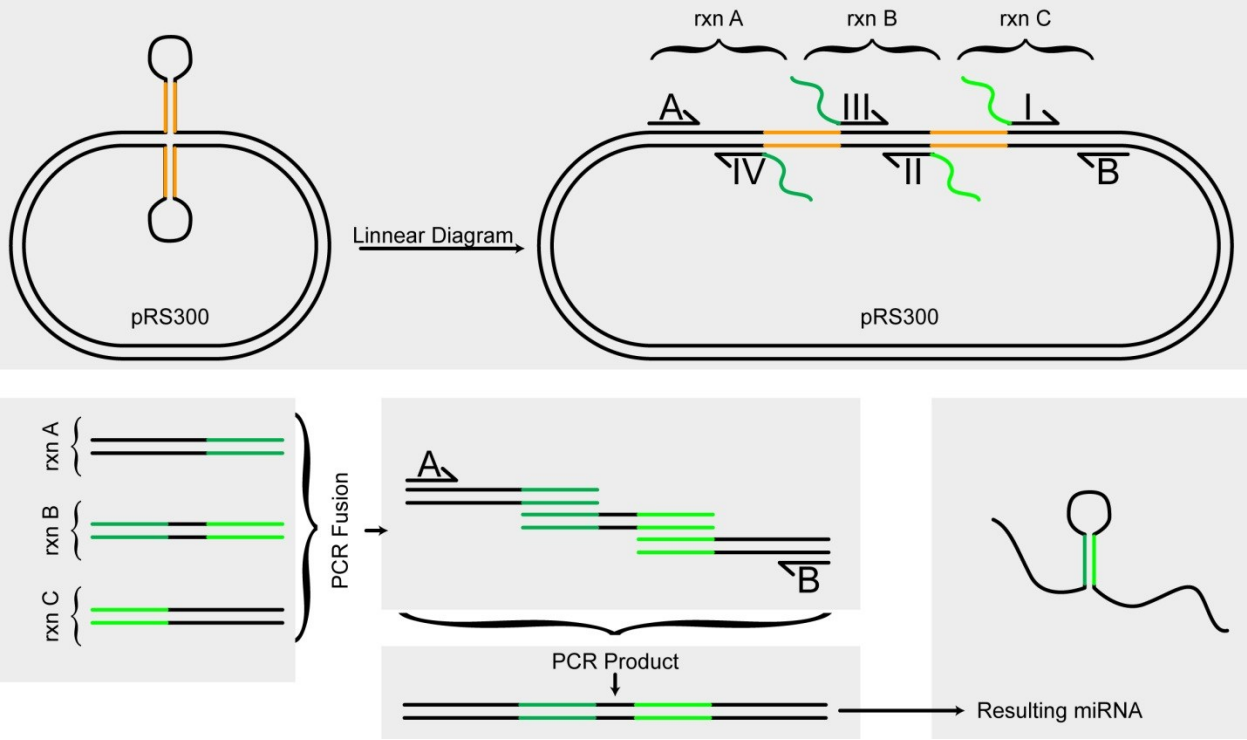

Figure A1- 3: amiRNAs are constructed using a PCR-fusion/site-directed-mutagenesis-like protocol.

pRS300 is a plasmid containing the sequence of the endogenous Arabidopsis "miR319a" micro RNA which normally targets a family of transcription factors involved in leaf development (Palatnik et al., 2003). A series of reactions are used to change the sequence of the hairpin region and thus change the intended targets. Primers A and B are standard primers used for making all amiRNAs. Primers I-IV are specific to each particular amiRNA and have long tails that will become the hairpin part of the new miRNA. First, three separate reactions are conducted (diagramed as rxn A, B, and C). Then, the PCR products are combined using PCR fusion with primers A and B. In this step, the amplicons serve as primers on each other to create the larger fusion product. This final PCR product is then cloned into plant expression vectors. Once transformed into plants, it will give rise to amiRNAs with a double-stranded hairpin region complementary to your gene of interest.

After the PCR amplicon has been made through PCR fusion (Product size=701bp) (Figure A1- 3), the fragment is cloned into regular plant expression vectors. We cloned amiR-ctn-1 and amiR-ctn-2 into pENTR-D-TOPO by doing secondary nested PCR on our fusion product using these primers caccGAATTCCTGCAGCCCCA & GTGGATCCCCCCATGG (Product=437bp). The pENTR-D-TOPO reaction requires "cacc" on the 5' end of the fragment. Once the amiRNAs were in pENTR, gateway cloning was used to recombine them into plant binary vectors. We used pMDC32 (Curtis and Grossniklaus, 2003), which drives expression of the amiRNA with the strong and ubiquitous CaMV 35S promoter and terminates transcription with the nopaline synthase (NOS) terminator. We also put the amiRNA constructs into a root-cap-specific promoter as discussed in Box 2 about the root cap promoter.

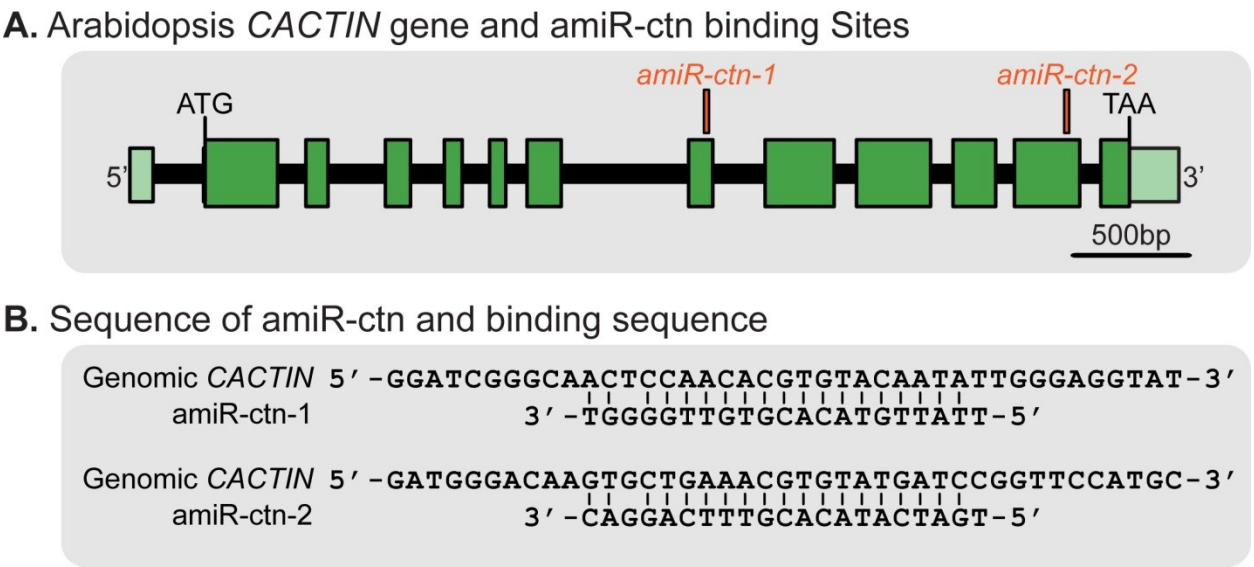

Figure A1- 4: amiR-ctn-1 and amiR-ctn-2 bind the CACTIN mRNA in different locations.
A. The locations of the amiRNA binding sites are shown on the genomic CACTIN gene for reference, although in the cell they would bind the mRNA. **B.** The sequence of the binding interaction is shown. Notice the strategic mismatches.

Box 1: The Root Cap Promoter

The RCP1 promoter is derived from a complicated region of the Arabidopsis genome

The *RCP1* promoter was discovered as being root-cap specific through a β-glucuronidase (GUS) enhancer trap insertional mutagenesis (Tsugeki and Fedoroff, 1999). One mutant line was found to show GUS expression in only the root tip, and tailed-PCR was used to sequence the flanking sequence of the insert. Tsugeki and Fedoroff cloned the 1.4kb *Sca*I restriction enzyme fragment upstream of the T-DNA insert in front of GUS and showed that it was sufficient to cause root cap specific expression of the reporter gene. This report was published before the complete Arabidopsis genome was sequenced and it turns out that this particular gene region is part of a recent tandem genomic duplication (Figure A1- 5). We can deduce that the duplication is quite recent because there is no sequence divergence between the two copies due to mutations. Tsugeki and Fedoroff noted that the actual "*RCP1*" *AT5G17520* gene is expressed in both the root tips and in leaves even though their promoter fragment drives expression in only the root cap. The full chromosome sequence reveals that their promoter fragment is not actually upstream of *AT5G17520*, but rather it is upstream of *AT5G17523*, which is a duplicated truncation of *AT5G17520*. The duplicated portion

directly upstream of the two genes are identical, but farther upstream they are different, which could be the cause of the different expression pattern. However, it could also be that factors outside of the promoter of *AT5G17520* are truly responsible for its expression in leaves. The 1.4kb "*RCP1*" promoter fragment may be a somewhat artificial promoter, but nonetheless it is a useful tool for controlling transgene expression.

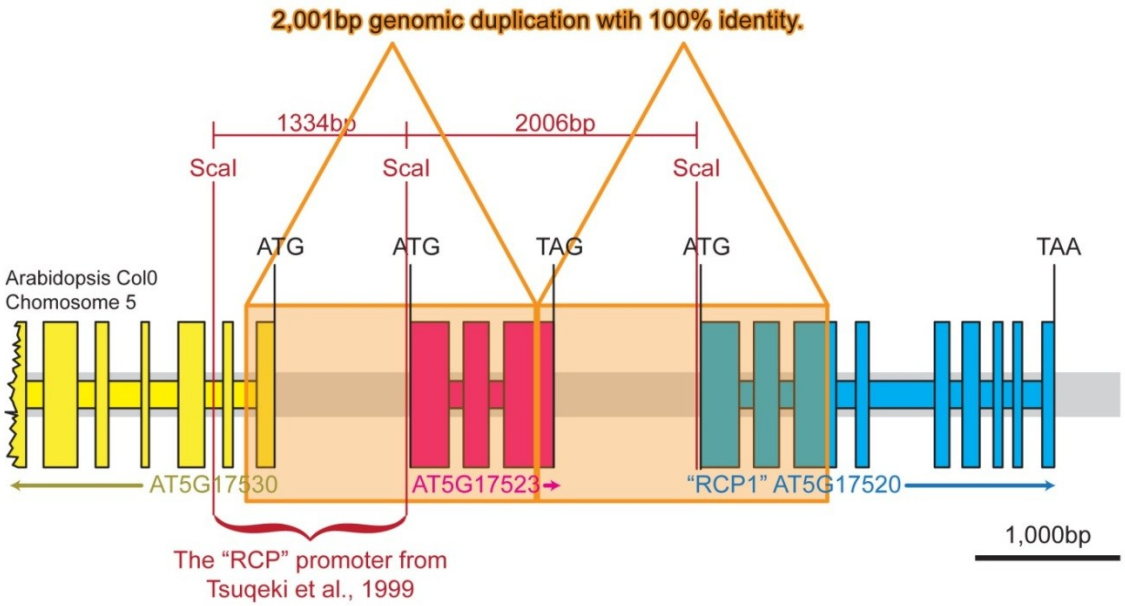

Figure A1- 5: The Root Cap Promoter (RCP) region is located in a complex region of the genome that contains a recent tandem duplication.
Within the two tandem copies of the 2,001bp duplication, the sequence is 100% identical. The TDNA insertion line that originally led to the identification of this promoter from Tsugeki and Fedoroff, 1999 was inserted just after the ATG of *AT5G17523* (magenta).

Multisite cloning of the RCP1 promoter

We cloned the 1.4kb *Sca*I genomic fragment described in Tsugeki and Fedoroff (1999) into a 2-element multisite gateway system. We chose this system because it would facilitate easy combinations of this promoter with different genes or amiRNAs. We amplified the described fragment from genomic Columbia DNA using these attB-winged primers: GGGGACAAGTTTGTACAAAAAAGCAGGCTTAagtactttctaaatgttg and GGGGACAACTTTTGTATACAAAGTTGTagtacttgggtttggaac. The amplicon was recombined into pDONR221-P1P5r (element 1 of 2-site system) using BP clonase. Simultaneously, both amiR-ctn-1 and amiR-ctn-2 were recombined into pDONR221-P5P2 (element 2 of 2-site system) using these attB-winged primers: GGGGACAACTTTGTATACAAAAGTTGccgaattcctgcagccccaa and GGGGACCACTTTGTACAAGAAAGCTGGGTAggatccccccatggcgat. The pDONR plasmid containing RCP, the pDONR plasmid containing the amiRNA, and a binary plant destination vector with a transcriptional terminator were combined with LR clonase II plus (Invitrogen #12538-120, Carlsbad, CA). This destination vector was made by Dr. Julian Verdonk by cutting the CaMV 35S promoter out of vector pK2GW7, but leaving the transcriptional terminator in place. This reaction resulted in the RCP promoter upstream of the amiRNA in a binary vector.

Estrogen-Inducible System

We recombined pENTR containing the amiRNAs into pDMC7, which contains the XVE estrogen inducible expression system (Zuo et al., 2000) (Figure A1- 13).

Plant transformation

Columbia wild type plants were transformed with the amiRNA constructs using Agrobacterium tumefaciens GV3101 (Clough and Bent, 1998).

qPCR analysis of CACTIN transcript silencing

Quantitative Real Time PCR (qRT-PCR or qPCR) was used to quantify the level of expression of the CACTIN-RFP transgene. The experiments using seedlings as a tissue source were grown as described in the “plant materials” section of methods, and harvested one week after sowing. The experiments using rosette leaves used the same weight of leaves from aged-matched flowering plants. Large, green leaves were selected from each plant. The tissue was harvested in liquid nitrogen and RNA isolated using RNeasy Plant Mini Kit (Qiagen74904, Hilden Germany). DNA was eliminated using RQ1 DNase (Promega M6101, Madison WI) and cDNA was synthesized using iScript Reverse Transcriptase (BioRad 170-8891 Hercules, California). Reactions were conducted in the Roche 480 light cycler in the University of Wisconsin Madison Gene Expression Center using SYBR Green I (Roche 4707516001, Basel, Switzerland). Primer sequences and general efficiencies are listed in Table A1- 1. Primer efficiencies and transcript abundance were calculated using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). LinRegPCR calculates the primer efficiency from the exponential region of the amplification curve for each sample in the reaction. This technique does not require superfluous cDNA-dilution-reactions for calculating the primer efficiency and it does not assume the same primer efficiency for different batches of cDNA. It is free, user-friendly, used by qPCR experts (Czechowski et al., 2005), integrates well with

Microsoft Excel, and it gives very consistent results across trials, which implies that it is calculating a genuine trait of that particular primer pair. We have made a guide for using this software with data from the Roche 480 light cycler that is available at www.K8Baldwin.com.

qPCR analysis of Candidate Gene Spliceoform

Genes with many spliceoforms were chosen as candidates for having altered relative spliceoform abundance in *CACTIN* silencing plants (Ali et al., 2007; Song et al., 2009; Zhang and Mount, 2009). Primers were designed to amplify a particular splice junction (Table A1- 2). Some amplicons correspond to an alternately included exon, and others span an alternately used junction. Amplicons were 60-180bp in length, which is ideal for qPCR. Previous publications had used a single primer pair for each candidate gene and then compared the relative intensity of the multiple bands obtained. We opted for a more quantitative method because there was great variability in relative band intensity from reaction to reaction.

Table A1- 1: Primers used in qPCR for quantifying *CACTIN* expression.

Gene	Forward Primer	Reverse Primer	Primer Eff
Reference Gene *AT1G58050*	CCATTCTACTTTTTGGCGGCT	TCAATGGTAACTGATCCACTC TGATG	1.83
Reference Gene *AT1G13320*	TAACGTGGCCAAAATGATGC	GTTCTCCACAACCGCTTGGT	1.89
CACTIN (AT1G03910)	TGGACCCGACTCTGATCTCT	CTGTCGGTCTTCCTTTTTGG	1.86

Reference genes are from (Czechowski et al., 2005).

Gene	Primer Sequence (5' to 3')	Amplicon Size	Approximate Primer Efficiency	Melt Curve
SRp34b, At4g02430	CACAAAATTCGATCCTGAGTTC	98bp	1.85	77.0C
	GGCGATATCCGTGAAAGAGA			
	GACATGAAATATGCGATAAAAAAAG	108bp	1.86	78.0C
	CTCCTCGAATCCCTCCTTG			
	CCTATCCGTGGTAGGAGCAA	91bp	1.91	79.0C
	TCTTCCAACAGACCCAGCTT			
	AGCAGGAGCAAGAGCAGAAG	119bp	1.84	83.0C
	CCTCGTGGAGACAGTGACCT			
	CACATGCGTAAAGGAGGAGA	115bp	1.88	78.0C
	CTTTTTTTATCGCATATTTCATGTC			
SRp34, At1g02840	CCGTCTCCAAGAAGATATGGA	127bp	1.8	77.0C
	TCCAAGTGATCCAATGTCTCC			
	CCGTCTCCAAGAAGATGGAT	124bp	1.88	78.0C
	CCAAGAGAACCCAGATTCCA			
	GAGGACATGAAGTATGCGCTGA	133bp	1.88	81.0C
	AATAGGATCTTCCCCGGCTA			
RSp31, At3g61860	CACTCTTTGTGTTGCGTTTTG	108bp	1.91	79.5C
	CGAGTTTGCGAATAGCGTCT			
	ACTTGTCGAACAACCGTTCC	113bp	1.91	81.0C
	CTTCTCCGATTGTTCAGAGGAAT			
	TGCAGCAGGTTAATGGACAG	75bp	1.91	77.0C
	GCAACAAAAGAAAGCACAAGG			
	GGAACGGTTGTTCGACAAGT	73bp	1.88	79.0C
	AAAGTACACAAAAGCATATCCAGATTT			
	GGAACGGTTGTTCGACAAGT	113bp	1.86	82.0C
	CGAGTTTGCGAATAGCGTCT			
	TGGACGAAGGAGTTCTGATT	83bp	1.5	79.5C
	TTCCTCTTGGGACTGGAGAA			
	GAGGATGAACGTGATGCTGA	94bp	1.9	80.0C
	CCTTTGCCCATTCAACTGAT			
	TGGAATGCCTCTACATCTCTCA	111bp	1.86	76.5C
	TGTCCATTAACCTGCTGCAT			
SOC1, AT2G45660	TGCAAATTGTGTAATATTCCAAGTAA	75bp	1.72	75.5C
	GCAAAAGTTTTAGATTCCAATTTGT			
	CAGCTCAAGCAAAAGGAGAA	88bp	1.65	79.5C
	TTGACCAAACTTCGCTTTCA			
	ATGAAAGCGAAGTTTGGTCA	100bp	1.75	78.5C
	CCAATGAACAATTGCGTCTCTA			

Table A1- 2: Table of spliceoform specific primers for candidate multi-spliceoform genes.

GFP-CACTIN as a reporter of CACTIN silencing

Genomic *CACTIN* from the start codon to the stop codon (containing introns, see TAIR: AT1G03910) was cloned in pENTR-D-TOPO using the primers: caccATGGGTTCTCATGGTAAGGGTAAGAG and TCGCCTGTACCTATGTCGTTTA. It was then recombined into pMDC43 (Curtis and Grossniklaus, 2003), which contains the CaMV 35S promoter and an N-terminal GFP tag.

GFP-CACTIN microscopy

Fluorescence microscopy was performed on seedlings that were grown on agar plates. The seedlings were placed on a slide in a drop of water and enclosed with a cover slip. We imaged the slides on a Leica DM LB2 microscope equipped with a mercury bulb and a Leica GFP fluorescence cube. Photographs were captured using an Olympus DP72 camera.

Results

Examining plants carrying amiRNAs under the CaMV 35S promoter

Promoter Choice

CaMV 35S promoter-driven vectors for plant expression are readily available, and generally result in strong ubiquitous expression of the transgene. However, the strength of expression is also very dependent on insertional position within the plant genome, which is thought to be random during agrobacterium-mediated transformation (Schubert et al., 2004). One caveat of using the 35S promoter to drive expression of *CACTIN*-targeted amiRNAs is that very strong silencing would likely result in plant death and might preclude embryo development or seedling from surviving selection. However, we hoped that some transformants would happen to express the amiRNA transgene at a level which would not kill the developing embryo but that would still result in informative phenotypes.

Some amiR-ctn-1 and amiR-ctn-2 plants have abnormal phenotypes

We observed similar, broad pleiotropic phenotypes for multiple transgenic lines from both of the constructs (amiR-ctn-1 and amiR-ctn-2). The phenotypes we observed only became abnormal as the plants started producing rosette leaves. Their severity varied from plant to plant, with no strong consistent pattern relative to transgenic line or generation. They consisted of lobed/serrated rosette leaves, sometimes with purple edging (Figure A1- 6, Figure A1- 7, Figure A1- 9). The abnormal plants also had a generally smaller size and much lower seed set (Figure A1- 6, Figure A1- 7). The segregation of these phenotypes within the T2 plants suggested that the causative gene

is dominant, which would be expected for amiRNAs (Figure A1- 7). However, none of the seeds from phenotypically abnormal T1, T2, or T3 plants ever gave rise to 100% abnormal offspring. This could be due to none of the plants being homozygous for the amiRNA transgene, silencing of the amiRNA in some offspring, or that the phenotype does not have a fully heritable genetic cause.

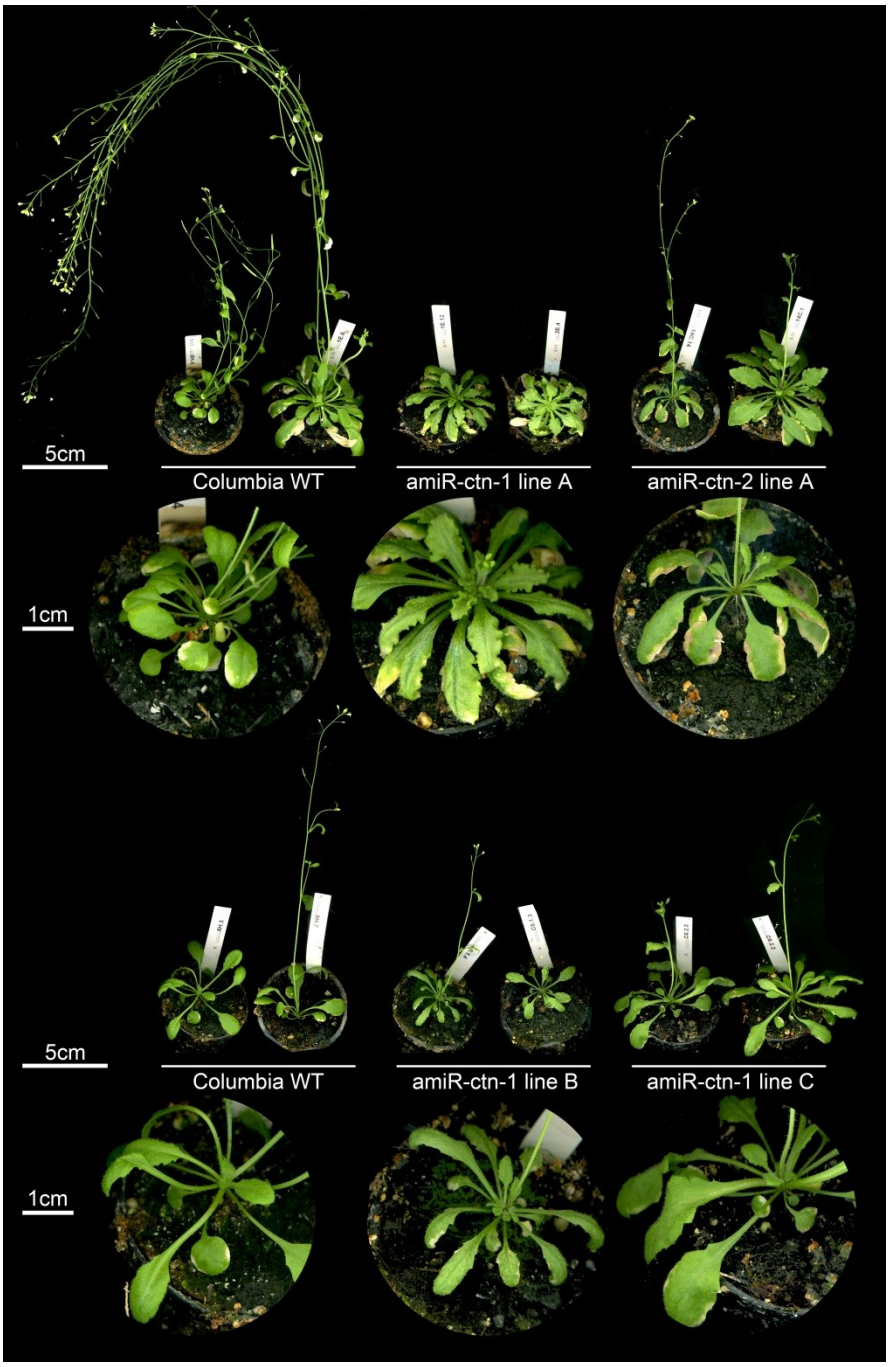

Figure A1- 6: Some of the plants carrying amiRNAs targeted against CACTIN have an abnormal phenotype.

Lower zoom-in panels of the first plant in each group show the leaf lobing on some of the abnormal lines. The severity of the phenotype varies within lines. The plants in the top group were grown at a different time than the plants in the lower group and were photographed at an older age.

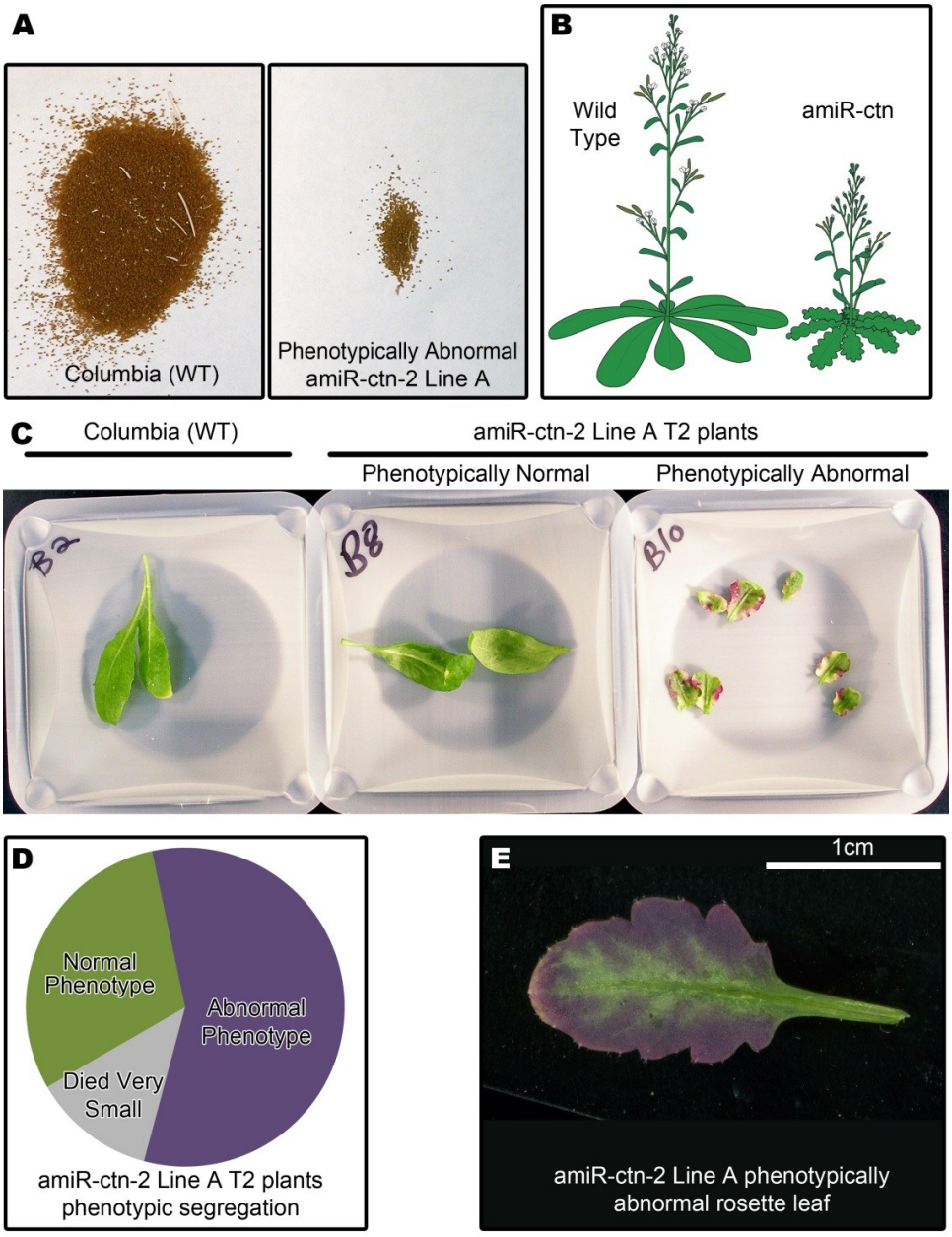

Figure A1- 7: Phenotypically abnormal amiR-ctn plants have low seed production, lobed purple leaves, and are inherited in a dominant-like fashion.

A. Seed set example from a single wild type plant and an amiR-ctn-2 Line A plant. Low seed set was a general trait of most phenotypically abnormal plants from all the lines. **B.** The phenotype of the abnormal plants is summarized as dwarf plants with lobed leaves and lower seed production. **C.** Rosette leaves from normal and phenotypically abnormal plants. **D.** Phenotype segregation ratios for T2 amiR-ctn-2 Line A plants. The parent was the T1 plant which received the T-DNA insertion and thus was hemizygous for any given insertion. N=40 plants total. **E.** Scan of a leaf from an abnormal plant showing lobing and purple coloring.

Plants carrying amiR-ctn have normal graviresponse and display wild-type patterns of spliceoform abundance for candidate alternatively spliced genes

Because CACTIN was identified in a screen for proteins that change in abundance in root tips after gravistimulation (see chapter 1), we tested the amiRNA seedlings for their gravicompetence using a 180° degree turn hairpin assay (Figure A1-8). The amiRNA seedlings were indistinguishable from wild type and responded to gravity normally.

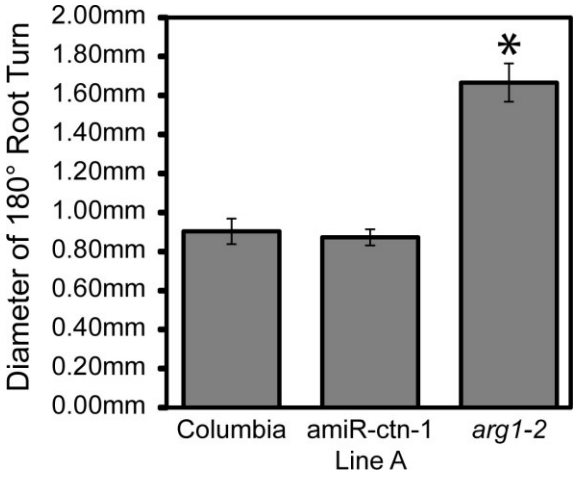

Figure A1- 8: Seedlings carrying amiRNAs targeted against CACTIN have a normal root gravitropic response.
Agar-grown seedlings were turned 180° degrees upside down. Wild type seedlings sense their change in orientation relative to gravity and their roots turn around as they grow towards the new 'down'. The diameter of the turn is reflective of the extent of gravity sensing and growth rate. We used the known gravity mutant, *altered response to gravity 1* (*arg1-2*), which has a gravity defect while still growing generally downwards. Seedlings descended from a phenotypically abnormal amiR-ctn-2 plant grew the same as wild type. N=29, 37, and 31.

The experiments described in chapter 2 have led us to propose that CACTIN is involved with the spliceosome. We used many spliceoform-specific qPCR primers to test for differences in relative spliceoform abundance of splicing-sensitive genes. For the genes we tested (*SRp34 [At1g02840], SRp34b [At4g02430], RSp31 [At3g61860], SOC1 [AT2G45660]*), we did not detect any reproducible differences in spliceoform preference in the amiR-ctn plants relative to wild type.

Splicing may be a temperature-sensitive process (Sablowski and Meyerowitz, 1998), and a slightly defective spliceosome may therefore be ‘rescued’ by lowering the temperature. We tested if the phenotypically abnormal amiR-ctn plants could be improved by growing them in cooler temperatures. The germination of all seeds was much lower at 16°C than at 24°C, however the phenotypes were not altered by the different temperatures.

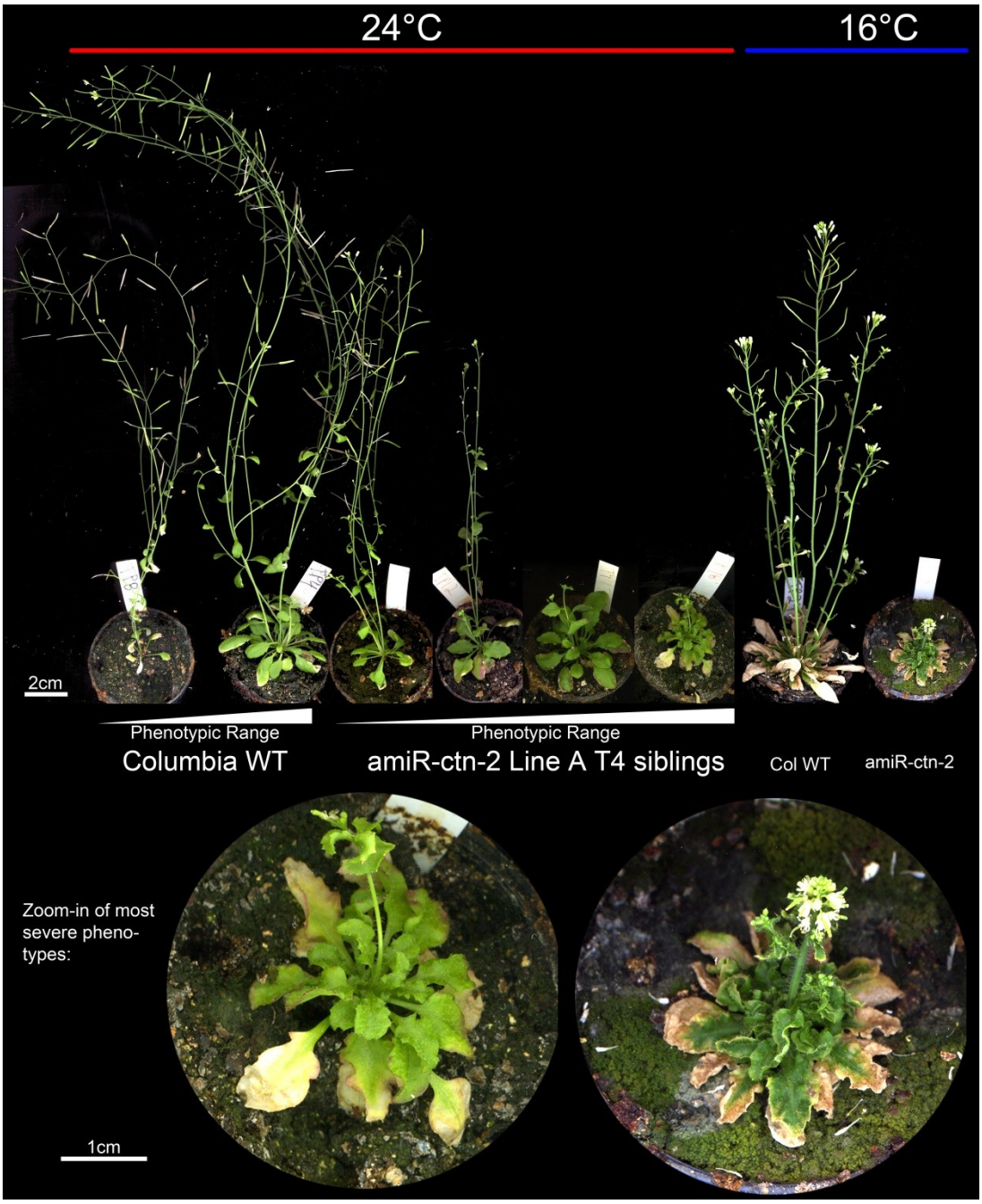

Figure A1- 9: The abnormal phenotype observed in some amiRNA lines is not remedied by cooler temperatures.
The range of phenotypes was narrower in the cooler temperature because significantly fewer seeds germinated.

amiR-ctn plants have lower levels of *CACTIN*, which do not correlate with the strength of phenotypes

Finding the same phenotype from multiple different amiRNAs targeted against the same gene suggest that the phenotype is meaningful. However, in this case the phenotype is quite broad and could be due to silencing of other genes or just generally sickly plants. One way to probe this question is to examine the correlation between phenotype and level of target-gene silencing. Because plant amiRNAs cause the cleavage and degradation of their target mRNAs, we used qRT-PCR to measure the degree of silencing of *CACTIN*. We observed significantly less *CACTIN* transcript in the seedlings and rosette leaves of amiR-ctn-1 and amiR-ctn-2 plants (Figure A1- 10A, Figure A1- 11). However, the degree of silencing was quite mild (over 20% of normal expression remaining). Importantly, we saw no correlation between the degree of silencing in whole rosette leaves and whether or not the plants showed the abnormal phenotype (Figure A1- 10B).

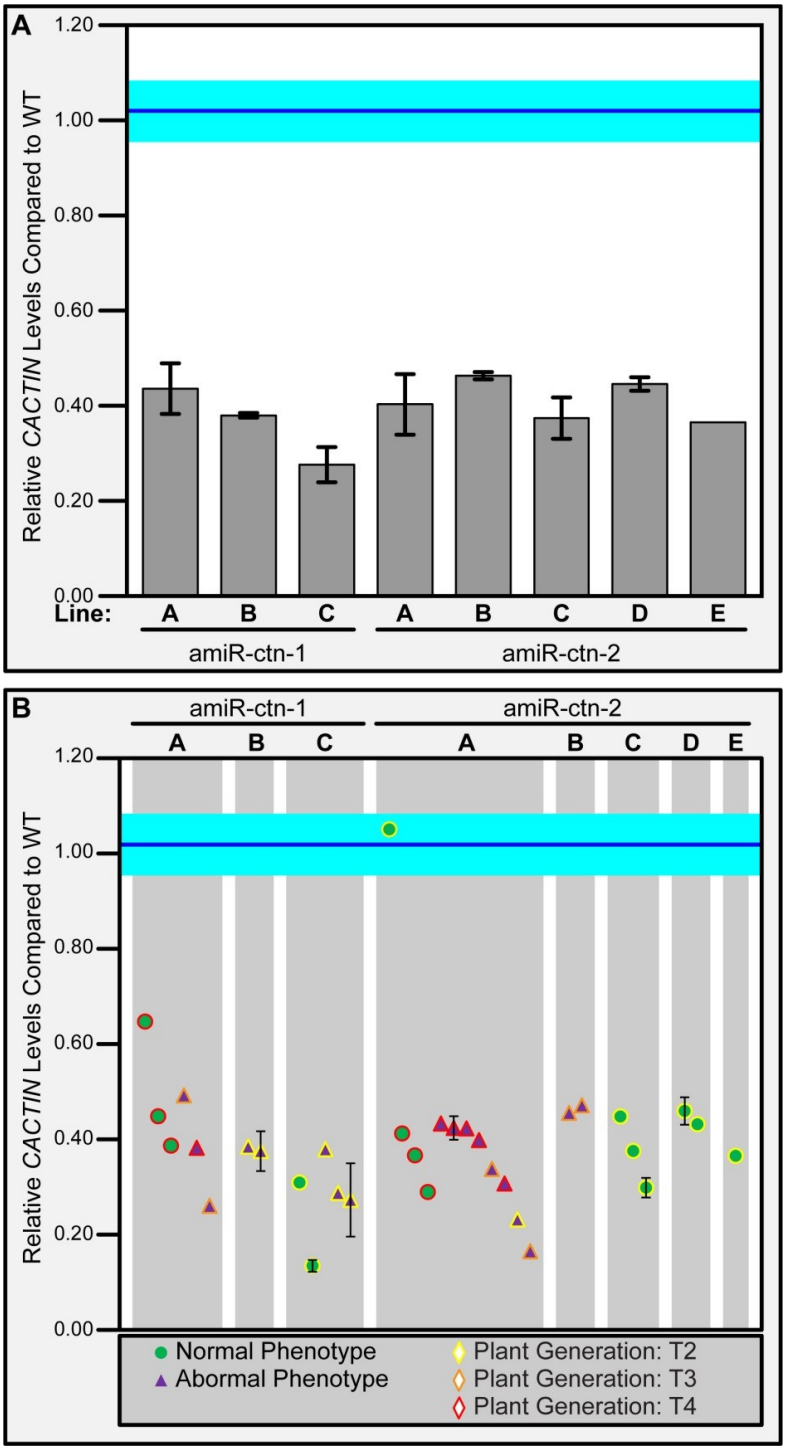

Figure A1- 10: Although the amiRNAs targeted against *CACTIN* do significantly lower *CACTIN* expression, it may not be lowered to a biologically relevant extent and there is no correlation between silencing level and plant phenotype.
A. qPCR of rosette leaves shows that amiRNA lines have less *CACTIN* expression than WT plants. All bars are statistically different from wild type (t-test $p<10^{-5}$). Error bars show standard error of multiple plants descended from the same T1 transformant line, and the number of samples range from 1 to 11 (see A below) . T2, T3, and T4 plants are included. For each of the qPCR experiments that were combined to make this figure, the data were presented relative to WT. Because there were technical replicates of WT in multiple experiments, the final averages of the separate wild type plants comes out slightly different from 1.00, which is why the blue line representing wild type is just above one. The cyan box shows the standard error of the wild type plants. The *CACTIN* levels were calculated relative to the reference gene *At1g58050*. Similar results were found relative to *AT1G13320* as an alternative reference.
B. Each point represents an individual plant, and they are grouped by the T1 transformant line that they are derived from. For samples with error bars, they represent the standard error of the technical repeats (multiple cDNA synthesizes from the same RNA.) This graph is made from the same data in A.

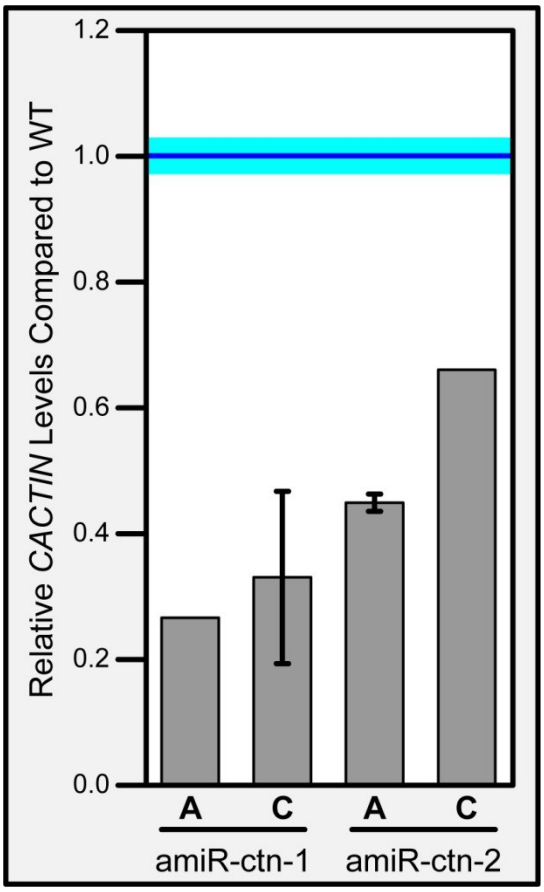

Figure A1- 11: Seedlings carrying amiRNAs targeted against *CACTIN* have reduced *CACTIN* levels. Although the amiRNA seedlings have a normal phenotype, they are silencing *CACTIN* to a similar extent to the mature plants. Error bars show standard error of different biological replicates. The number of sample varies from 8 to 1 for each genotype. WT seedlings are shown as a blue line at 1.0, and the cyan box is the standard error of different samples of Columbia.

Assaying *in-situ* silencing with a GFP-CACTIN reporter

To explore the possibility that there was strong silencing in just a certain cell type of the leaf that did correlate with the phenotype, we crossed the amiR-ctn plants to a line carrying *GFP-CACTIN*. This GFP transgene would be expected to be silenced in the same way as endogenous *CACTIN*, however it provides a visual marker for which cells are silencing. All of the F1 plants which contained *GFP-CACTIN* based on PCR results also showed at least some visible GFP signal in the seedling root, and we often saw signal in only some cell files of the root. However, we found that the intensity and pattern of the GFP signal was extremely variable cell-type-to-cell-type, plant-to-plant,

and organ-to-organ, even in plants without amiRNAs. The variability was so high in otherwise wild type plants that the effect of the amiRNA was not distinguishable. There was no GFP signal visible in the leaves of any of the plants (including plants that didn't carry amiRNAs), although leaf microscopy is more challenging than root, and low intensity signal could be undetectable (data not shown).

A tissue-specific promoter may allow for identification of stronger silencing lines

Very strong silencing of *CACTIN* throughout the plant would be expected to be lethal and not survive transformation selection. In contrast, silencing in just one organ may result in a plant that is still alive, but showing informative phenotypes in the organ in question. Although microarray databases predict that *CACTIN* is expressed in essentially all tissues, they also show that *CACTIN* is more highly expressed in the root tip than many other parts of the plant (Zimmermann et al., 2004). We chose the "RCP1" root cap promoter because we are interested in root growth and it has been used in the literature to express transgenes only in the root tip.

Results: Seedlings carrying RCP::ami-R-ctn show normal phenotypes

Columbia wild type plants were transformed with the RCP::ami-R-ctn constructs. No abnormal phenotypes were observed. To test for subtle gravitropism defects, T2 seedlings were screened on kanamycin and the resistant seedlings were tested for gravicompetence in a 180° turn hairpin assay (Figure A1- 12). Again, no abnormal phenotypes were observed.

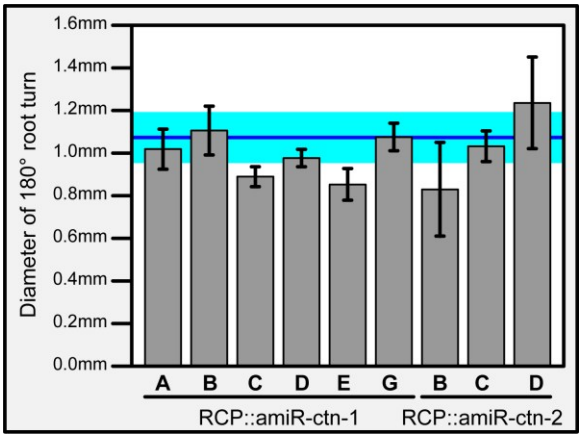

Figure A1- 12: RCP::ami-R-ctn plants show normal root growth and gravitropism. The diameter of the turn is reflective of the extent of gravity sensing and growth rate. The blue shows Columbia wild type with the cyan box showing standard error. Error bars show standard error of seedling turn diameter. All seedlings used for this graph were grown simultaneously. None are statistically different from Columbia (t-test $p<0.10$; N=8-29).

One reason that these lines may not show overt abnormal phenotypes is that they may not be successfully silencing *CACTIN*. We used a scalpel to cut off the lower 1-2 mm of roots and isolated RNA for qPCR. We found no significant differences from wild type for *CACTIN* expression in RCP::amiR-ctn1 and RCP::amiR-ctn2 lines. The root cap itself is too small to cut off with a scalpel, so the silencing signal is inevitably diluted by the other root tissues. Because of the tissue dilution issue, there still could be mild silencing in the root cap of these plants that we were unable to detect.

Inducible promoter systems drive amiRNA expression on demand

The root cap is an important organ of the plant, so it is possible that we were still selecting against strongly-silenced lines by picking the largest plants off of hygromycin selection. Another approach is to use an inducible promoter. We cloned amiR-ctn-1 and amiR-ctn-2 into an estrogen-inducible expression system vector (pMDC7) (Curtis and Grossniklaus, 2003). In this system, a region of the human estrogen receptor was fused to a bacterial DNA-binding protein and also to a herpes virus transcriptional activator protein domain. The net result is a new chimeric protein that can bind to the LexA operator sequence and promote expression of downstream transgenes only in the presence of estrogen (Figure A1- 13).

Figure A1- 13: (Figure next page) With domains from human estrogen receptor, viral VP16, and bacterial LexA, XVE is able to bind the LexA operator sequence in an estrogen-dependent manner. Estrogen binding causes XVE to dimerize, thus allowing the LexA domain to bind the LexA operator sequences upstream of the transgene of interest. The human herpes virus VP16 transactivation domain binds components of RNA polymerase II which are highly conserved between plants and animals (Hall and Struhl, 2002). The plant destination vector, pMDC7, is diagramed showing the region of the plasmid that is inserted into the plant genome after Agrobacterium transformation. The LexA operator sequence is shown as red ovals just upstream of where the gene of interest is inserted after the gateway reaction.

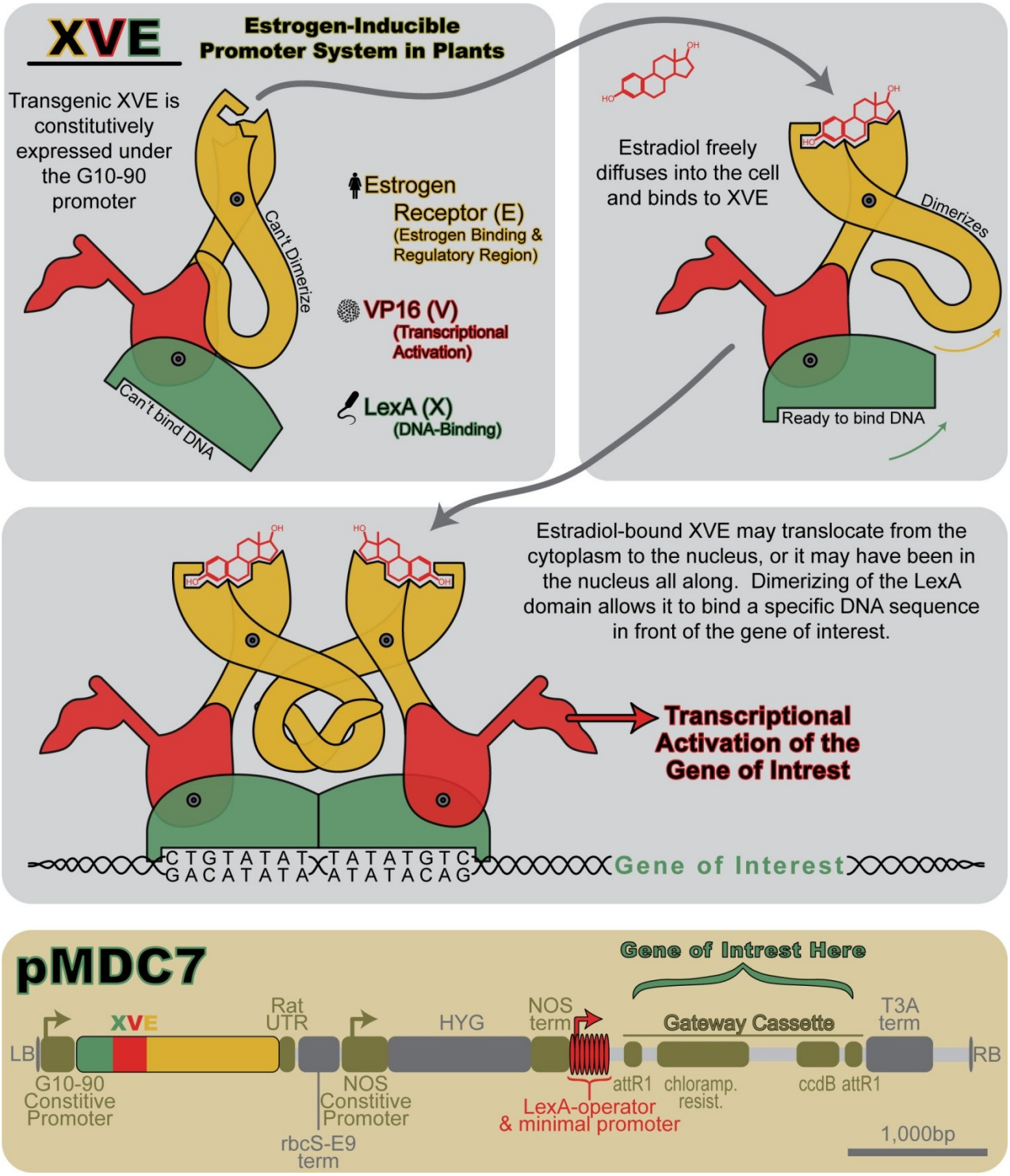

Figure A1- 13: Chimeric XVE is an estrogen-inducible transcription factor.
Legend: previous page.

Wild type Arabidopsis plants were transformed with estrogen inducible ami-R-ctn-1 and ami-R-ctn-2 constructs. The T2 seedlings were selected for hygromycin resistance (indicating that they carry the transgene) and then transferred to estrogen and vehicle control agar plates. We expected to see morphological abnormalities in the seedlings growing on estrogen plates because *CACTIN* is expressed throughout plant development and is likely important for cellular growth. For the first week, the seedlings on inductive and control media appeared morphologically the same. However, after a week of growth, differences became apparent for some lines. We found that 36% of the *CACTIN* silencing lines had shorter average root length after prolonged growth on Estrogen (T-test $p<0.002$) (Figure A1- 14). The four control lines, which contained the estrogen inducible system driving the *β-glucuronidase* (*GUS*) gene, did not grow statistically significantly differently on the estrogen media verses vehicle alone media (Figure A1- 14). The next step would be to test if the estrogen-induced root length correlates with the degree of *CACTIN* silencing using qPCR. If only 36% of the lines are actually silencing *CACTIN*, then this is a smaller percentage of effective amiRNA transformants than have been observed in the literature for amiRNAs targeted against non-essential genes (Schwab et al., 2005). One possible explanation is that some percentage of transformant lines had leaky expression in the absence of estrogen resulting in sick seedlings that we did not recover though selection. As a result, the percentage of unsuccessful silencing transformants could become enriched.

If some of our lines shown in Figure A1- 14 are strongly silencing *CACTIN* when exposed to estrogen, then these plants may be good candidates for testing for splicing

defects. This could be conducted by assaying candidate genes, such as SR proteins, which have many spliceoforms and their relative abundance is sensitive to alterations in the spliceosome. A more thorough way to assay these plants for splicing problems would be whole transcriptome sequencing. A time course after estrogen activation would help suggest which RNA changes might be a direct consequence of reduced *CACTIN*.

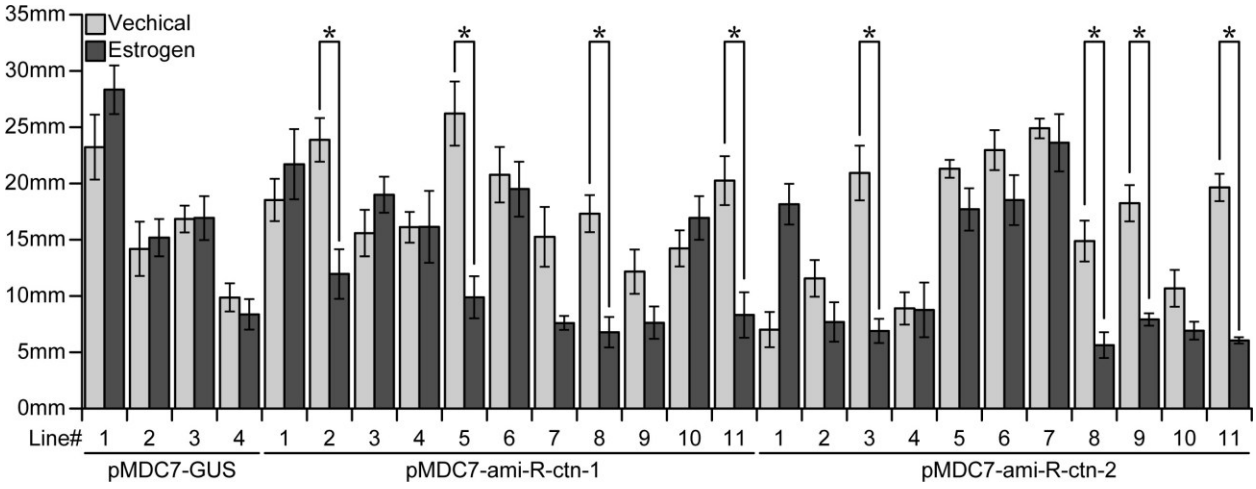

Figure A1- 14: Some lines of estrogen inducible CACTIN amiRNA plants show reduced root growth on inductive media than control media.
Seeds were germinated on hygromycin agar plates in the dark, and after 3 days the resistant seedlings were moved to new plates. For each line, half of the resistant seedlings were moved to estrogen plates, and half were moved to vehicle control plates. No obvious differences in seedling morphology between the two treatments were observed until eight days after the seedlings were transferred to the inductive plates. The plates were scanned and the root lengths were measured using the NeuronJ plugin for ImageJ (Meijering et al., 2004). Asterisk indicates that the average root length for that line was different on estrogen verses vehicle control plates (T-test $p<0.002$, which is 0.05 Bonferroni corrected for 22 comparisons.)

Discussion

The CaMV 35S-driven amiRNAs produced an abnormal phenotype

We observed an abnormal phenotype in multiple lines expressing two amiRNAs targeting *CACTIN* transcripts. The low-degree of silencing and lack of correlation between phenotypes and *CACTIN* transcript levels suggested that these plants are not ideal models to investigate the role of CACTIN in plant growth, development and response to the environment. However, it is possible that *CACTIN* silencing within just a certain cell type or a small group of cells within the developing leaf and/or its primordium is responsible for the abnormal phenotype. Because mRNA was extracted from whole rosette leaves of flowering plants for qPCR, the correlative silencing in only a small group of cells could have been diluted and lost. Another possibility is that the amiRNA constructs were successfully inhibiting translation of the *CACTIN* transcript but not causing its degradation. One way to test both of these possibilities was to use a visual reporter of CACTIN protein levels. We crossed the amiR-ctn lines to plants carrying a GFP-reporter of *CACTIN* silencing, but the baseline signal was too variable to allow careful evaluation of the results. The cause of the abnormal phenotype could hypothetically be further investigated by transforming the plants carrying amiR-CTN with mutated versions of the *CACTIN* gene that have altered codon-usage at the site where the amiRNA binds the mRNA, thus making them resistant to amiRNA binding. If the phenotype is truly due to silencing of *CACTIN* in a particular tissue of the plant, then these altered transgenes should “rescue” the phenotype.

The plants carrying 35S::amiR-CTN may not have silenced CACTIN sufficiently

As noted in Figure A1- 10, the amiRNA lines maintained 20-50% of *CACTIN*. amiRNAs targeted against other genes have been able to silence them to undetectable transcript levels (Schwab et al., 2006). Because *CACTIN* is an essential gene (chapter 2), we did not expect to find successful transformants with undetectable *CACTIN* levels, but we had hoped for lines with very low levels. The high levels of residual *CACTIN* expression that we found may suffice for normal plant growth. We attempted to address this using root cap expression of the amiRNA, but we still found no abnormal phenotypes and were unable to detect *CACTIN* silencing. We further attempted to successfully silence CACTIN in a non-lethal way using estrogen-inducible expression of the amiRNAs. However, contrary to expectations, these seedlings grew normally on estrogen containing plates. More investigation is needed to determine if they still may be a viable tool for researching CACTIN.

amiRNA experiments did not yield information about *CACTIN*

Because the *cactin-1* insertional mutant resulted in early embryo arrest, a silencing strategy was a rational technique to implement. Using a ubiquitous (CaMV 35S) and a root cap-specific (RCP) promoters, we obtained no strongly silenced lines with decreased *CACTIN* RNA levels correlating with a discreet phenotype. This could be due to selection against strong-silencing lines because of the importance of CACTIN, or because our amiRNAs were not very effective at silencing CACTIN. More work will be needed to evaluate these possibilities.

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Appendix 2: Gravity Sensing and Signal Transduction in Vascular Plant Primary Roots[3]

Katherine L. Baldwin, Allison K. Strohm, Patrick H. Masson

Abstract

During gravitropism, the potential energy of gravity is converted into a biochemical signal. How this transfer occurs remains one of the most exciting mysteries in plant cell biology. New experiments are filling in pieces of the puzzle. In this review, we introduce gravitropism and give an overview of what we know about gravity sensing in roots of vascular plants, with special highlight on recent papers. When plant roots are reoriented sideways, amyloplast re-sedimentation in the columella cells is a key initial step in gravity sensing. This process somehow leads to cytoplasmic alkalinization of these cells followed by relocalization of auxin efflux carriers (PINs). This changes auxin flow throughout the root, generating a lateral gradient of auxin across the cap that upon transmission to the elongation zone leads to differential cell elongation and gravibending. We will present the evidence for and against the following players having a role in transferring the signal from the amyloplast sedimentation into the auxin signaling cascade: mechanosensitive ion channels, actin, calcium ions, inositol trisphosphate, receptors/ligands, ARG1/ARL2, spermine, and the TOC complex. We also outline auxin transport and signaling during gravitropism.

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[3] This chapter is a modified version of the publication: Baldwin KL, Strohm AK, Masson PH. (2013) Gravity sensing and signal transduction in vascular plant primary roots. Am J Bot. Jan;100(1):126-42.

Introduction to Gravitropism

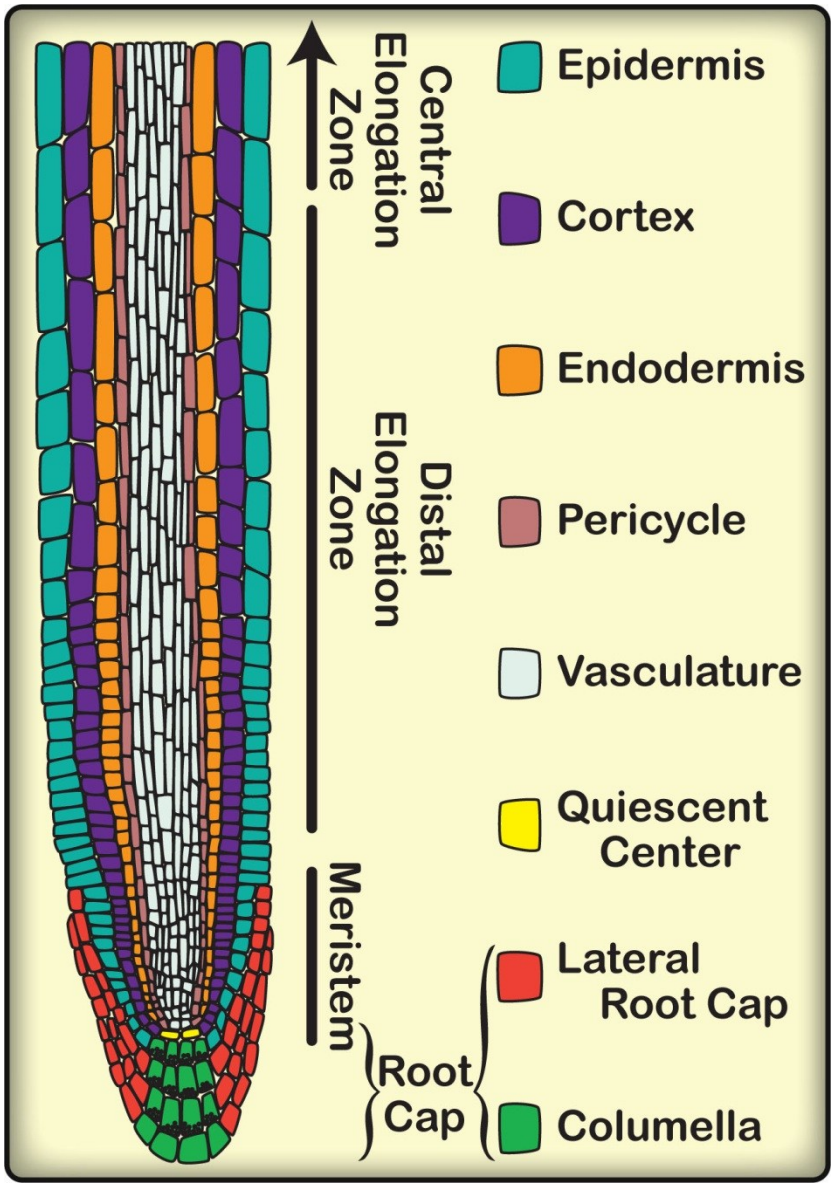

Figure A2- 1: Diagram of root cell types in an Arabidopsis root.

Plants sense gravity and guide their growth with respect to the gravity vector in a process called gravitropism. This is exemplified by turning a plant on its side (reorientation) and watching the roots bend downwards and the stem curve upwards, even in the absence of light. Gravitropism interacts with other tropisms, such as phototropism, hydrotropism, and thigmotropism to control adaptive plant growth and development. Primary roots generally grow down, and stems generally grow up, but lateral roots and branches grow at genetically specified, yet physiologically, developmentally and environmentally adapted, set-point angles relative to gravity (Digby and Firn, 2002).

As discussed in detail below, in roots the key site of gravity sensing (the columella cells of the root tip) is spatially separated from the elongation zone where the gravibending takes place (Figure A2- 1). This makes roots a good organ to characterize when trying to understand the early phases of gravity sensing, versus the later stages of differential growth. A better understanding of gravitropism has the capacity to lead to engineered plants for severe or foreign environments such as space. Control over gravitropism could help with traditional agricultural or horticultural problems as well, such as improving the upward bending of crop stems after prostration during storms or preventing stem bending during transport.

Researchers have been trying to understand gravitropism for over 200 years (Knight, 1806; Darwin and Darwin, 1880). Despite ingenious screens and excellent genetic tools, the mechanism by which the potential energy of gravity is converted to a biochemical signal in vascular plants is still unknown. However, a lot of information has been gathered, and researchers are filling in the missing puzzle pieces every day. This review aims to describe the current understanding of gravity sensing and signal transduction in vascular plant roots and to highlight the still-missing information. The mechanism of gravity sensing in plants is one of the most fascinating questions in molecular biology, and because of the new availability of systems biology tools and high-throughput computer-based growth analysis, this is an energizing time in this field.

Plants Sense Gravity

Root gravibending occurs in the elongation zones

Root tips are covered by a cap, which protects the root from obstacles in the soil. Adjacent to the cap is the root meristem, where cells surrounding a small quiescent center differentiate into initials, which divide slowly and asymmetrically, reconstituting themselves while yielding another cell that will divide multiple times before leaving the meristem. Shootward to the meristem are the distal and central elongation zones, where the cells elongate massively through anisotropic expansion. The maturation region of the root is shootward to the elongation zones, where cell growth is complete. There, cells differentiate into the various cell types that will constitute the mature zone. The elongation zones are the critical sites of root bending during gravitropism. When a seedling is turned on its side, the first detectable curvature is located in the distal elongation zone, which is the region of the root shootward to the meristem extending up to where the cell elongation rate is 30% of its maximum rate (Ishikawa and Evans, 1993; Miller et al., 2007). The central elongation zone is located shootward of the distal elongation zone and it is where cells reach their maximum elongation rate (Ishikawa and Evans, 1993). As the root bends downward, the region of curvature extends both shootward and slightly more rootward to encompass the entire distal elongation zone and some of the central elongation zone (Miller et al., 2007).

The root columella cells sense gravity

Although root bending occurs in the elongation zones, they are not the key sites of gravity sensing. Roots of monocot and dicot plants use columella cells within the cap as specialized gravity sensing cells, or statocytes (Figure A2- 1). There are only a couple dozen of these cells in an Arabidopsis root tip. They have a particularly “open”

cell structure, with small vacuoles and a cortical ER (Zheng and Staehelin, 2001; Leitz et al., 2009). They are polarized with the nucleus positioned towards the shootward edge of the cells (Leitz et al., 2009). The most notable feature of these cells is their sedimenting amyloplasts. Amyloplasts are specialized plastids filled with dense starch. As discussed below, the amyloplasts and the columella cells are very important for gravity sensing.

Stems sense and respond to gravity within the same organ

The statocytes of stems and hypocotyls are significantly different from roots in that they are located throughout the same region of the organ where bending occurs. In the stems and hypocotyls of dicot plants, the endodermal cells contribute to gravity sensing (Fukaki et al., 1998). There are thousands of endodermal cells in the stems and hypocotyls of dicots, and like columella cells, they contain dense sedimenting amyloplasts. Monocot stems also sense and respond to gravity within the same organ. Pulvini are knee-like swellings in the stems that contain cells with sedimenting amyloplasts. During gravistimulation the pulvini can expand asymmetrically, resulting in stem bending.

The starch-statolith hypothesis and the Cholodny-Went theory attempt to explain some aspects of gravitropism

The starch-statolith hypothesis proposes that in vascular plants, the starch-filled amyloplasts of gravity-sensing cells act as statoliths, which fall within cells, thereby signaling the direction of gravity (Haberlandt, 1900; Němec, 1900). During root gravitropism, the sedimentation of the amyloplasts initiates a signaling cascade that is

not completely understood but it results in changes in auxin concentration on either side of the root. This differential auxin flow is thought to be initiated by a change in distribution of auxin efflux facilitators (PIN-FORMED (PIN) proteins) within the columella cells. The resulting lateral auxin gradient is then transported to the elongation zone where differential elongation occurs. As stated in the Cholodny-Went theory, gravibending is the result of differential accumulation of auxin on opposite sides of the elongation zone, resulting in differential growth and tip curvature (Went, 1926; Cholodny, 1927).

Many experiments demonstrate the importance of sedimenting amyloplasts in gravitropism

Sedimentation of dense, starch filled amyloplasts is a key first step in gravitropism. The importance of starch can be illustrated with starchless mutants, such as *pgm* (Sack and Kiss, 1989.). These mutants lack a starch synthesis enzyme, phosphoglucomutase, and have a severely attenuated gravity response (Kiss and Sack, 1989; Band et al., 2012). Further evidence comes from the necessity of the starchy-amyloplast-containing cells in the root, the columella cells. Physical elimination of columella cells though various means, such as laser or genetic ablation, greatly decreases the gravity response (Blancaflor et al., 1998; Tsugeki and Fedoroff, 1999).

Changes in statolith density are also sufficient for gravibending. The *sex1* (starch excess) mutant in Arabidopsis lacks a starch degradation enzyme and thus contains too much starch in some tissues (Vitha et al., 2007). Depending on the organ and the culture conditions, the amyloplasts of *sex1* are sometimes larger than WT,

particularly in hypocotyls of light grown seedlings. Generally, the organs that display, and conditions that result in, larger amyloplasts are also associated with greater gravisensitivity (Vitha et al., 2007).

More support for statolith movement being sufficient for gravisensing comes from magnetophoresis experiments. In these studies, high gradient magnetic fields are used to displace laterally the diamagnetic amyloplasts within the statocytes of vertical plant organs. This shift in amyloplasts position is sufficient to make roots curve towards the direction of plastid movement along the field, and hypocotyls and stems to bend away from it (Kuznetsov and Hasenstein, 1996; Kuznetsov and Hasenstein, 1997).

There is an alternative system of gravity sensing in roots

Although there is a lot of evidence that statoliths in columella cells are very important for root gravitropism, there is also growing evidence for an additional, secondary mechanism of gravity sensing that might be independent of starch and might be located outside of the root cap.

Starchless mutants retain some response to gravity

Some of the same experiments that have highlighted the importance of amyloplasts in plant gravitropism also reveal the possible existence of another gravity sensing mechanism. The *pgm* mutants discussed above have a greatly attenuated gravitropism response, but they do eventually reorient their roots downward and stems upward. Some groups have reported that their amyloplasts are completely starchless and that the amyloplasts never settle (Caspar and Pickard, 1989). This suggests that in

the absence of amyloplasts falling, there is a secondary mechanism that can still direct the root downward. However, other groups have found that the *pgm* mutant amyloplasts still contain some starch under their growth conditions (Saether and Iversen, 1991) and could perhaps respond to gravity through the canonical pathway, albeit in an extremely attenuated fashion.

Does the distal elongation zone of roots also contribute to gravity sensing?

One tool that has allowed researchers to dissect the graviresponse is the ROTATO device. This device holds predefined regions of corn and Arabidopsis roots at specific angles from the gravity vector using an automated camera and motorized stage. The ROTATO has been used to analyze the gravitropic response of Arabidopsis *pgm* mutants; these experiments found support for a secondary mechanism of gravity sensing (Wolverton et al., 2011). In wild-type plants, there is a positive relationship between the rate of root curvature and the constant angle at which the root tip is held. This means that the more extreme the angle of displacement, the faster the rate of curvature. In contrast, the *pgm* mutants always respond at the same slow rate no matter which tip angle is chosen (Wolverton et al., 2011). This suggests that the type of gravity sensing in *pgm* plants is fundamentally different from that of wild type; it is not just an attenuated normal response.

ROTATO experiments that hold the root tip vertical while gravistimulating the elongation zone have suggested that 20% of the gravity response comes from a site located within the distal elongation zone, not the root tip (Wolverton et al., 2002). Since

in roots the statoliths are only found in the columella cells, this suggests an entirely different type of gravity sensing.

Roots that have their caps removed respond to gravity very slowly (Mancuso et al., 2006). The residual gravity response may be due to regrowth of a new root cap with columella cells. However, decapped corn roots respond very differently to gravistimulation in the presence of actin polymerization-inhibiting drugs than do intact corn roots (Mancuso et al., 2006). In these experiments, the gravibending of intact roots was not affected by actin-destabilizing drugs, and the decapped roots showed faster gravibending. This suggests that there is another site of gravity sensing outside of the root cap that depends on actin differently than the columella-based mechanism. Because root cells outside of the cap do not seem to contain sedimenting amyloplasts, their mechanism of gravity sensing should differ from the starch-statolith model discussed above.

It is interesting to note that actin destabilization increases the graviresponse of intact Arabidopsis roots, as it does to the decapped corn roots (Staves et al., 1997b; Hou et al., 2003, Mancuso et al., 2006). It will be helpful to have these decapping and actin-destabilizing experiments repeated in Arabidopsis so mutants will be available for dissecting this phenomenon further.

Could some plant cells also sense gravity using the hydrostatic pressure exerted by their protoplast on the wall?

If the distal elongation zone cells of vascular plant roots can sense gravity despite lacking statoliths, the starch-statolith model discussed above is insufficient. The

large internodal cells of the green algae, Chara, do not contain statoliths, and yet they seem to use gravity to control the polarity of cytoplasmic streaming (Staves et al., 1997a). These cells may give some clues to the nature of alternative gravity-sensing machinery. The Chara internodal cells seem capable of sensing the difference in hydrostatic pressure between their top and bottom sides, a process that may lead to the opening of stretch-activated mechanosensitive ion channels within the plasma membrane. The resulting increase in cytosolic Ca^{2+} levels would ultimately trigger the observed change in cytoplasmic-streaming polarity.

While it is tempting to postulate that this hydrostatic pressure model of gravity sensing might also function in higher plant roots, an assumption that seems supported by experiments carried out on gravistimulated rice roots exposed to high-density media (Staves et al., 1997c), it should still be cautioned that the Chara internodal cells, which are about three centimeters long, are much larger than most vascular plant statocytes, and thus there would be a larger pressure differential between the upper and lower sides.

Gravity Signal Transduction in the Columella Cells

These proposed secondary sites of gravity sensing that are outside of the root cap or that do not require starch are very exciting because so little is known about them. In contrast, there is more known about the signal transduction that occurs after amyloplast sedimentation in the columella cells of the root cap, although some intriguing

questions remain. The following sections discuss the mechanisms that take place in the columella cells of the roots.

How do sedimenting amyloplasts signal the direction of gravity?

After a plant is rotated on its side, the amyloplasts of the columella cells begin to fall to the new bottom sides of the cells. The experiments discussed above have shown that this is a key step in gravity sensing, but how the falling amyloplasts signal a change in subcellular localization of columella cell auxin efflux carriers is unknown. Researchers are testing different mechanisms to explain how amyloplasts activate a signaling cascade that leads to relocalization of PIN proteins in the statocytes.

Leitz *et al*. 2009 used electron tomography to demonstrate that falling amyloplasts bend and distort the ER where they make contact. This deformation is enough to hypothetically open stretch-activated mechanosensitive ion channels. These authors also demonstrated very close contact between the amyloplasts and the ER, suggesting that protein-protein interactions between the amyloplasts and the ER are possible (Leitz et al., 2009). Interestingly, two hypotheses have been proposed to explain gravity susception upon amyloplast sedimentation: one involves the opening of mechanosensitive ion channels; the other proposes a ligand-receptor interaction.

Do mechanosensitive ion channels contribute to gravity sensing?

Under the mechanosensitive ion channel hypothesis of signal transduction, sedimenting amyloplasts distort plasma or ER membranes either through direct pressure or by pulling on actin filaments connected to membranes. This membrane

deformation opens stretch-activated mechanosensitive ion channels, changing the localized concentration of ions, most likely Ca^{2+}, in that region of the cell. The localized Ca^{2+} could then trigger a repolarization of the statocytes, along with a relocalization of PINs and changes in auxin transport.

If this hypothesis were correct, one would expect that pharmacological agents that disrupt the opening of mechanosensitive ion channels would inhibit gravitropism. Furthermore, such channels should be identified and unambiguously assigned a role in gravity signal transduction. One should also be able to detect fast changes in cytosolic Ca^{2+} levels in the statocytes early in response to gravistimulation, and compounds that inhibit cellular transducers of cytosolic Ca^{2+} signals, including calmodulin and/or Ca^{2+}-dependent protein kinases, should also interfere with gravitropism. If the channels were opened by tugging on actin, then actin-destabilizing drugs would be expected to inhibit the graviresponse. However, as discussed below, while some of these expectations have been met, others have not, casting some doubt on the central role of mechanosensitive ion channels in gravity sensing.

Inhibitors of mechanosensitive ion channels inhibit gravitropism

The lanthanide elements, lanthanum and gadolinium, are general inhibitors of cation-selective stretch-activated ion channels (Caldwell et al., 1998). These elements inhibit gravitropism in seedlings, and the magnitude of the effect of various lanthanides correlates with their effect on ionic plasma membrane channels as measured by patch

clamp experiments in onion protoplasts (Ding and Pickard, 1993). These findings are consistent with a lanthanide-sensitive channel playing a role in gravitropism.

The plant mechanosensitive channel genes identified so far do not contribute to gravitropism

Many of the mechanosensitive ion channels known in other organisms do not have clear orthologs in plants. These include the metazoan DEG/ENaC channels (named for *C. elegans degenerins* and mammalian Epithelial Na+ Channel), the mammalian TWO-PORE DOMAIN K^+ (K2P) channels, the opisthokont (fungal and animal) TRANSIENT RECEPTOR POTENTIAL (TRP) channels, and the bacterial MECHANOSENSITIVE CHANNELS OF LARGE CONDUCTANCE (MscL) (Haswell and Meyerowitz, 2006; Arnadottir and Chalfie, 2010).

However, orthologs of the bacterial MECHANOSENSITIVE ION CHANNEL OF SMALL CONDUCTANCE, MscS, are currently being studied in Arabidopsis. Two members of this family (MSL2 and MSL3) localize to plastids and are involved in maintaining plastid shape during hypoosmotic stress (Veley et al., 2012). Other family members, MSL9 and MSL10, are localized to the plasma membrane, and double mutants show defects in patch-clamp tests for stretch-activated channel activity (Haswell et al., 2008). Surprisingly, these mutants show no abnormal phenotypes at the whole-plant level, and quintuple mutants (*msl4 msl5 msl6 msl9 msl10*) made from this family show no gross phenotypes and have normal gravitropism (Haswell et al., 2008).

Another stretch-activated ion channel from Arabidopsis was found in a screen for cDNAs that rescue a conditional yeast mutant called *mating pheromone-induced death*

(*mid1*). The Arabidopsis gene MID1-COMPLEMENTING ACTIVITY (MCA1) has very little similarity to the yeast channel. Studies on Arabidopsis plants carrying mutations in this gene suggest that it is involved in thigmomorphogenesis, but there is no evidence yet that it, or its paralog *MCA2*, are involved in gravitropism (Nakagawa et al., 2007). There may be other plant-specific mechanosensitive ion channels that have not been identified yet and that are involved in gravitropism.

Are actin filaments needed for gravity sensing?

It has also been proposed that tugging on actin filaments could open mechanosensitive channels in membranes. Indeed, there is a network of actin filaments surrounding the amyloplasts in columella cells (Collings et al., 2001). If pulling on actin filaments were truly responsible for transducing the gravity signal, then actin-destabilizing drugs would be expected to decrease the plant's response to gravity. However, treating roots with drugs like latrunculin B and cytochalasin D promotes gravitropism in various vascular plants, rather than inhibiting it, suggesting that actin is not a positive regulator of gravity sensing (Staves et al., 1997b; Hou et al., 2004). In fact, latrunculin B-treated corn seedlings that were exposed to defined gravistimuli and then placed on a rotating clinostat that randomized their orientation within the gravity field displayed an extended curvature relative to untreated controls, suggesting that actin might be a negative regulator controlling over-bending (Blancaflor et al., 2003; Hou et al., 2004).

Similarly, the roots of germinating flax seeds acquire the ability to sense gravity only after starchy amyloplasts have formed in their statocytes but before the formation of a fully developed actin cytoskeleton (Ma and Hasenstein, 2006). Together, these results do not favor actin playing a major role in early gravity sensing in columella cells.

Early cytosolic Ca^{2+} responses to gravistimulation have not been detected in statocytes

An involvement of cation-selective mechanosensitive ion channels in gravity signal transduction would imply the occurrence of cytosolic Ca^{2+} changes in the statocytes early in response to gravistimulation. Considering the implications of the starch statolith hypothesis, one would further expect such changes to occur locally on the bottom side of the statocytes, where sedimenting amyloplasts lie on top of the peripheral ER, deforming their membranes (Leitz et al., 2009). Pressure- and/or curvature-dependent opening of mechanosensitive ion channels would allow a local increase in cytosolic Ca^{2+} levels, potentially leading to a rerouting of the vesicular trafficking pathway to favor the accumulation of auxin efflux facilitators within the bottom membrane.

Several investigators have attempted to detect gravity-induced changes in cytosolic Ca^{2+} levels in response to gravistimulation. Unfortunately, attempts at observing such changes specifically in the statocytes have failed. For instance, the columella cells of gravistimulated Arabidopsis seedlings did not show a detectible increase in cytoplasmic Ca^{2+} in experiments relying on indo-1 as the calcium sensitive detection system (Legue et al., 1997). However, it remains possible that gravity-induced

Ca^{2+} changes in the root tip are too minute to be detected by such technologies. Calmodulins are known to be highly expressed in columella cells of roots (Stinemetz et al., 1987), implying that small or highly localized gravity-induced changes in intracellular Ca^{2+} levels could affect cell signaling while remaining below the limit of resolution of the detection strategies used to date. A more complete discussion on the latest advances on Ca^{2+} changes in the columella cells is provided in a review by Toyota and Gilroy on mechanoperception (Toyota and Gilroy., 2013)

Although Ca^{2+} changes have not been observed in gravistimulated columella cells, they have been observed elsewhere in the plants, suggesting that Ca^{2+} plays a role downstream in the graviresponse. Experiments using transgenic AEQUORIN to form an *in situ* calcium-sensitive luminescent complex with added coelenterazine detected a biphasic Ca^{2+} response to gravistimulation when several Arabidopsis seedlings were gravistimulated in front of a luminometer or a photon-counting charge-coupled-device camera (Plieth and Trewavas, 2002; Toyota et al., 2008). The first peak, which reached a maximum within 4 sec of gravistimulation, was insensitive to the angle of rotation whereas the second, more prolonged peak, which reached its maximum after 40 – 60 sec, was responsive to the angle of stimulation. These data were interpreted to suggest that only the second peak might be specifically modulated by gravistimulation whereas the first could result from the mechanostimulus that accompanies seedlings' reorientation within the gravity field.

Unfortunately, the Ca^{2+} peaks discussed above derived only from hypocotyls and petioles, not from roots (Toyota et al., 2008). Furthermore, their amplitudes were found to decrease when auxin transport inhibitors were added, suggesting they might occur in response to auxin signaling. Yet, more recent data showing that the initial rate of the second peak's increase remains unchanged when seedlings are exposed to auxin transport inhibitors suggest that this cytosolic Ca^{2+} response, or at least some part of it, might still precede auxin signaling during gravity signal transduction, whereas its final amplitude might be modulated by auxin signaling (Toyota et al., 2008). Sadly, the Ca^{2+} detection system used in these experiments did not allow cell-specific resolution of the signal.

Ca^{2+} inhibitors do not clarify the role of Ca^{2+} in gravitropism

Various Ca^{2+} chelators have been applied to gravitropic plant tissues and shown to affect gravibending (Bjorkman and Cleland, 1991; Belyavskaya, 2001). However, these drugs may have broad disruptive effects on the plant, and the results are equally consistent with Ca^{2+} playing either an early role or a downstream function in gravitropism. Similarly, inhibiting calmodulins (calcium binding messenger proteins) with pharmacological agents at low concentrations that do not detectably reduce the root elongation rate still inhibits gravibending in Arabidopsis (Sinclair et al., 1996). Furthermore, inhibiting multifunctional Ca^{2+}/calmodulin-dependent protein kinases with the KN-93 pharmacological agent inhibited light-dependent root gravibending in the *Merit* cultivar of corn (Lu et al., 1993). Recent work from Monshausen et al., 2011 suggests that Ca^{2+} is downstream of auxin signaling and is involved in converting auxin

signals into extracellular pH changes, which we will discuss further in the auxin section. In summary, a role for Ca^{2+} in the earlier stages of gravitropism remains enigmatic.

Inositol trisphosphate ($InsP_3$) is a signaling molecule

Does plant InsP3 modulate cytoplasmic Ca^{2+}?

Some support for an involvement of Ca^{2+} in gravity signal transduction may be found in investigations addressing a role for inositol 1,4,5-trisphosphate ($InsP_3$, also known as IP_3) in gravitropism, although there is ambiguity on its role in the process. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a membrane lipid that can be cleaved into two fragments: $InsP_3$, and diacylglycerol (DAG). Both of these cleavage products act as signals in eukaryotic cells undergoing a diverse set of stimuli, as reviewed in (Berridge, 2009; Munnik and Nielsen, 2011). In animal cells, the soluble $InsP_3$ activates an ER localized receptor channel to release Ca^{2+} into the cytosol (Berridge, 2009). However, plants do not have a clear ortholog of the $InsP_3$ activated-Ca^{2+} channel receptor (Munnik and Nielsen, 2011). There is evidence that $InsP_3$ is still connected to Ca^{2+} in plants because injection of a photoactivatable $InsP_3$ into the stomata guard cells results in increased cytoplasmic Ca^{2+} levels and stomata closure in the vetch plant dayflower (Blatt et al., 1990; Gilroy et al., 1990). However, another signaling lipid, myo-inositol hexakisphosphate ($InsP_6$), can activate Ca^{2+} spikes in potato with 100 times higher efficiency than $InsP_3$, perhaps suggesting that $InsP_6$ is the biologically relevant inositol molecule for signaling Ca^{2+} changes in plants (Lemtiri-Chlieh et al., 2003). Furthermore, it is still unclear if the effects of $InsP_3$ and $InsP_6$ on Ca^{2+} in plant cells are direct.

$InsP_3$ has a role in gravitropism

Although the mechanism of $InsP_3$ signaling and whether or not it involves Ca^{2+} in plants is still ambiguous, it is clear that $InsP_3$ is a signaling molecule in plants and that it has a role in the graviresponse. A first clue that $InsP_3$ is involved in gravitropism derived from experiments measuring its levels during early gravitropic responses of plant organs. In these experiments, $InsP_3$ was found to first fluctuate and then increase detectably at the bottom half of oat and Arabidopsis stems early upon gravistimulation (Perera et al., 1999; Perera et al., 2001; Perera et al., 2006). Roots were not examined in these experiments. Interestingly, over-expression of a human-derived constitutively-active inositol trisphosphatase, which hydrolyzes $InsP_3$, caused gravitropic defects in Arabidopsis roots, stems, and hypocotyls (Perera et al., 2006). Furthermore, inhibiting the formation of $InsP_3$ using the aminosteriod U73122 resulted in abnormal root morphology and impaired graviresponse (Andreeva et al., 2010).

Microarray experiments conducted on plants expressing the constitutively active inositol trisphosphatase as well as plants treated with $InsP_3$ inhibitors during gravistimulation found that many of the transcripts that change in abundance during gravistimulation do not change as much when $InsP_3$ is depleted, implying that $InsP_3$ signaling is important for a normal graviresponse (Salinas-Mondragon et al., 2010). Importantly, these authors also found that many of the $InsP_3$-dependent transcription changes that occur during gravitropism also happen during phototropism in an $InsP_3$-dependent way (Salinas-Mondragon et al., 2010). They concluded that $InsP_3$ may be involved in the crosstalk between light and gravity responses.

The experimental perturbations discussed above decreased both the amount of $InsP_3$, and the graviresponse of treated organs. On the other hand, Arabidopsis mutants in the *INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 13* gene (*5PTase13*) lack one of the enzymes involved in breaking down $InsP_3$, and thus would be expected to have higher levels of $InsP_3$. In concordance with the experiments discussed above, these plants have an enhanced gravitropic response (Wang et al., 2009). These mutants also have altered responses to exogenously applied auxin transport inhibitors, suggesting that $InsP_3$ plays a role in auxin transport and/or signaling (Wang et al., 2009). *5PTase13* mutants are less sensitive to brefeldin A, a drug that affects endomembrane trafficking, and less PIN protein can be found in association with the brefeldin-A-induced endosomes in the root cortex of the mutant relative to wild type plants. Furthermore, the PIN2 protein displayed a broader expression pattern in the root tips of mutant plants than in the wild type (Wang et al., 2009).

Taken together, these data suggest that $InsP_3$ signaling could be involved in relocalizing PIN proteins through modulating the endomembrane system after root reorientation within the gravity field. Whether this process is Ca^{2+}-dependent or not remains to be investigated.

Could plastid- and ER-associated receptors and ligands transduce the gravity signal?

An alternative way to transduce the information derived from a repositioning of amyloplasts within the statocytes into a cellular signal is through a receptor-ligand interaction between proteins on the outer surface of the sedimenting amyloplasts and

proteins on the ER or plasma membrane upon which the amyloplasts eventually settle. When the ligands make contact with the receptors, the interaction may stimulate phosphorylation or some other form of protein activation in a localized region of the cell that signals the direction of gravity. Conceptually, this protein-protein interaction could also stimulate some of the same secondary messengers discussed above, like Ca^{2+} or $InsP_3$.

Insights from green algae

Careful studies on the statolith-containing rhizoid cells of the green algae, Chara, suggest that their statoliths may need to merely contact the membrane for signal transduction to occur and that increasing the pressure of the statoliths on the membrane does not increase signal transduction (Limbach et al., 2005). This observation is consistent with a ligand-receptor contact model. However, the proteins involved in this interaction have not been identified or confirmed, and Chara are distant relatives of vascular plants; their $BaSO_4$-containing statoliths do not share ancestry with amyloplasts. Yet this report introduced a novel model of statolith-based gravity sensing that does not rely on the involvement of stretch-activated ion channels and provides some supportive evidence for it using a model system with optimal characteristics for this initial physiological and cell biological investigation.

What are the physiological outputs of gravity signal transduction in the root statocytes?

Although the actual gravity receptor has not been identified yet, several physiological responses to gravistimulation have been reported to occur in the root

statocytes as a consequence of activation of the gravity signaling pathway: a fast alkalinization of the cytoplasm, and a change in cellular polarity involving redistribution of auxin efflux carriers (PINs) to the bottom flanks of these cells. This pH change might be part of the trigger that causes relocalization of the PIN protein because mutants defective in gravity-induced columella cell alkalinization also fail to show PIN relocalization, but the mechanism that could facilitate this is unknown (Boonsirichai et al., 2003; Harrison and Masson, 2008b).

Gravistimulation promotes a fast alkalinization in the cytoplasm of statocytes

Cytoplasmic pH changes have been observed by microinjection of a pH-sensitive dye into the cytoplasm of columella cells in Arabidopsis seedlings or by using a pH-sensitive form of transgenic GFP. By one minute of gravistimulation, the pH in tiers 2 and 3 of columella cells went from 7.2 up to about 7.6 (Fasano et al., 2001; Boonsirichai et al., 2003) (Figure A2- 2). A similar alkalinization of the statocytes' cytoplasm upon gravistimulation was observed in the pulvini of maize (Johannes et al., 2001). So far, this gravistimulus-associated pH change has been noted throughout the columella cell cytosol, not localized within cellular compartments. Furthermore, concomitant with this cytoplasmic alkalinization of the columella cells of Arabidopsis roots, a pH decrease also occurred in their wall/apoplastic region, decreasing from about pH 5.4 to about 4.7 within just a few minutes of gravity simulation. It took hours for the wall pH to return to its resting 5.4 value (Fasano et al., 2001). This is consistent with the protons being removed from the cytoplasm of columella cells and pumped outside the cell into the apoplast.

In an interesting test of the importance of these observed pH changes, Fasano et al. 2001 used microinjection of UV-activated caged protons to counteract the gravistimulated loss of protons in the cytoplasm of columella cells of Arabidopsis roots. This treatment significantly decreased the gravicurvature of roots suggesting that cytoplasmic pH change is important for the graviresponse. Because this cytoplasmic pH change happens in the statocytes very quickly after gravistimulation, it may be part of the early steps in gravity sensing, possibly facilitating auxin transport. How it plays into the pathway is not clear, but it functions downstream of amyloplast sedimentation because starch-deficient mutants display either no, or greatly reduced, cytoplasmic alkalinization of the columella cells upon gravistimulation (Fasano et al., 2001).

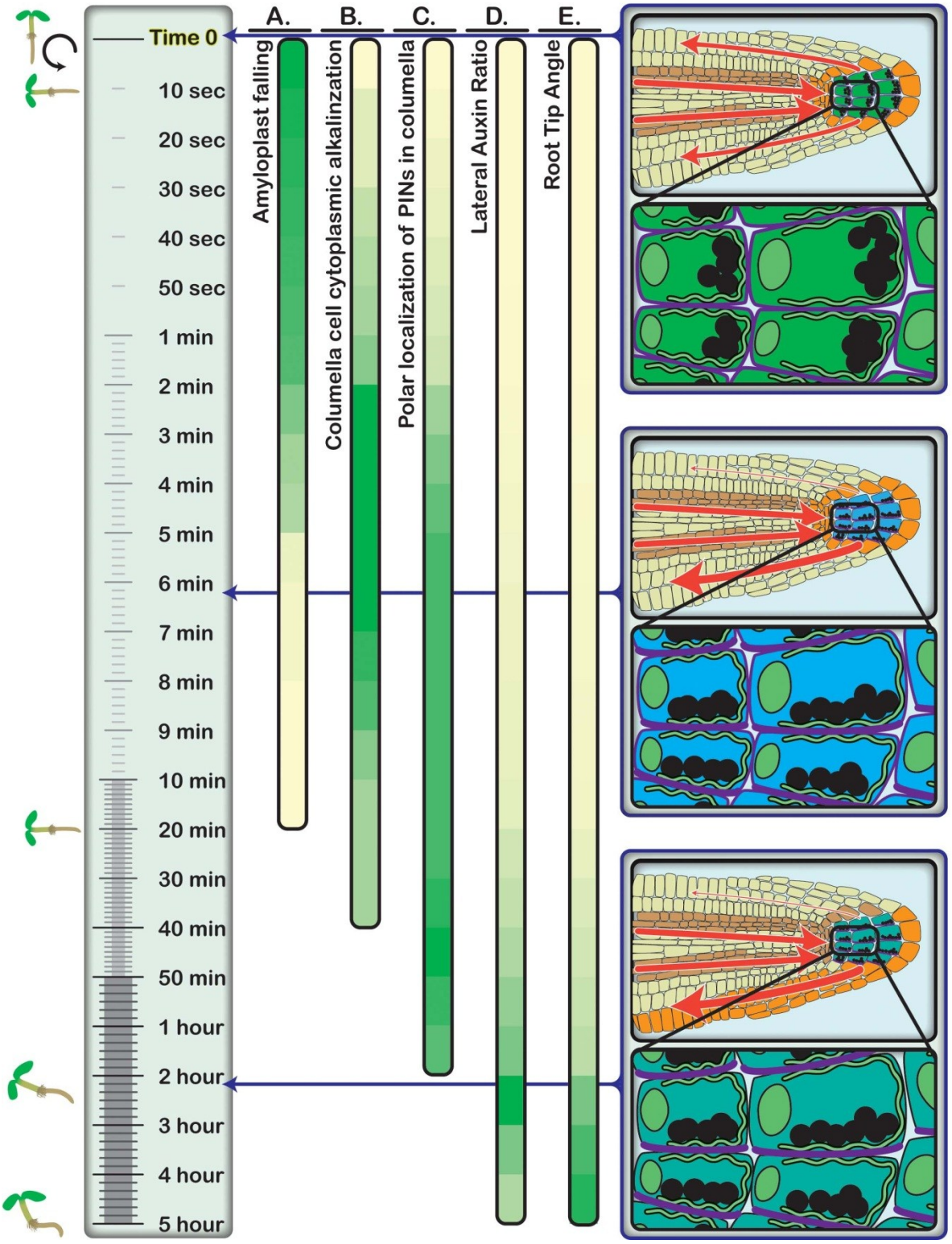
A.
B.
C.
D.
E.
Amyloplast falling
Columella cell cytoplasmic alkalinzation
Polar localization of PINs in columella
Lateral Auxin Ratio
Root Tip Angle
Time 0
10 sec
20 sec
30 sec
40 sec
50 sec
1 min
2 min
3 min
4 min
5 min
6 min
7 min
8 min
9 min
10 min
20 min
30 min
40 min
50 min
1 hour
2 hour
3 hour
4 hour
5 hour

Figure A2- 2 Flow of events following gravistimulation of an Arabidopsis root.
The top of the figure shows time zero when the seedling is rotated 90°. Note that the time scale is non-linear. The heat-map ribbons depict the degree of change of the listed parameters. The sources that inspired these generalizations are cited: A. Distance of the top amyloplast from the new bottom of the cell in Wassilewskija Arabidopsis (Stanga et al., 2009). B. Columella cell pH as visualized by injection of a pH sensitive dye (Fasano et al., 2001). This cytoplasmic pH change is accompanied by an extracellular apoplastic pH change not shown here (Fasano et al., 2001; Monshausen et al., 2011). C. PIN3 or PIN7 was scored on its polar verses non-polar localization from various sources. The early time points were inspired by (Friml et al., 2002b, figure 5), which used antibodies to localize PIN3. The later points were inspired by (Kleine-Vehn et al., 2010, figure 1), which used GFP fusions to PIN3 and PIN7, and also by (Harrison and Masson, 2008b, figure 3 which used a PIN3 antibody. D. Auxin concentration was indirectly measured using the DII-VENUS reporter from (Band et al., 2012). The heat ribbon illustrates the fluorescence ratio between the upper and lower sides of a gravistimulated root as shown in figure 3B of Band et al. 2012. E. Root tip angle was inspired by (Lewis et al., 2007). Note that the rate of root curvature is extremely variable depending on experimental conditions. Root Insets: Flow of auxin according to the reverse fountain model (Swarup and Bennett, 2003) is shown in vermilion arrows. Thicker arrows indicate more flow. The relative auxin concentration in cells is shown in orange. Columella cells of the root tip are shown in green. Amyloplasts are shown as black circles. The nuclei and ER are shown in lighter green. The localization of PIN3 and PIN7 are diagrammed as a purple outline on the plasma membrane of the columella cells. The alkalinization of the cytoplasm of the columella cells is diagrammed as blue.

Genetics identifies new early gravity signal transducers functioning in the statocytes

ARG1 and one of its paralogs are involved in early gravisensing

How the plastids signal pH changes is not clear, but genetic screens are filling in the gaps. Arabidopsis plants carrying mutations in *ALTERED RESPONSE TO GRAVITY 1* (*ARG1*) and its paralog, *ARG1-LIKE 2* (*ARL2*) are slower to reorient their hypocotyls and roots when gravistimulated (Sedbrook et al., 1999; Boonsirichai et al., 2003; Guan et al., 2003). *ARG1* and *ARL2* are non-redundant because the double mutant has the same phenotype as each single mutant, and these proteins may physically interact (Harrison and Masson, 2008a).

There are a number of findings that suggest that these genes are involved in the earliest phases of the gravity response. *arg1* and *arl2* mutants have normal phototropic responses and respond normally to exogenously applied hormones, suggesting that they are not core components of the auxin pathway (Guan et al., 2003). *arg1* mutants do not show some of the earliest signs of a gravity response such as alkalinization of columella cells, relocalization of the PIN3 auxin efflux facilitator to the lower membranes of the statocytes, and lateral auxin transport to the lower flank of gravistimulated roots (Boonsirichai et al., 2003; Harrison and Masson, 2008b).

The columella amyloplasts of *arg1* mutants contain normal amounts of starch as determined by starch staining and by transmission electron microscopy (Fukaki et al., 1997; Sedbrook et al., 1999; Stanga et al., 2009). The amyloplast sedimentation kinetics of *arg1 toc132* (a synergistic double mutant that will be discussed in the next section) were examined in live reoriented root columella cells and were indistinguishable from wild type (Stanga et al., 2009). This suggests that *ARG1* functions in the signal transduction phase of gravitropism in columella cells, downstream of plastid sedimentation. However, Shiva Kumar et al., 2008 found retarded amyloplast sedimentation in fixed endodermal cells in the hypocotyls of *arg1* seedlings. The ARG1 pathway may be regulated differently in roots and hypocotyls. One piece of evidence supporting this is that *arg1 pin3* double mutants show enhancement of the gravitropism defects of the single mutants in hypocotyls but not roots (Harrison and Masson, 2008b). Alternatively, these differences may have been due to the significant differences in growth conditions. If the gravicompetence of *arg1*

is truly dependent on specific growth conditions or the availability of certain nutrients, it could shed light on the possible functions of this gene.

ARG1 and *ARL2* encode membrane associated J-Domain proteins, and GFP fusions show them localized to the endomembrane system (Sedbrook et al., 1999; Boonsirichai et al., 2003). Therefore they are in the right location to play a role in the proper targeting of proteins such as PINs or proton transporters between intracellular compartments and the plasma membrane.

Expression of *ARG1* in only the root cap is sufficient to rescue the root mutant phenotype, and likewise, expression of *ARG1* in only the endodermis rescues the hypocotyl gravity defect. These results suggest that *ARG1* is active in the key sites of gravity sensing (Boonsirichai et al., 2003). Therefore, it seems likely that ARG1 and ARL2 are involved in the canonical statolith-dependent gravisensing step. Perhaps they constitute one wing of the sedimenting-statolith response pathway. However, there is strong enhancement of the gravitropism defect in *arl2 pgm* double mutants compared to either single mutant (Guan et al., 2003), suggesting that *ARG1* and *ARL2* are actually in a separate genetic pathway from *PGM*. This also suggests that the gravitropism defects in *arg1* are not due to the conditionally abnormal amyloplast sedimentation kinetics. This could mean that *ARG1* and *ARL2* are involved in an amyloplast-independent pathway that also occurs in the columella cells. Alternatively, a simpler explanation is that the *pgm* mutants retain some statolith sedimentation and that signal transduction is further affected by the loss of ARG1 or ARL2.

Plastids have a role in gravitropism that is separate from amyloplast sedimentation kinetics

Two genetic enhancers of the *arg1* phenotype were found through a forward screen: *toc132* and *toc75*. Both mutations affect components of the Translocon of the Outer envelope of Chloroplasts (TOC) complex, which is found on the surface of plastids. The *arg1 toc132* and *arg1 toc75* double mutants are totally agravitropic (Stanga et al., 2009). The *toc132* mutants are likely knockouts whereas the *toc75* mutant discovered in this screen is a hypomorph. *toc132* single mutants are only sometimes slightly pale, whereas the *toc75* hypomorph has pale leaves, and *toc75* knockout mutants are lethal (Ivanova et al., 2004; Kubis et al., 2004; Baldwin et al., 2005). For this reason, more analysis has been conducted on *toc132*.

Importantly, the columella cell amyloplasts are morphologically normal in *arg1 toc132* double mutants; they contain normal amounts of starch, and they sediment with normal kinetics (Stanga et al., 2009).

The implications of these mutations are still being investigated. TOC132 may facilitate the amyloplast localization of a ligand that could function as modeled in the receptor ligand hypothesis discussed above. Alternatively, these mutants may indicate that plastids are important for gravitropism in a role that is separate from amyloplast sedimentation and that we do not understand yet. It is not known yet if *TOC132* expression is necessary in the columella cells for proper gravitropism in the *arg1* background, or if it is needed in other parts of the plant. Additional work, including

identification of new genetic modifiers, is being conducted and will help to resolve the TOC complex's role in gravitropism.

Spermine has a role in gravitropism

Young *et al.* 2006 found that Adenosine Kinase 1 (ADK1) levels increase in root tips after gravistimulation using a 2-dimensional gel based proteomic screen in Arabidopsis. *adk1* mutants show growth defects but also slow root gravitropic responses. ADK1 is a key enzyme in the SAM/adoMet/methylation cycle, which is involved in the synthesis and/or methylation of many cellular compounds and signaling molecules. These observations suggest that one of the products of this cycle may play a role in gravitropism. Importantly, gravity-induced relocalization of the PIN3 auxin efflux facilitator in the columella cells of the *adk1* mutant does not occur. This suggests that ADK1 is necessary for early gravisensing. However, the roots of the *adk1* mutants also have morphological abnormalities, so it cannot be ruled out that some of the phenotypes are indirect consequences of abnormal structure. Most intriguingly, spermine, a polyamine product of the AdoMet cycle, was able to partially rescue the gravitropic defect of the *adk1* mutant (Young et al., 2006). Polyamines are synthesized by both eukaryotic and prokaryotic cells, but their full roles are not understood. Spermine may therefore be part of the gravity signaling cascade in roots. It is not known whether ADK1 or spermine are needed in the columella cells or other parts of the root.

Auxin as a Gravitropic SIgnal

Gravitropism signal transduction converges on auxin signaling

Although the mechanisms of gravity sensing and signal transduction are not completely understood, it is clear that the signal converges on auxin signaling. One of the only loss-of-function completely agravitropic single mutants in Arabidopsis is *aux1*, which encodes an auxin influx carrier (Marchant et al., 1999). Mutations in many core auxin pathway members, such as PIN proteins and Aux/IAA proteins, confer reduced gravicompetence (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998; Nagpal et al., 2000). Inhibition of rootward auxin transport using the drug, 1-N-naphthylphthalamic acid (NPA), also inhibits gravibending (Rashotte et al., 2000). Although this review is focused on gravity sensing and early transduction, we will give an overview of auxin signaling in gravitropism. For more information, see these recent reviews on auxin transport (Peer et al., 2011), PIN relocalization (Friml, 2010), and auxin receptors and response (Scherer, 2011).

Our understanding of auxin transport during gravitropism has benefitted from *in vivo* auxin reporters. An auxin-responsive synthetic promoter (DR5rev) paired with reporter genes such as GUS (beta-glucuronidase) and GFP (green fluorescent protein) has been an extremely useful tool (Ulmasov et al., 1997). However, there are several disadvantages. Transcription of auxin-responsive genes requires a cascade of signaling, and this reporter is thus quite indirect. Also, GFP requires slow post-translational modifications before it matures into a fluorophore, thus further delaying visualization of auxin changes. In order to address both of these problems, Brunoud et al., 2012 designed a new auxin reporter construct that provides a better temporal and

spatial resolution of auxin levels in plant tissues. This reporter will be described in the 'new tools' section of this review.

Auxin is the message that the columella cells send to the elongation zone

Fountain stream of auxin

Auxin flows through plants by being passed from cell to cell along vertical columns, or files, of cells. Plasma membrane localized auxin influx and efflux carriers control this flow. Coordinated, polarized expression of these auxin carriers makes auxin flow down the center of the root from the above-ground tissues to the tip where it is diverted up along the outer layers of the root in what has been termed a reverse 'fountain' model (Swarup and Bennett, 2003) (Figure A2- 2). Columella cells are located right in the middle of the fountain where the flow changes from rootward to shootward (Figure A2- 2). During normal growth, the columella redirects the auxin symmetrically on all sides of the root. After the root is turned on its side, the columella senses the change in its orientation within the gravity field and redirects the flow of auxin asymmetrically to the new lower side of the root. The lateral root cap cells and epidermal cells faithfully transmit this signal shootward to the elongation zone, where differential growth occurs. Auxin carrier proteins are critical for both phases of this response: initial establishment of the differential auxin flow in the columella and transmission of the message through the lateral cap and epidermal cells.

The auxin influx carrier, AUX1, is required for transmission of the gravity signal

Auxin is allowed into cells by both diffusion (depending on its charge state) and auxin influx carriers such as AUX1 and its family members (Michniewicz et al., 2007). AUX1 is critical for gravitropism (Marchant et al., 1999) but seems to be localized symmetrically in the plasma membranes of columella cells (Swarup et al., 2001), where its expression is not necessary for root gravitropism (Swarup et al., 2005). This suggests that AUX1 is critical for the downstream steps of communicating the auxin gradient up the root to the elongation zones. Indeed, AUX1 expression seems to be most important in the lateral root cap and epidermal cells of the elongation zone for it to function in root gravitropism (Swarup et al., 2005). AUX1 is localized symmetrically in these cells also (Swarup et al., 2001).

Auxin efflux carriers dynamically control auxin flow during gravitropism

Conversely, the PIN proteins shuttle auxin *out* of the cell (Petrasek et al., 2006). The PIN family of proteins was named after mutations in its founding member, PIN1, were shown to result in pin-formed stems with no inflorescences on top and thus look like knitting needles (Kappert, 1959). PIN proteins are important for all aspects of the fountain flow. PIN1, PIN3, PIN4, and PIN7 proteins of the central cylinder are always localized rootward within the plasma membrane, and they send auxin downward towards the columella cells of the root tip (Geldner et al., 2001; Friml et al., 2002a). PIN3 and PIN7 are key players in the columella cells to establish the initial change in auxin flow upon gravistimulation (Friml et al., 2002c; Kleine-Vehn et al., 2010). PIN2 is critical for transmission of this differential flow through the lateral cap and up through the elongation zones.

Establishment of the auxin gradient occurs in the columella via PIN3 and PIN7

In vertically growing roots, PIN3 and PIN7 are localized symmetrically around the plasma membranes of columella cells, and auxin flows symmetrically on the lateral sides of the root (Friml et al., 2002b; Kleine-Vehn et al., 2010). During gravistimulation, these PINs relocalize in the columella cells to accumulate on the new bottom side of the cell (Friml et al., 2002b; Kleine-Vehn et al., 2010) (Figure A2- 2). Consequently, auxin flow changes such that more auxin is shuttled to the lower side of the root.

In the columella, PIN3 and PIN7 appear to act redundantly as the *pin3 pin7* double mutant has a more defective gravity response than either single mutant, although it is not completely agravitropic (Kleine-Vehn et al., 2010). How PIN3 and PIN7 are able to change localization in less than 10 minutes (Friml et al., 2002b; Kleine-Vehn et al., 2010)) is not completely understood, but the body of information is growing. *De novo* synthesis of PINs does not seem to contribute to relocalization of PIN3, as illustrated by photo-bleaching experiments (Kleine-Vehn et al., 2010). Instead, existing PINs seem to be recycled from the plasma membrane into the endomembrane system and then redelivered to the membrane in a selective manner resulting in polar relocalization (Kleine-Vehn et al., 2011).

Like PIN3 and PIN7, ARG1 and ARL2 are active in the columella cells and membrane-localized (Boonsirichai et al., 2003). Furthermore, *arg1* and *arl2* mutants do not show gravity-induced relocalization of PIN3 in the columella (Boonsirichai et al., 2003). This could suggest that ARG1 and ARL2 regulate localization of PINs, or some

element necessary for regulating PINs. Spermine, or a related molecule may also be involved in the establishment of an auxin gradient because *adk1* mutants do not show PIN3 relocalization in the columella cells of gravistimulated roots, and this can be partially rescued by exogenous spermine (Young et al., 2006).

PIN2 is necessary for transmission of the auxin signal to the site of gravibending

Following the flow of the fountain, the auxin of the root cap is shuttled shootward through the outer layers of the root (the epidermis), facilitated by PIN2. In the lateral-cap and epidermal cells of the root tip, plasma membrane localized PIN2 is found on the shootward side of the cells during normal downward growth as well as during gravistimulation. This brings auxin into the elongation zone from the root tip, along cell files, and is critical for gravibending (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998).

PIN localization is controlled by multiple cellular mechanisms

Polarized PIN localization is critical for both establishing the auxin gradient as well as transmitting it. In the columella cells, PINs change localization quickly after gravistimulation (Friml et al., 2002b; Kleine-Vehn et al., 2010). In contrast, the PINs of the lateral cap and epidermis must always maintain a set polar localization. Recent work has added a lot of understanding to how polar localization of PINs is established and maintained.

Clathrin endocytosis is important for PIN2 localization

Mutations in *PIN2* that are thought to affect its clathrin-mediated endocytosis, as well as drugs inhibiting clathrin endocytosis, show PIN2 has a less-defined polar localization (Kleine-Vehn et al., 2011). This, combined with microscopy-based computer modeling, suggests that PINs may be endocytosed in a selective way to keep them contained to one side of the membrane (Kleine-Vehn et al., 2011).

The phosphorylation state of PINs affects their localization

Several lines of evidence suggest that phosphorylation of PIN proteins is important for their polar localization and activity. The proper localization of PIN2 is disrupted by protein kinase inhibitors (Sukumar et al., 2009). Overexpression of the kinase PINOID changes the localization of PIN2, and *pinoid* mutants have an impaired gravity response and a pin-formed inflorescence phenotype (Friml et al., 2004; Sukumar et al., 2009). Mutations in genes encoding components of the protein phosphatase 2A (PP2A) complex (also called *ROOTS CURL IN NAPHTHYLPHALAMIC ACID 1* (*RCN1*)) also affect gravitropism and localization of PINs (Michniewicz et al., 2007). The PINOID kinase and PP2A phosphatase complex appear to have antagonistic activity because inhibiting phosphatases in the *pinoid* mutant as well as inhibiting kinases in the phosphatase mutant (*rcn1*) improve gravicompetence (Sukumar et al., 2009). Both these treatments are detrimental to gravitropism of wild type plants. Generally, PINOID activity seems to increase the rootward localization of PINs, and phosphatase activity seems to increase their shootward localization (Friml et al., 2004; Michniewicz et al., 2007).

Recently, Ganguly *et al.*, 2012 made five coincident point changes in *PIN3* that may prevent it from being phosphorylated *in vivo*. This is an excellent approach to find the important phosphorylation sites; however at this time it remains possible that the mutations disrupted the protein to such an extent that it could not be properly localized to the same cellular location as its kinases. *PIN3* with the five point changes was unable to rescue the gravity defects of the *pin3* mutant and showed different patterns of expression and localization than wild-type PIN3. These interesting results may suggest a role for PIN3 phosphorylation in early phases of gravity signal transduction in the statocytes.

Auxin affects PIN localization

Auxin itself also regulates PIN localization resulting in a positive feedback loop that could help to propagate a differential auxin concentration gradient (Paciorek et al., 2005). Using formation of brefeldin A-induced endosomes as an indicator of endocytosis, Paciorek *et al.*, 2005 showed that auxin application could inhibit endocytosis in general, and also endocytosis of PIN1, PIN2, and PIN4 in particular. The ability of auxin to inhibit endocytosis is dependent on the auxin receptor, AUXIN BINDING PROTEIN 1 (ABP1) (Robert et al., 2010). During the graviresponse, the high levels of auxin that accumulate on the lower side of the elongation zone of the root may contribute to PIN2 remaining localized to the plasma membrane. On the upper side of the root, where auxin levels are lower, PIN2 is more effectively endocytosed, resulting in less PIN2 at the membrane (Paciorek et al., 2005). This further heightens the

differential flow of auxin on the bottom versus the top of the root, thus enhancing the auxin gradient.

Small GTPases control rootward localization of PINs

Small GTPases (small G proteins) have broad roles in cell signaling, including controlling selective vesicle trafficking. Their activity is regulated by GDP/GTP Exchange Factor (GEF) proteins, which remove GDP from the GTPase and allow it to be re-stocked with GTP. The *GNOM* gene (named for the embryo-defective knock-out mutant phenotype) encodes a GEF that is expected to modulate the activity of ADP-ribosylation-factor family (ARF) GTPases. GNOM is important for the localization of some PINs including PIN3 in columella cells, and indeed weak allele mutants show gravitropism defects (Geldner et al., 2004 Kleine-Vehn et al., 2009). The GTPase that operates in columella cells to facilitate the proper localization of PIN3 and PIN7 and that is presumably the target of GNOM has not been identified. Brefeldin A seems to affect PIN localization by inhibiting GNOM, and it disrupts the localization of rootward-localized PINs more than shootward-localized PINs (Geldner et al., 2003; Kleine-Vehn et al., 2009).

Recently, another GEF important for gravitropism was identified. SPIKE1 was found through a forward genetic screen for extra root hair and gravitropism mutants (Lin et al., 2012). SPIKE1 encodes a GEF protein in the family of DEDICATOR OF CYTOKINESIS HOMOLOGY REGION 2 Rho. The *spike1-1* mutant shows increased sensitivity to brefeldin A, perhaps because it relies on GNOM more heavily in the

absence SPIKE1. The *spike1* phenotype is very similar to that of *pin2* mutants, and *spike1* fails to show accumulation of plasma membrane-localized PIN2 on the lower side of the root during gravistimulation. This led the authors to propose that SPIKE1 functions by affecting PIN2 localization at the plasma membrane. Indeed the *spike1 pin2* phenotype is the same as the single mutants. Lin *et al.*, (2012) identified a mutant in a GTPase gene that has a similar phenotype to *spike1* and *pin2* called *Rho-like GTPase from plants 6* (*rop6*). *In vitro* and *in vivo* pull down assays show that SPIKE1 binds to the inactive form of ROP6. This suggests that SPIKE1 is the GEF for the GTPase ROP6 and that both inhibit PIN2 endocytosis in roots.

PIN2 rootward localization in the cortex cells of the distal elongation zone is also important for root gravitropism

PIN2 is also expressed in the root cortex, a cell layer that is located between the central cylinder and the epidermis. Unlike the epidermal cells, the cortex cells in the distal elongation zone show a rootward localization of PIN2. Recently, Rahman *et al.*, 2010 showed that this PIN2 localization is important for gravitropism and auxin flow. The authors identified a concentration of brefeldin A that disrupted the shootward localization of PIN2 in the cortex but not PIN1 in the vasculature. These low levels of brefeldin A affected auxin flow as well as gravibending. Knockout mutants for *pin2* were not affected by low levels of brefeldin A, suggesting that these experiments were indeed testing a specific effect of PIN2. The rootward localization of PIN2 in the cortex compared to the epidermal cells may be important for preventing diffusion of the auxin gradient.

Other auxin efflux carriers may also affect auxin flow

Recently, Barbez et al*.,* 2012, found a novel family of putative auxin carriers that localize to the ER called PIN-LIKES (PILs). The PIL proteins were found through *in silico* searches for proteins with similar topology to PINs, although they do not show pronounced sequence identity to PINs. Over expression of PILs in root hairs causes shorter root hairs, a phenotype similar to that derived from over-expressing PINs1-4 (Ganguly et al., 2010). This suggests that expression of the PILs in a cell decreases auxin signaling in that cell. Perhaps the ER serves as a cache compartment, and pumping auxin into it keeps the auxin out of the cytoplasm. Endogenous *PIL2-PIL7* are up-regulated by exogenous auxin application, and so they may be negative feedback regulators of auxin signaling (Barbez et al., 2012). Their role in root gravitropism is yet to be examined but may yield interesting findings.

The ATP-BINDING CASSETTE (ABC) family of transporters also includes several important auxin carriers. ABC transporters use ATP hydrolysis to mediate the import and export of a wide variety of compounds, including auxin, across membranes. Consequently, ABC transporters homologous to *MULTIDRUG RESISTANT* (*MDR*) genes that encode P-GLYCOPROTEINS (PGPs) are important for creating the auxin gradient that directs gravitropic curvature. PGP1 and PGP19/MDR1/ABCB19 function directly as auxin transporters in protoplast assays and when expressed in yeast and mammalian cells (Geisler et al., 2005; Yang and Murphy, 2009). *pgp19* and *pgp19 pgp1* mutants exhibit reduced basipetal auxin transport (Noh et al., 2001; Lewis et al., 2007). Interestingly, mutations in these genes result in enhanced gravitropism and

phototropism, possibly because the reduction in polar auxin transport allows lateral auxin gradients to form more readily (Noh et al., 2003; Lin and Wang, 2005). PGP19 may form a complex with PIN1, and PIN1 is mislocalized in *pgp19* mutants (Noh et al., 2003; Blakeslee et al., 2007). A molecule called Gravacin interferes with these complexes and blocks their auxin transport activity, resulting in reduced root and hypocotyl gravitropism (Rojas-Pierce et al., 2007). Based on these experiments, PGPs and PINs are likely to function in an independent but coordinated manner to regulate auxin transport.

The message is delivered to the elongation zone

Establishing an auxin gradient across the root is critical for gravitropism, and the elongation zone seems to be the key destination of the auxin message. This has been illustrated by blocking the auxin response in these cells with targeted expression of a semidominant negative-regulator of auxin signaling (*auxin resistant 3*-1) which completely blocks gravibending (Swarup et al., 2005). There are two known types of auxin receptors: AUXIN BINDING PROTEIN 1 (ABP1), and TRANSPORT-INHIBITOR-RESISTANT1 (TIR1) (Hertel et al., 1972; Lobler and Klambt, 1985; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). ABP1 is an extracellular protein that binds auxin and mediates auxin's effect on cells. The mechanism of ABP1 function is not clear, but it may have a role in inhibiting endocytosis when bound to auxin (Robert et al., 2010). The other auxin receptor, TIR1, is an E3 ubiquitin ligase that is activated by auxin binding to ubiquitinate AUX/IAA (AUXIN-RESPONSIVE PROTEIN/INDOLEACETIC ACID-INDUCED PROTEIN) transcriptional repressors, causing their degradation

(Dharmasiri et al., 2005; Kepinski and Leyser, 2005). When present, AUX/IAA proteins interact with defined AUXIN RESPONSE FACTORS (ARFs), interfering with their ability to either up- or down-regulate the expression of auxin-responsive genes. In the presence of auxin, these repressors are ubiquitylated and degraded, thereby allowing the corresponding ARFs to modulate the transcription of their targeted auxin-responsive genes. Auxin also has immediate effects on cells that do not require transcription or translation of new proteins, and the mechanisms are not understood yet (Scherer, 2011).

Interestingly, in gravistimulated hypocotyls and stems, auxin also accumulates on the lower side of the organ but it has the opposite effect: growth is inhibited on the side with less auxin resulting in upward curvature (Rakusova et al., 2011). Presumably this is due to a difference in auxin response in above ground tissues verses root cells.

Auxin may affect extracellular pH through Ca^{2+} signaling

Changes in auxin concentration throughout the root during gravistimulation may cause extracellular pH fluxes, which in turn affect the elongation rate. Using a pH-sensitive fluorescent dye in the media around roots, Monshausen *et al.*, 2011 found that the pH increases on the upper side of the root during gravistimulation. The pH difference goes from the tip region up through the distal elongation zone. Exogenous application of auxin also causes pH increases, and as the auxin is transported through the root, there is a corresponding wave of pH increases along the outside of the root. Plants carrying mutations in the auxin influx carrier gene, *AUX1*, show a greatly reduced

response. The extracellular pH increase is accompanied by a slight increase in epidermal cell cytosolic Ca^{2+}, and blocking Ca^{2+} channels with a lanthanide element inhibits the pH changes when auxin is added (Monshausen et al., 2011). This suggests that Ca^{2+} is acting as a secondary messenger in converting the auxin signal into a pH change. The root pH change may function as a feedback mechanism on cellular auxin uptake, which is a pH dependent process (Monshausen et al., 2011).

Extracellular calcium may facilitate gravity-signal transmission

Transmission of the auxin signal may also involve extracellular Ca^{2+} changes. Changes in extracellular Ca^{2+} levels during gravistimulation have been found in corn roots using Ca^{2+}-specific microelectrodes (Bjorkman and Cleland, 1991). In order to have the statocytes closer to the electrodes, Bjorkman et al. removed the cap from corn roots and allowed them to only partially regrow. They found a 1.7 fold decrease in Ca^{2+} levels on the upper side of the gravistimulated root and a similar amount of increase on the bottom side. These changes have not been directly connected to auxin signaling, but they are likely to be downstream of auxin transmission. Unfortunately, whether these changes have anything to do with the potential changes in cytosolic Ca^{2+} levels discussed above remains unknown. Yet, these gravity-induced changes in extracellular Ca^{2+} were proposed to either serve in cell-to-cell communication during gravity signal transmission, or to help propagate auxin signals.

Auxin may cause the formation of reactive oxygen species, which may in turn cause root curvature

Auxin may affect root bending through reactive oxygen species (ROS). ROS increase in the root tip of gravistimulated corn, and exogenous application of hydrogen peroxide on one side of the root is sufficient to make the root turn towards it (Joo et al., 2001). Also, exogenous auxin application is sufficient to cause ROS generation in corn roots. Even when auxin transport was inhibited by application of the drug NPA to the roots, H_2O_2 caused root curvature. Thus, it is possible that auxin causes root curvature by producing ROS. Consistent with this, the application of antioxidants to the outside of the root inhibited gravibending (Joo et al., 2001). Additionally, mitogen-Activated Protein (MAP) kinase inhibitor drugs are able to inhibit the effects of H_2O_2, suggesting that maybe ROS affects root curvature through the MAP kinase pathway (Liu et al., 2009).

Peptide regulators affect root growth and auxin transport

Recently, members of the ROOT GROWTH FACTOR (RGF), also known as GOLVEN, family of small peptides have been found to be important in root development and gravitropism (Matsuzaki et al., 2010; Whitford et al., 2012). These secreted peptides are modified by tyrosine sulfation and proline hydroxylation. Application of *in vitro* synthesized peptides within the media is sufficient to trigger abnormal root meristem morphology and slow graviresponse in wild type plants. Plants carrying mutations in the transcription factor *PLETHORA* (Matsuzaki et al., 2010) and in the auxin efflux carrier *PIN2* (Whitford et al., 2012) are immune to some of these treatments suggesting that some RGF peptides work through transcription factors and auxin

pathways. How these peptides affect root growth and their mode of action is yet to be understood.

Dissolution of the auxin gradient during root bending and resetting

Recently, Band *et al.* 2012 used the new, more temporally-sensitive auxin reporter DII-VENUS (described below) to investigate the redistribution of auxin during the graviresponse to a 90° reorientation of Arabidopsis seedlings. They found that the auxin gradient across the root dissipated after the root had only turned about 40°. This is also the angle at which the amyloplasts make the transition from contact with the side of the columella cells back to the tip-side of the columella cells. This observation suggests that the second half of the root reorientation may be accomplished without the presence of an auxin gradient. It also suggests that amyloplast-mediated resetting after gravity bending has begun might be more like a digital tipping switch than an organic rheostat (Band et al., 2012). This may imply that the return to a non-gravistimulated state occurs like an on/off switch at a root-tip angle of 45°. In contrast, gravity activation (before bending) can take place at reorientation angles as low as 15° (Mullen et al., 2000). It will be fascinating to see if future work can build on this observation.

Lateral Roots Sense Gravity

In most plant species, lateral roots that emerge from the side of primary roots also sense gravity. In some species, lateral roots grow at a set-point angle relative to gravity (Digby and Firn, 2002). In Arabidopsis, lateral roots generally emerge perpendicularly to the downward growing root and then turn downwards over time (Kiss

et al., 2002; Guyomarc'h et al., 2012). Lateral roots develop columella cells and amyloplasts similarly to primary roots (Kiss et al., 2002).

Recently, Guyomarc'h et al. 2012 performed a very careful analysis of different markers during lateral root emergence and growth. They found expression differences for some key auxin carriers between primary and lateral root tips. Interestingly they also found that downward curvature began before starch was visible by Lugols staining (when the lateral roots had emerged approximately 210μm). This could mean that the initial stages of lateral root gravitropism do not require starch or merely that the level of starch is too low to detect with Lugols staining. And indeed, previous studies using electron microscopy (a more sensitive technique) have shown that some starch grains are visible in amyloplasts much earlier, when the lateral root has emerged only 100μm (Kiss et al., 2002).

Also addressing the relevance of starch in lateral root gravitropism, Bai *et al.*, 2011 tested the graviresponses of lateral roots in the *pgm* mutant. Interestingly, they found the same response to gravity as wild-type lateral roots (Bai and Wolverton, 2011). This is different from the findings of Kiss et al. 2002, who found a slower response in the lateral roots of *pgm* mutants. Both Bai et al., 2001 and Kiss et al., 2002 found that there was little or no starch (by iodine and light microscopy respectively) in the amyloplasts of the lateral roots of the *pgm* mutant, like the primary roots of this mutant. The cause of the conflicting graviresponse results is almost certainly the difference in experimental conditions, including gravity stimulation angle, light intensity, and light direction. The

lateral roots of *pgm* seedlings have been shown to have a strong phototropic response, so light is likely to have a large effect on their growth (Kiss et al., 2002). It would be very interesting to measure the curvature rates of the lateral roots of a *pgm* mutant in total darkness, although this would no doubt entail some technical hurdles.

The cellular organization and eventual presence of starch in the tips of Arabidopsis lateral roots is very similar to primary roots, and yet they initially grow sideways before bending downwards. A recent study has offered some insight as to why they grow more horizontally than primary roots. Rosquete et al 2013 found that some of the PIN proteins that are key to establishing the initial auxin gradient across the columella of primary roots (PIN 7 and PIN4) are not expressed in lateral roots. Another PIN protein, PIN3, is still expressed. Perhaps fewer PINs in the columella result in a weaker gravisignal so that the lateral roots grow at a more sideways angle than primary roots. As the lateral roots mature, they begin to express PIN7 and PIN4, and they begin to grow straight down (Rosquete et al., 2013).

Lateral root implications for primary roots

Lateral roots have not been as well studied as primary roots. If more work bears out the hypothesis that Arabidopsis lateral roots have a stronger starch-independent gravisensing pathway than primary roots, then lateral roots could serve as a good system for exploring the starch-independent mechanism. As discussed above, there is evidence for a starch-independent mechanism operating in primary roots, but it has proved difficult to identify.

New Tools Will Help Further Dissect Gravisensing

DII-VENUS is an *in vivo* auxin sensor

Visualizing hormone flux during a dynamic process is a challenge that has restricted experimental design in plant biology. DR5 promoter-based auxin reporters have been invaluable tools for overcoming this hurdle; however, they represent a very indirect measure of auxin concentration, functioning at the very end of complex auxin signaling pathways. In order to address some of the problems associated with the DR5 reporter system, Brunoud et al., 2012 recently designed a new auxin reporter construct. Instead of relying on a transcriptional response, it takes advantage of the more upstream step of AUX/IAA degradation. The domain of the AUX/IAA protein that confers its TIR1- and auxin-dependent degradation was fused to a fast-maturing yellow fluorescent protein called “VENUS” and a nuclear localization signal under the constitutive cauliflower mosaic virus 35S promoter. When expressed in Arabidopsis, this reporter is responsible for a yellow fluorescence nuclear signal in cells with low auxin signaling. Upon auxin treatment, this nuclear fluorescence decreases in an auxin-dependent fashion.

DII-VENUS responds as expected to changes in auxin concentration and has a large range of linear response in fluorescence intensity to changes in auxin concentration (Brunoud et al., 2012). For example, Brunoud et al. 2012 show that the VENUS signal disappears from the bottom side of gravistimulated roots but remains bright on the upper side. DII-VENUS appears to be an improved new tool that will allow for greater temporal resolution in future experiments. This tool might help resolve some

cause-and-effect questions that remain about the various observed phenomena in gravity signaling such as the relationships between statolith falling, columella cell alkalinization, PIN relocalization, auxin gradient formation, and tip bending (Figure A2-2).

Auxin precursor-, catabolite-, and conjugate-detection by spectrometry

Novak et al., 2012 has developed protocols for total quantification of most auxin-related compounds in Arabidopsis tissue samples as small as 20mg. The technique relies on solid phase extraction, evaporation in a vacuum, and analysis by liquid chromatography tandem mass spectrometry. Novak et al., 2012 used this technique to separately assay roots and hypocotyls, but it could be used on smaller segments of tissue. This novel approach may allow gravitropism researchers to measure the auxin-conjugates that result from the auxin gradient in the elongation zone after gravistimulation. It may also allow more sensitive detection of auxin synthesis in the root tip (Ljung et al., 2005).

GFP-based *in vivo* calcium sensors introduce a new level of sensitivity

Ca^{2+} is a ubiquitous cellular regulator that can modulate dynamic events by being released into the cytoplasm in brief spikes representing concentration increases. Detecting and studying transient phenomena like cytoplasmic Ca^{2+} spikes demands *in-vivo* sensing tools like yellow cameleons (Miyawaki et al., 1997). This class of Ca^{2+} sensor fuses a calmodulin to the M13 peptide that calmodulin binds to only when it is

activated by Ca^{2+} binding. On either side of the calmodulin and M13 peptide are two different fluorescent proteins capable of fluorescence resonance energy transfer (FRET) such as cyan florescent protein and yellow florescent protein. Genetically encoded sensors do not rely on even-absorption or injection of a dye, and they can be targeted to a specific organelle by addition of a signal peptide or retention sequence. An increasing number of variable-sensitivity and organelle-targeted cameleons have been developed, as recently reviewed (Swanson et al., 2011). Of particular interest to gravitropism may be the "YC-Nano" cameleon sensor developed by Horikawa et al., 2010. YC-Nano has a very high affinity for Ca^{2+}, and thus it is very sensitive to low-level changes in Ca^{2+} (Horikawa et al 2010). Experiments using this particular Ca^{2+} sensor in plants have not yet been published. New Ca^{2+} sensors provide the best tool yet available to detect localized Ca^{2+} spikes in the columella during gravitropism, which are predicted to exist if gravitropism signaling involves a mechanosensitive Ca^{2+} ion channels to infer the falling of amyloplasts.

Global analysis tools allow detection of new phenomena

In recent years, global analysis tools have added new ways to dissect the gravitropism pathway. Genetics and broad transcriptomic and proteomic screens together will pave the next frontier of cellular biology. Microarray experiments on gravistimulated and mechanostimulated seedlings have yielded rich databases of genes whose expression patterns change during gravistimulation (Kimbrough et al., 2004; Moseyko et al., 2002).

The Benfey lab has done a number of global analyses not directly related to gravitropism but still very helpful to the field. They produced protoplasts from roots carrying cell-type specific GFP markers and then performed flow cytometry cell sorting to isolate only the GFP-expressing cells. Then microarray experiments were done on these isolated cell types (Nawy et al., 2005; Brady et al., 2007). Because gravity sensing takes place in key cells of the root, the Benfey lab data is helpful for finding candidate genes expressed in different cell types involved in key steps of the graviresponse, including those occurring in the columella cells. Recently, they expanded these experiments to also include proteomic characterization of these cell types (Petricka et al., 2012). As more proteomic experiments are conducted, it is clear that transcript abundance and protein abundance only loosely correlate. Thus emerging proteomic experiments are of utmost importance for finding the missing links in gravity sensing and signal transduction.

New emerging techniques will allow for the detection of changes that have been missed in past experiments. For example, total mRNA sequencing techniques will allow researchers to detect splicing changes during gravistimulation as well as transcripts not present on microarray chips. Small RNA sequencing could also reveal a role for microRNAs in gravitropism. Additionally, improvements in mass spectrometry have made it easier to identify individual proteins and post-translational modifications in small samples. These techniques, combined with traditional genetics, will help detangle the mechanism of gravity sensing.

Computer vision and robots collect phenotypic data more comprehensively than humans alone

Another emerging technology that will aid in future discoveries is robotic-assisted and computer-processed phenotyping (Spalding, 2009). Miller et al., 2010 showed that time lapse computer analysis was much more sensitive at finding abnormal root growth phenotypes than traditional hand measuring of stagnate photos (Miller et al., 2010). Robotic time-lapse photography opens up possibilities for large-scale high-throughput phenotypic screens. When robotic/computer vision tools are combined with genetic tools like recombinant inbred line populations and/or large collections of genotyped natural variants, researchers can use association mapping to identify new genes involved in root growth that may be difficult to find using traditional mutagenesis due to functional redundancy and/or early developmental lethality of the corresponding knockout mutants. Robotic image analysis can also allow a small number of researchers to process data from a large number of candidate mutants more quickly. For a review on mathematical analysis of plant growth, see (Moulia and Fournier, 2009).

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