DIVERSE LOCALIZATION OF 14-3-3 PROTEINS IN Arabidopsis thaliana

By

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This thesis is dedicated to all the friends and family who have helped me over the years.

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of the University of Florida in Partial Fulfillment of the
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Chairman: Dr. Robert J. Ferl
Major Department: Plant Molecular and Cellular Biology

The 14-3-3 family of proteins was discovered in the late 1960s during an extensive study of cytosolic bovine brain proteins. Once thought to be limited to mammalian brain tissue, studies over the last 15 years revealed 14-3-3s to be ubiquitous. The 14-3-3s are a family of acidic, soluble proteins that are commonly found as multi-isoform families within an organism. Specifically in plants, 14-3-3s are associated with many essential processes including transcription complexes, carbon and nitrogen metabolism, and protein-protein interactions. The Arabidopsis thaliana family of 14-3-3 proteins consists of ten distinct and well-characterized isoforms as well as three newly recognized isoforms. The question of functional specificity among the isoforms has led us to our current project. Using a set of isoform-specific antibodies and C-terminal green fluorescent protein fusions, we show that individual 14-3-3s differ in subcellular localization in Arabidopsis thaliana root tissue.

CHAPTER 1
INTRODUCTION

This is truly an exciting era for science and technology. Buzzwords such as genomics, cloning, and proteomics are regularly used by non-scientists and have nearly become household terms. The last year brought huge advancements in sequencing projects. We began the millennium with a grand feat in sequencing the entire human genome (Lander et al., 2001). For the first time, humans will be able to study in great detail genes that make us human. Many lessons were learned during the human genome project: some scientific and some political, but none more important than 'if we work hard enough we can accomplish anything.'

The human genome project also sparked many other sequencing projects. The Arabidopsis thaliana genome project is of particular interest to many scientists because it is used as a model organism for plant studies. Its relatively small genome size, ease of propagation, and short life cycle make it a prime candidate for study by a wide range of scientists with very different interests. The completion of the Arabidopsis thaliana genome project late last year finally gives scientists the opportunity to speak with confidence about the genome as a whole (Arabidopsis Genome Initiative, 2000).

Arabidopsis thaliana also provides us with the opportunity to study the 14-3-3 proteins. The 14-3-3s are a multi-isoform, acidic, soluble family of proteins that were once thought to be found only in mammalian brain tissue. However, more extensive studies have proven 14-3-3s to be ubiquitous among eukaryotes. The 14-3-3s perform

multiple functions within the cell and are grouped under an overall theme of protein-protein interaction. Plant 14-3-3s are known to associate with kinases, to regulate key enzymes of carbon and nitrogen metabolism, and even to associate with transcription factors.

The goal of this thesis is to characterize the subcellular localization of nine specific isoforms within the root tip of Arabidopsis thaliana using laser scanning confocal microscopy. Isoform-specific antibodies and in-frame green fluorescent protein fusions are used.

In characterizing the subcellular localization of these isoforms, we are hoping to answer several questions. However, the key question is this: do the isoforms have functional specificity or do they play redundant roles within the cell? If the isoforms do have specificity, then we expect to see different localization patterns within the cell among the nine isoforms. If there is no isoform specificity, then we expect to see a general localization pattern emerge for all the isoforms.

CHAPTER 2
REVIEW OF LITERATURE

History

The 14-3-3 proteins are a family of acidic, soluble proteins that were discovered in 1967 by Blake Moore and Vernon Perez during an extensive study of cytosolic bovine brain proteins (Moore and Perez, 1967). The 14-3-3 proteins were named based on fractionation on DEAE cellulose and electrophoretic mobility in starch gel electrophoresis (Moore and Perez, 1967). Further studies over the next twenty years revealed that the 14-3-3 family of proteins are conserved among mammals. Still it was thought that the 14-3-3s were primarily limited in their location and function to brain tissue. However, upon extended examination starting in the late-1980s, 14-3-3s were found to be present in a plethora of organisms and tissues (Ichimura et al., 1991). To date, 14-3-3 members have been discovered in many organisms including cow (Moore and Perez, 1967), sheep (Token et al., 1990), chicken, human, rat, mouse, turtle, frog, goldfish (Ichimura et al., 1991), spinach (Hirsch et al., 1992), Arabidopsis thaliana (Lu et al., 1992), maize (de Vetten et al., 1992), yeast (van Heusden et al., 1992), barley (Brandt et al., 1992), *Drosophila* (Swanson and Ganguly, 1992), rice (Kidou et al., 1993), tomato (Laughner et al., 1994), tobacco (Chen et al., 1994), oat (Korthout and de Boer, 1994). 14-3-3s have been found in a variety of tissues as well, including testis, intestine, liver, kidney, and skeletal muscles of mammals (Ichimura et al., 1991). It is important to note

that 14-3-3s are present in different organisms and tissues in varying concentrations and locations.

Nomenclature

The nomenclature regarding 14-3-3s has been difficult and often confusing for several reasons. First, many scientists characterized and named proteins based on function or other characteristics; only later discovering the protein to be a 14-3-3 family member. Therefore, the protein or group of proteins lacks the 14-3-3 moniker and is thus misnamed. Second, the speed at which proteins are being associated as 14-3-3 family members has and continues to be rapid. The rate of discovery combined with the lack of a formal naming scheme has led to much confusion in the literature regarding 14-3-3 proteins. In the late 1980s, 14-3-3 members found in the bovine brain were further characterized and designated as Greek letters starting at the beginning of the alphabet (Ichimura et al., 1988). Seven 14-3-3 members were identified in that study and were named based upon elution during reverse-phase HPLC. The mammalian isoforms are designated alpha, beta, gamma, delta, epsilon, zeta, and eta. The list of mammalian isoforms grew again to include tau and sigma in 1991 and 1993, respectively (Nielsen, 1991; Leffers et al., 1993). Additionally, further studies revealed the alpha and delta isoforms to be the phosphorylated forms of beta and zeta, respectively (Aitken et al. 1995). In keeping with the mammalian trend, the Arabidopsis thaliana 14-3-3s are designated starting with the end of the Greek alphabet and working forward as they are discovered. However, there is not a strict adherence to the naming system in arabidopsis. Therefore, overlap in designations does exist between mammalian and arabidopsis 14-3-3 isoforms. The Arabidopsis thaliana 14-3-3 isoforms are omega, psi, chi, phi, upsilon,

rho, pi, omicron, nu, mu, lambda, kappa, and epsilon (Lu et al., 1994a; Wu et al., 1997; Rosenquist et al., 2000; DeLille et al., 2001). Table 2-1 lists the mammalian and Arabidopsis thaliana 14-3-3 Greek names and symbols for reference.

Function

The 14-3-3 family of proteins is found in every eukaryotic organism where this specific family is sought (Robinson et al., 1994). The conservation of 14-3-3 proteins among a wide variety of species implies an essential and necessary role, yet 14-3-3 members are associated with a variety of functions. Two general themes have emerged regarding the roles of 14-3-3s; protein kinase activities and protein-protein interactions. The earliest function of 14-3-3s was recognized in 1987 by Ichimura et al. during a study of acidic brain proteins (Ichimura et al., 1987). The 14-3-3s Ichimura studied were remarkably similar in amino acid sequence to a protein known to activate tyrosine and tryptophan hydroxylases. Further studies revealed the activator protein and the 14-3-3s to be indistinguishable in amino acid sequence. Thus, the first role of 14-3-3s was established as an activator associated with the kinase-dependent regulation of enzyme activity. The 14-3-3s are also associated with other protein kinase activities including the regulation of protein kinase C, stimulation of calcium-dependent exocytosis in adrenal chromaffin cells, activation of c-Raf-1, and the regulation of Bcr-Abl (Aitken, 1995; Morgan and Burgoyne, 1992; Fantl et al., 1994; Reuther et al., 1994).

The protein-protein interaction theme is more general and therefore less well defined. The 14-3-3 family of proteins has been shown to be associated with large complexes. These complexes involve protein kinases, DNA binding complexes and chaperones (Ferl, 1996). Multiple recognition motifs have been proposed and the

common theme among them is the presence of a phosphoserine. Two of the motifs are RX(Y/F)XpSXP, RXXSXpSXP, where X is any amino acid and pS denotes a phosphoserine. However, other binding motifs have also been proposed that do not contain a phosphoserine, such as WLDLE, RXSX(S/T)XP, XXXSXXSXXXSXXSX (Pan et al., 1999).

Specifically in plants, 14-3-3s are associated with several specialized functions including a role in fusicoccin binding sites and the regulation of nitrate reductase and sucrose phosphate synthase (Korthout and de Boer, 1994; Finnie et al., 1999). Fusicoccin (FC) is a fungal toxin excreted by Fusicoccum amygdali that targets the plant plasma membrane H^+-ATPase proton pump (Chung et al., 1999). Studies have shown that 14-3-3s complex with the H^+-ATPase proton pump and thus constitute the FC receptor (Sehnke and Ferl, 2000). FC binding to the receptor causes the proton pump to stay "on," thus causing turgor pressure to decrease in guard cells and the plant leaves to wilt.

Key enzymes of nitrogen and carbon metabolism are also regulated by 14-3-3s, and currently form the paradigm for 14-3-3 regulation of enzyme activity. Nitrate reductase (NR) and sucrose phosphate synthase (SPS) are up-regulated during photosynthesis. However, in the dark, NR is inactivated via a two-step mechanism (Figure 2-1) (Chung et al., 1999). In the first step, a specific serine is phosphorylated in the target enzyme. In the second step, Mg^{2+} binds an inhibitor protein, causing a conformational change, thus allowing the inhibitor protein to bind to the target enzyme. One or more 14-3-3s make up this inhibitor protein (Bachmann et al., 1996). Sucrose phosphate synthase is also regulated by light and phosphorylation. However, 14-3-3

binding to SPS has resulted in both activation (Moorhead et al., 1999) and inactivation (Toroser et al., 1998).

The 14-3-3s in plants are also associated with a class of transcription factors called G-box factors (GBFs) (Lu et al., 1992). G-box factors are a class of bZIP transcriptional activators involved in stress and abscisic acid activation of genes. Additionally, Arabidopsis thaliana 14-3-3s have been shown to interact with other transcription factors, TBP and transcription factor IIB (Pan et al., 1999).

The overall amino acid conservation of 14-3-3s is relatively well conserved both within and among species. The N- and C-terminal regions are the most variable in both mammals and plants. The Arabidopsis thaliana isoforms share a core region in which 51% of the amino acids are conserved. The amino acids in the N-terminal region are only conserved to a degree of 14%, while there is no amino acid conservation in the C-terminal region among the isoforms (Chung et al., 1999).

Arabidopsis thaliana 14-3-3 Proteins

One of the largest and best-characterized 14-3-3 families in plants is found in Arabidopsis thaliana. The well-characterized Arabidopsis thaliana 14-3-3 isoforms are omega, psi, chi, phi, upsilon, nu, mu, lambda, kappa, and epsilon (Lu et al., 1992; Lu et al., 1994b; Wu et al., 1997). The 14-3-3 family in Arabidopsis thaliana was initially discovered while studying the regulation of the alcohol dehydrogenase gene and a cis regulatory element called the G-box (Lu et al., 1992). Using monoclonal antibodies against the partially purified G-box binding factor, Lu et al. isolated cDNA clones that encoded 14-3-3 isoforms omega, psi, chi, phi, and upsilon (Lu et al., 1992; Lu et al., 1994b). Using the chi isoform as bait in a yeast two-hybrid screen, Wu et al. identified

the kappa, lambda, and epsilon isoforms (Wu et al., 1997). Finally, the isoforms mu and nu were identified by searching the Arabidopsis thaliana EST database using the eight known isoforms as a query (Wu et al., 1997). Recently, 3 new isoforms, omicron, rho, and pi, were identified by database analysis (Rosenquist et al., 2000; DeLille et al., 2001). A full-length cDNA has been identified for omicron and a truncated cDNA has been identified for the rho isoform. The third isoform, pi, remains putative at this point as no cDNA or EST has been found.

Determining the subcellular localization of the individual 14-3-3s is essential to understanding their true roles in the cell. Bihn et al. reported the general subcellular location of the Arabidopsis thaliana 14-3-3s (Bihn et al., 1997). The authors used a suite of three monoclonal antibody cell lines and localized 14-3-3s to the nucleus and cytoplasm. However, the localization of specific isoforms was not revealed.

The14-3-3s are generally found as multi-isoform families in eukaryotic organisms. This begs the question: Do all the isoforms play the same role or does functional specificity exist among the isoforms? Studies have identified subsets of isoforms in various organelles within the cell. For example, in addition to being present in the cytoplasm, epsilon, mu, nu and upsilon are also present in chloroplasts (Sehnke et al., 2000). Functional specificity can also be seen with nitrate reductase. Omega, chi, and upsilon demonstrate decreasing affinity, while the isoforms psi and phi have no affinity for the enzyme.

The goal of this thesis is to determine the localization patterns of nine well-characterized arabidopsis 14-3-3 isoforms using a combination of isoform specific antibodies and in-frame green fluorescent (GFP) fusions. It is the hope that differences in

localization patterns among the isoforms do indeed occur, thus providing evidence for functional specificity.

Table 2-1. Mammalian and Arabidopsis thaliana 14-3-3 Proteins. Currently nine are found in mammalian tissue and thirteen in arabidopsis. Mammalian isoforms are designated starting at the beginning of the Greek alphabet. Arabidopsis 14-3-3s are designated from the end of the alphabet, working forward. Some overlap in naming does exist between mammalian and arabidopsis isoforms. The Greek names and symbols are listed for reference.

Mammalian 14-3-3 Proteins		Arabidopsis 14-3-3 Proteins	
Greek Letter	Greek Name	Greek Letter	Greek Name
α	alpha	ε	epsilon
β	beta	κ	kappa
γ	gamma	λ	lambda
δ	delta	μ	mu
ε	epsilon	ν	nu
ζ	zeta	ο	omicron
η	eta	π	pi
τ	tau	ρ	rho
σ	sigma	υ	upsilon
		ϕ	phi
		χ	chi
		ψ	psi
		ω	omega

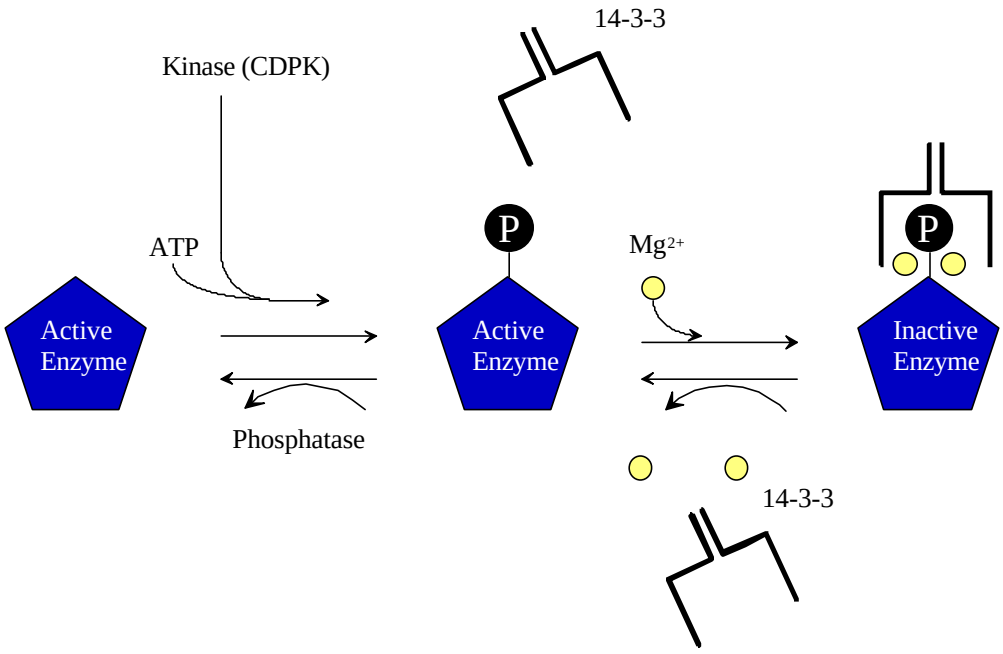


Figure 2-1. Key enzymes in carbon and nitrogen metabolism in plants in a reversible two-step mechanism. The first step is the phosphorylation of a specific serine in the target enzyme by calmodulin-domain protein kinase (CDPK). In the second step, Mg^{2+} binds 14-3-3 proteins causing a conformational change that allows the 14-3-3 to bind the target enzyme and thus complete the inactivation. ATP = adenosine triphosphate.

CHAPTER 3
Arabidopsis thaliana 14-3-3 FAMILY OF SIGNALING REGULATORS

The 14-3-3 family of proteins has received much attention in the literature during the last 10 years. The current interest is not surprising given the number of diverse organisms in which 14-3-3s have been identified and the important role that they play in signal transduction. Moore and Perez initially catalogued the 14-3-3 proteins in 1967 during an extensive study in which bovine brain proteins were given numerical designations based on column fractionation and electrophoretic mobility (Moore and Perez, 1967). The 14-3-3 family was thought to be limited to nervous tissue and largely conserved among mammals during the late 1960s and 1970s. However, studies over the last 15 years have proven 14-3-3s to be ubiquitous, found in virtually every eukaryotic organism and tissue (Ichimura et al., 1987; Robinson et al., 1994). In any given organism, the 14-3-3 family usually consists of multiple genes and protein isoforms. Multiple isoforms and multiple functions, coupled with the large number of different organisms that have been studied, have led to potential confusion regarding 14-3-3 nomenclature and function. (The 14-3-3s are currently designated by Greek letters, with the mammalian isoform names generally chosen from the beginning of the alphabet and the plant isoforms chosen from the end of the alphabet). Recent completion of the Arabidopsis thaliana genome project provides a unique opportunity to examine a complete 14-3-3 family within a single higher eukaryotic organism and to present a framework to codify the understanding of plant 14-3-3 functional diversity and constraint.

The 14-3-3 proteins play key functional roles in many critical physiological pathways that are regulated by phosphorylation. Their role is to complete the signal transduction process by binding to the phosphorylated target, which completes a change in structure that regulates activity. This core functional characteristic is deeply engrained in the highly conserved structural core of the 14-3-3 dimer, which provides grooves for binding two specifically phosphorylated peptides. The primary diversity among 14-3-3 isoforms lies in the N- and C-termini, with the C-terminal region potentially able to form a flexible hinge guarding access to the central core region (Sehnke and Ferl, 2000).

Plants require a battery of regulators and corresponding responses to deal with complex environmental and developmental changes, a situation that seems consistent with the presence of a large and diverse 14-3-3 family. Localization of 14-3-3 family members inside organelles such as the chloroplast (Sehnke et al., 2000), nucleus (Bihn et al., 1997) and mitochondria (Sehnke and Ferl, 2000), in addition to the cytoplasm (Bihn et al., 1997), further demonstrates both their global regulatory potential and their apparent need for diversity in expression and function. The list of the processes controlled by 14-3-3s includes the fundamental nitrogen and carbon assimilation pathways, which are executed by the light and substrate-regulated metabolic enzymes nitrate reductase and sucrose phosphate synthase (Sehnke and Ferl, 2000). Other enzymes under the control of 14-3-3s include starch synthase (Sehnke et al., 2001), glutamate synthase, F1 ATP synthase, ascorbate peroxidase, and affeate O-methyl transferase (Finnie et al., 1999). Additionally, the control of the plant's turgor pressure via regulation of at least one form of a plasma membrane H^+ ATPase is accomplished by 14-3-3 proteins (Korthout and deBoer, 1994; Marra et al., 1994; Oecking et al., 1994). Less understood, yet equally

bona fide 14-3-3 binding partners include transcriptional machinery such as the G-Box complex, core transcription factors; TBP, TFIIB and EMBP (Chung et al., 1999). The specific 14-3-3 isoforms required by each of these pathways has not been fully characterized, however a conserved mechanism of plant 14-3-3s binding is the requirement for divalent cations to "charge" the 14-3-3s via a structural reorientation of the C-termini (Lu et al., 1994b). Interestingly, only a subset of the Arabidopsis thaliana 14-3-3 isoforms possess this EF hand-like divalent cation-binding motif in the C-terminal region.

The Arabidopsis thaliana genome project for the first time provides reasonable certainty about the number and diversity of 14-3-3 family members within a plant species. The Arabidopsis thaliana 14-3-3 family consists of 13 members. Ten of the members, omega, phi, chi, psi, upsilon, nu, mu, lambda, kappa, and epsilon are well characterized and present as ESTs and cDNAs (Lu et al., 1992; Lu et al., 1994b; Wu et al., 1997). Three of the members, omicron, rho, and pi are recently recognized 14-3-3 family members. The omicron isoform was identified by Rosenquist et al. (Rosenquist et al., 2000) and subsequently a full-length cDNA was found by the same authors (AC # AF323920). A C-terminally truncated cDNA relative to the genomic sequence has been found for the rho isoform (AC# AF335544). The pi isoform remains a putative 14-3-3 member at this point as no cDNA or EST has been found. Two other putative members exist, however one isoform is badly truncated and would likely not be functional (Accession # AC007264), and the other isoform is annotated in Genbank as containing a one base pair intron (Accession #AC068562). We believe the Genbank entry for the later isoform is annotated incorrectly and the intron is actually a single base pair insertion that

causes a frame shift and the loss of the last 45 amino acids. Thus we are designating both of these isoforms as "14-3-3-like proteins." Figure 3-1 represents the intron-exon structures of all the Arabidopsis thaliana 14-3-3s. All of the reference information regarding Arabidopsis thaliana 14-3-3s is found in Table 3-1.

An alignment of the thirteen isoforms reveals some interesting information (Figure 3-2). The isoforms range in length from 241-286 amino acids. The isoforms all share a conserved core region, with the N-termini and C-termini being the most divergent. In fact, the amino acids in the N-termini are conserved to a degree of only 14% and there is very little amino acid conservation at the C-termini (Chung et al., 1999).

Phylogenetic analyses based on amino acid sequence data and gene structure provides a robust tree upon which to hang descriptions of family member function and localization (Figure 3-3). The family members break into two major evolutionary branches, the Epsilon group and the Non-Epsilon group. This clear delineation at the trunk of the tree is ubiquitous among plants and animals possessing multiple isoforms, indicating that the initial formation of two isoforms is a fundamental and ancient divergence. The Epsilon group is itself split into the isoforms epsilon, mu, omicron, rho, and pi. The Non-Epsilon group is made up of the isoforms kappa, lambda, phi, chi, omega, psi, nu, and upsilon. The Epsilon group breaks into two sub-branches, with epsilon and pi on one sub-branch; and omicron, rho and mu in the second sub-branch. The Non-Epsilon group comprises three very distinct sub-branches. Kappa and lambda make up one sub-branch: phi, chi, and omega make up a second sub-branch; and psi, nu, and upsilon make up the third sub-branch. The Non-Epsilon group members contain the previously mentioned EF hand-like divalent cation-binding motif (Lu et al., 1994b).

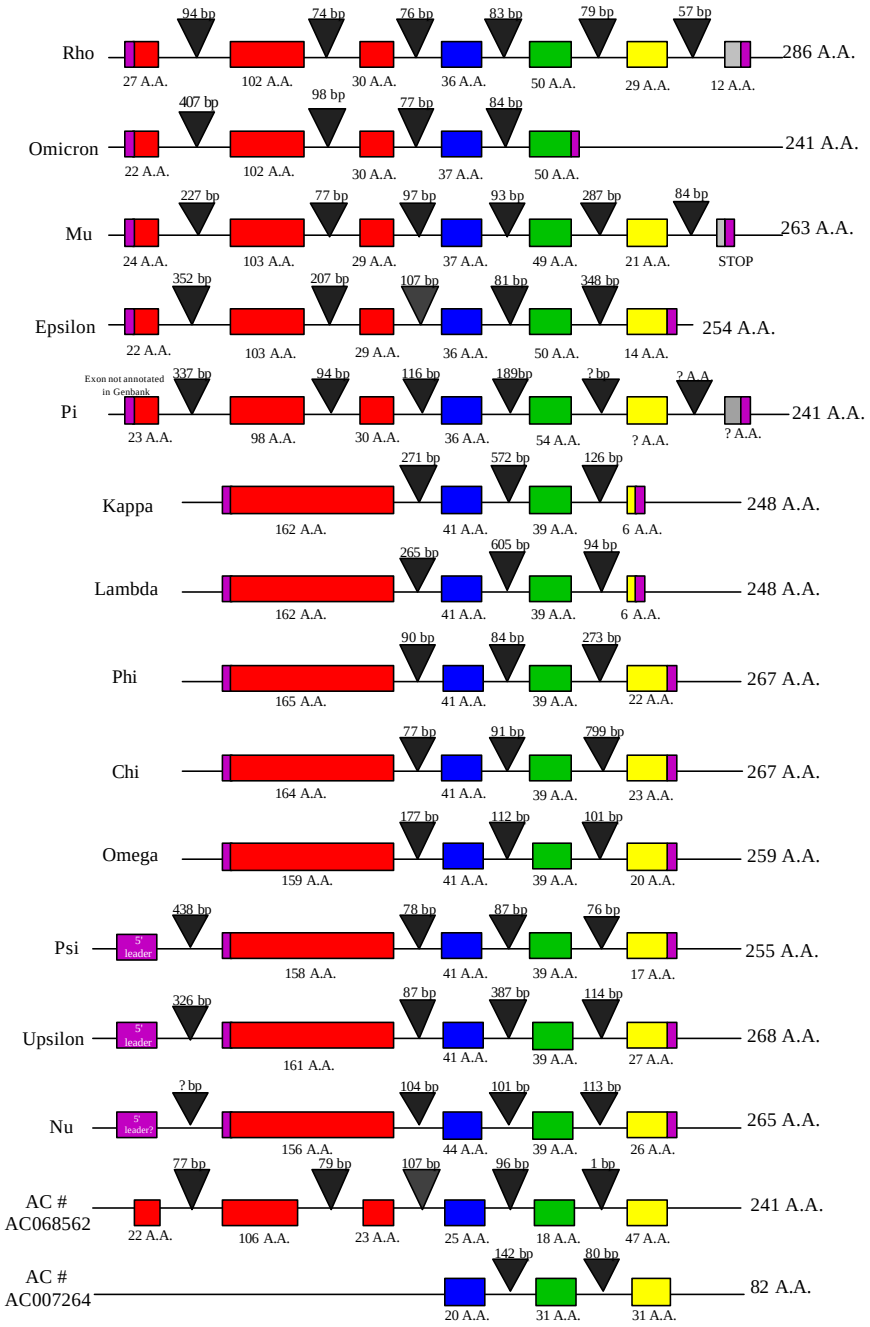
The Non-Epsilon and Epsilon groupings are also well supported by intron-exon structure. The Non-Epsilon members all contain four exons and three introns that are highly conserved in placement. Psi, nu and upsilon contain an extra intron in the 5' leader (Wu et al., 1997). The Epsilon members all possess an intron-exon structure distinct from the Non-Epsilon group, having two additional N-terminal exons. (Presently, the pi isoform contains five exons and four introns. The first N-terminal exon is not annotated in Genbank; however it is indeed present on inspection.) Several genes of the Epsilon group also appear to have additional C-terminal exons. However, the extreme divergence of the C-terminal regions prohibits exon identification based solely on sequence data. The pi isoform is not present as cDNA or an EST. Therefore its structure remains putative at this point and the cDNA available for rho is believed to be truncated.

The complexity of this phylogenetic tree raises an important question. Why are so many 14-3-3 genes present within a single organism? One possible answer is that there is a need to ensure that 14-3-3 activity is present in every compartment of every cell of the organism, suggesting that diversity is simply a reflection of developmental evolution and sophistication. Using current prediction programs, there are no obvious subcellular targeting signals associated with any of the isoforms. So the large number of isoforms is not obviously linked to diversifying the subcellular location. It has been observed, however, that unicellular organisms contain relatively few isoforms while multicellular organisms have many; and certain organelles contain only subsets of the isoforms (Rosenquist et al., 2000). Another possible answer is that each isoform plays a specific and essential biochemical role, suggesting that general diversity reflects functional divergence. They all share a relatively conserved core region, which could point to the

conservation of a general theme, yet subtle changes in the core and the divergent termini could give each isoform its specific function by dictating affinity over a range of possible targets.

The structure of this tree does provide an evolutionary perspective that should contribute to answers to these questions based on emerging data. For example, only epsilon, mu, nu, and upsilon are present in chloroplast stroma, in addition to the cytoplasm (Sehnke et al., 2000), demonstrating that subcellular localization could be consistent with their position on the phylogenetic tree. Omega, chi, and upsilon demonstrate decreasing affinity for nitrate reductase, while phi and psi show no affinity (Bachmann et al., 1996). Isoforms omega, kappa, and lambda demonstrate a decreasing affinity for the proton ATPase (Rosenquist et al., 2000). These examples provide evidence that functional affinity for targets could also be consistent with the phylogenetic tree, but both the localization and function data sets are far from complete. The Arabidopsis thaliana 14-3-3 family should provide a well-developed and inclusive framework for comparative 14-3-3 biology.

Figure 3-1. Arabidopsis thaliana 14-3-3 gene structures and reference information. Colored boxes represent exons, which are drawn to scale and color coded as to their similarity between genes. The thin lines represent introns, which are not to scale and are shown to denote position only. The purple boxes represent 5' leader and 3' trailer sequences. The asterisks identify putative exons, cases where cDNA sequences are not yet available. Current information is outlined to the right of each 14-3-3 gene. Rho, omicron, and pi are the least well characterized of the Arabidopsis thaliana 14-3-3s, thus very little information is available. An expanded version of this table is available at http://www.hos.ufl.edu/ferllab/. (a) Genomic structures of the Arabidopsis thaliana 14-3-3 family. (b) Genomic structures of the epsilon group members containing rho, omicron, mu, epsilon and pi. (c) Genomic structures of one branch of the non-epsilon group containing kappa and lambda. (d) Genomic structures of one branch of the non-epsilon group containing phi, chi, and omega. (e) Genomic structures of one branch of the non-epsilon group containing psi, upsilon and nu members. (f) Genomic structures of the "14-3-3 like" isoforms.



Legend:

- Triangles Represent Introns

- Colored Boxes Represent Exons

Figure 3-1a

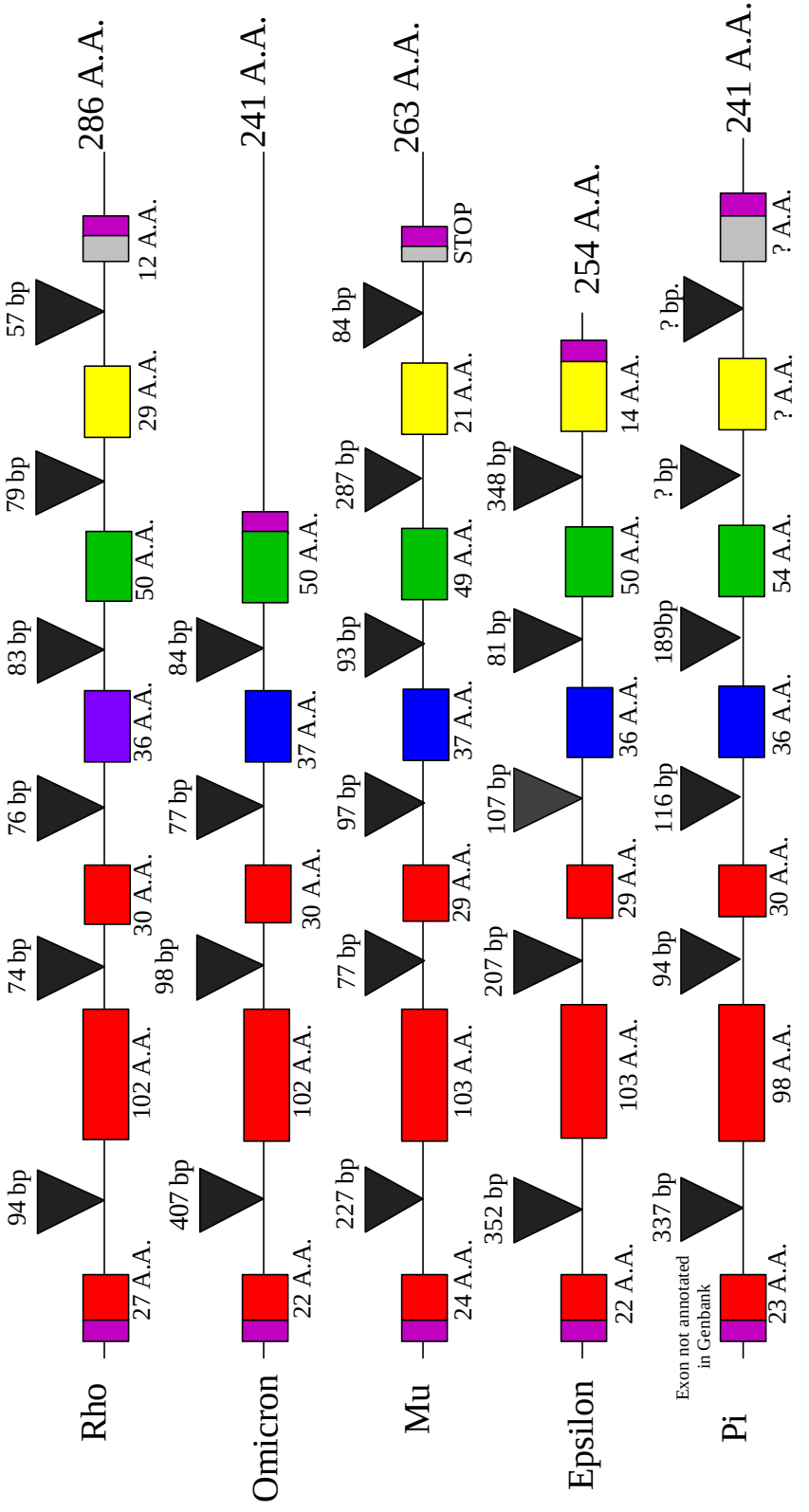
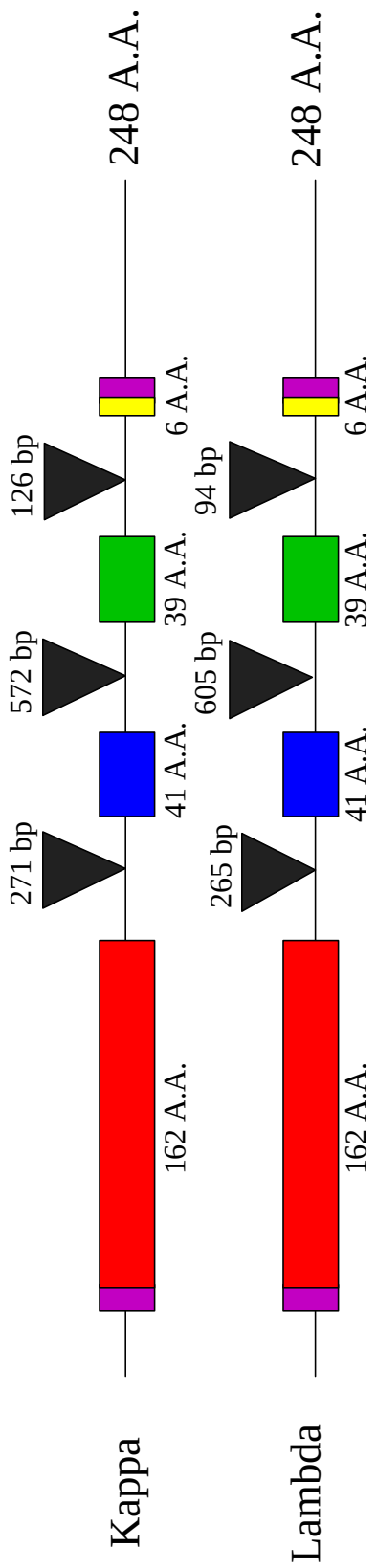

Rho
27 A.A.
94 bp
102 A.A.
74 bp
30 A.A.
76 bp
36 A.A.
83 bp
50 A.A.
79 bp
29 A.A.
57 bp
12 A.A.
286 A.A.
Omicron
22 A.A.
407 bp
102 A.A.
98 bp
30 A.A.
77 bp
37 A.A.
84 bp
50 A.A.
241 A.A.
Mu
24 A.A.
227 bp
103 A.A.
77 bp
29 A.A.
97 bp
37 A.A.
93 bp
49 A.A.
287 bp
21 A.A.
84 bp
STOP
263 A.A.
Epsilon
22 A.A.
352 bp
103 A.A.
207 bp
29 A.A.
107 bp
36 A.A.
81 bp
50 A.A.
348 bp
14 A.A.
254 A.A.
Exon not annotated in Genbank
Pi
23 A.A.
337 bp
98 A.A.
94 bp
30 A.A.
116 bp
36 A.A.
189bp
54 A.A.
? bp
? A.A.
? bp.
? A.A.
241 A.A.
Legend:
- Triangles Represent Introns
- Colored Boxes Represent Exons

Figure 3-1b.


Kappa
Lambda
162 A.A.
162 A.A.
271 bp
265 bp
41 A.A.
41 A.A.
572 bp
605 bp
39 A.A.
39 A.A.
126 bp
94 bp
6 A.A.
6 A.A.
248 A.A.
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Legend:
- Triangles Represent Introns
- Colored Boxes Represent Exons

Figure 3-1c.

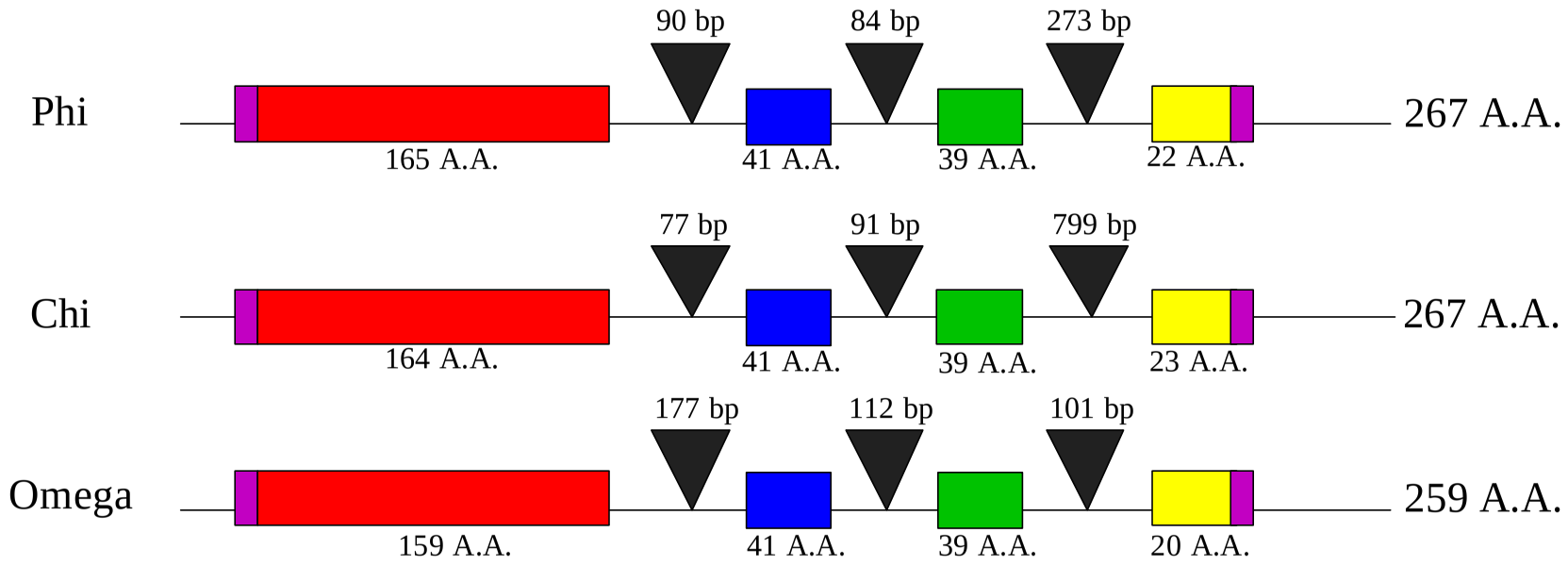

Phi
90 bp
84 bp
273 bp
165 A.A.
41 A.A.
39 A.A.
22 A.A.
267 A.A.
Chi
77 bp
91 bp
799 bp
164 A.A.
41 A.A.
39 A.A.
23 A.A.
267 A.A.
Omega
177 bp
112 bp
101 bp
159 A.A.
41 A.A.
39 A.A.
20 A.A.
259 A.A.
Legend:
- Triangles Represent Introns
- Colored Boxes Represent Exons

Figure 3-1d.

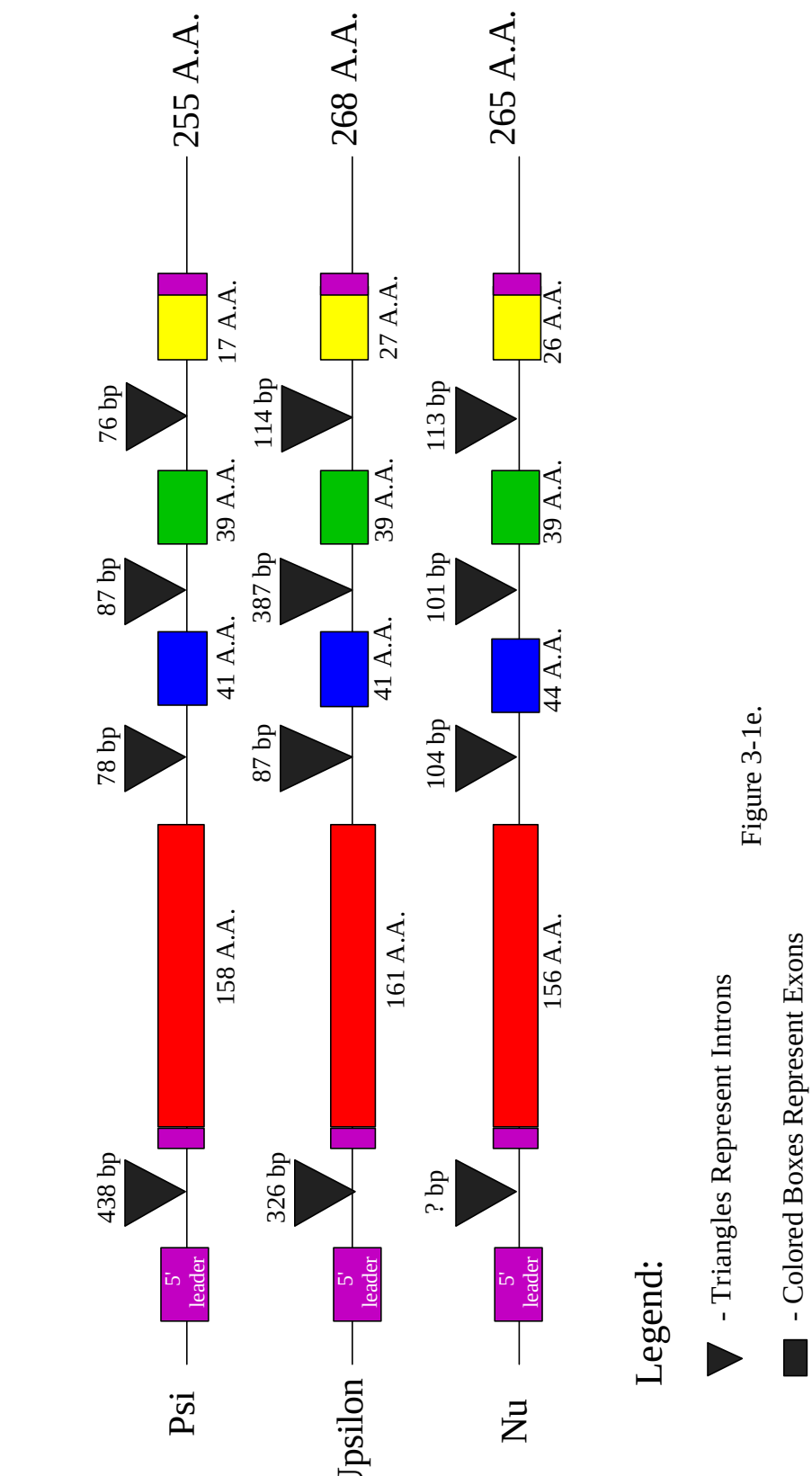
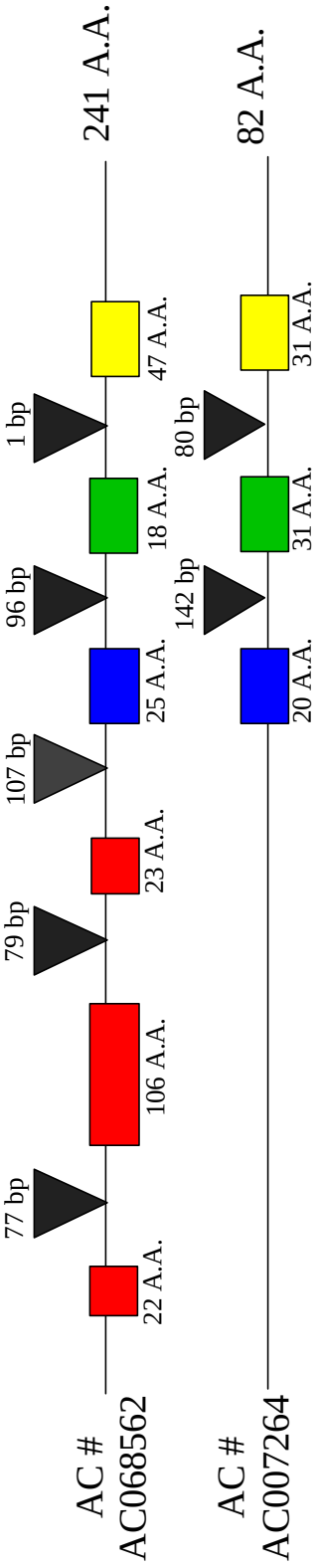

Psi
5' leader
438 bp
158 A.A.
78 bp
41 A.A.
87 bp
39 A.A.
76 bp
17 A.A.
255 A.A.
Upsilon
5' leader
326 bp
161 A.A.
87 bp
41 A.A.
387 bp
39 A.A.
114 bp
27 A.A.
268 A.A.
Nu
5' leader
? bp
156 A.A.
104 bp
44 A.A.
101 bp
39 A.A.
113 bp
26 A.A.
265 A.A.
Legend:
- Triangles Represent Introns
- Colored Boxes Represent Exons

Figure 3-1e.



Legend:

- Triangles Represent Introns

- Colored Boxes Represent Exons

Figure 3-1f.

Table 3-1. Reference information for the Arabidopsis thaliana proteins and genes. A truncated cDNA is available for rho. Pi remains a putative family member at this point.

14-3-3 PROTEINS AND GENES							
Greek Letter	Greek Name	GRF Gene and Accession #	Genbank Accession # for BAC	Genbank Locus # for cDNA clone	cDNA Designation	Genbank Accession # for cDNA	Original Publication
ε	epsilon	GRF10 AF145302	AC068562	ATU36446 1100 bp	2hybr 231	U36446	Wu et al. 1997
κ	kappa	GRF8 AF145300	AB011479	ATU36447 1023 bp	2hybr 31	U36447	Wu et al. 1997
λ	lambda	GRF6 AF145298	AL353995	ATU68545 1040 bp	2hybr 171	U68545	Jarillo et al. 1994
μ	mu	GRF9 AF145301	AC007087	ATU60444 1103 bp	EST D	U60444	Wu et al. 1997
ν	nu	GRF7 AF145299	AC021640	ATU60445 1069 bp	EST H	U60445	Wu et al. 1997
ο	omicron	GRF 11	AC007894			AF323920	Rosenquist et al. 2000
π	pi		AC012680			Not Available	
ρ	rho	GRF 12	AC013427			AF335544	
υ	upsilon	GRF5 AF001415	AL391145	ATHGFUPS 990 bp	GF14-19	L09109	Lu et al. 1994
φ	phi	GRF4 AF001414	AC079605	ATHGFPHI 1126 bp	GF14-10	L09111	Lu et al. 1994
χ	chi	GRF1 U09377	AL161513	ATHGFCHI 1126 bp	GF14-5	L09112	Lu et al. 1994
ψ	psi	GRF3 U09375	AB005248 AB005231	ATHGFPSI A 1108bp	GF14-4	L09110	Lu et al. 1994
ω	omega	GRF2 U09376	AC013430	ATHGF14A 1133 bp	GF14-14	M96855	Lu et al. 1992, 1994
	14-3-3 LIKE PROTEINS						
			AC007264				Rosenquist et al. 2000
			AC068562				

Figure 3-2**.** ClustalW alignment of all thirteen functional 14-3-3 proteins. The proteins range in size from 241-286 amino acids. The blue color denotes a match with the consensus sequence. The red color denotes an identical match among all the isoforms.

```
  Epsilon    (1) ------MENEREKQVYLAKLSEQTERYDEMVEAMKKVAQ---LDVELTVEERNLVSVGYKNVIGARRASWRI
    Kappa    (1) ----MATTLSRDQYVYMAKLAEQAERYEEMVQFMEQLVSGATPAGELTVEERNLLSGAYKNVIGSLRAAWRI
   Lambda    (1) ----MAATLGRDQYVYMAKLAEQAERYEEMVQFMEQLVTGATPAEELTVEERNLLSVAYKNVIGSLRAAWRI
       Mu    (1) ----MGSGKERDTFVYLAKLSEQAERYEEMVESMKSVAK---LNVDLTVEERNLLSVGYKNVIGSRRASWRI
       Nu    (1) ------MSSSREENVYLAKLAEQAERYEEMVEFMEKVAK-TVDTDELTVEERNLLSVAYKNVIGARRASWRI
  Upsilon    (1) ----MSSDSSREENVYLAKLAEQAERYEEMVEFMEKVAK-TVETEELTVEERNLLSVAYKNVIGARRASWRI
      Phi    (1) MAAPPASSSAREEFVYLAKLAEQAERYEEMVEFMEKVAE-AVDKDELTVEERNLLSVAYKNVIGARRASWRI
      Chi    (1) -MATPGASSARDEFVYMAKLAEQAERYEEMVEFMEKVAK-AVDKDELTVEERNLLSVAYKNVIGARRASWRI
      Psi    (1) -------MSTREENVYMAKLAEQAERYEEMVEFMEKVAK-TVDVEELSVEERNLLSVAYKNVIGARRASWRI
    Omega    (1) ------MASGREEFVYMAKLAEQAERYEEMVEFMEKVSA-AVDGDELTVEERNLLSVAYKNVIGARRASWRI
  Omicron    (1) ------MENERAKQVYLAKLNEQAERYDEMVEAMKKVAA---LDVELTIEERNLLSVGYKNVIGARRASWRI
      Rho    (1) -MSSSGSDKERETFVYMAKLSEQAERYDEMVETMKKVAR---VNSELTVEERNLLSVGYKNVIGARRASWRI
       Pi    (1) ------MENEREKLIYLAKLGCQAGRYDGMRKVCELDIE-------LSEEERDLLTTGYKNVMEAKRVSLRV
Consensus    (1)            RE FVYLAKLAEQAERYEEMVEFMEKVA      DELTVEERNLLSVAYKNVIGARRASWRI

  Epsilon   (64) LSSIEQKEESKGNDENVKRLKNYRKRVEDELAKVCNDILSVIDKHLIPSSNAV-ESTVFFYKMKGDYYRYLA
    Kappa   (69) VSSLEQKEESRKNEEHVSLVKDYRSKVETELSSIFSGILRLLDSHLIPSATAR-ESKVFYLKMKGDYHRYLA
   Lambda   (69) VSSIEQKEESRKNDEHVSLVKDYRSKVESELSSVCSGILKLLDSHLIPSAGAS-ESKVFYLKMKGDYHRYMA
       Mu   (66) FSSIEQKEAVKGNDVNVKRIKEYMEKVELELSNICIDIMSVLDEHLIPSASEG-ESTVFFNKMKGDYYRYLA
       Nu   (66) ISSIEQKEESRGNDDHVSIIKDYRGKIETELSKICDGILNLLDSHLVPTASLA-ESKVFYLKMKGDYHRYLA
  Upsilon   (68) ISSIEQKEDSRGNSDHVSIIKDYRGKIETELSKICDGILNLLEAHLIPAASLA-ESKVFYLKMKGDYHRYLA
      Phi   (72) ISSIEQKEESRGNDDHVTTIRDYRSKIESELSKICDGILKLLDTRLVPASANG-DSKVFYLKMKGDYHRYLA
      Chi   (71) ISSIEQKEESRGNDDHVSLIRDYRSKIETELSDICDGILKLLDTILVPAAASG-DSKVFYLKMKGDYHRYLA
      Psi   (65) ISSIEQKEESKGNEDHVAIIKDYRGEIESELSKICDGILNVLEAHLIPSASPA-ESKVFYLKMKGDYHRYLA
    Omega   (66) ISSIEQKEESRGNDDHVTAIREYRSKIETELSGICDGILKLLDSRLIPAAASG-DSKVFYLKMKGDYHRYLA
  Omicron   (64) LSSIEQKEESKGNEQNAKRIKDYRTKVEEELSKICYDILAVIDKHLVPFATSG-ESTVFYYKMKGDYFRYLA
      Rho   (69) MSSIEQKEESKGNESNVKQIKGYRQKVEDELANICQDILTIIDQHLIPHATSG-EATVFYYKMKGDYYRYLA
       Pi   (60) ISSIEKMEDSKGNDQNVKLIKGQQEMVKYEFFNVCNDILSLIDSHLIPSTTTNVESIVLFNRVKGDYFRYMA
Consensus   (73) ISSIEQKEESRGNDDHVSLIKDYR KVETELS IC GIL LLDSHLIPSASSG ESKVFYLKMKGDYHRYLA

  Epsilon  (135) EFSSGAERKEAADQSLEAYKAAVAAAENGLAPTHPVRLGLALNFSVFYYEILNSPESACQLAKQAFDDAIAE
    Kappa  (140) EFKSGDERKTAAEDAMIAYKAAQDVAVADLAPTHPIRLGLALNFSVFYYEILNSSEKACSMAKQAFEEAIAE
   Lambda  (140) EFKSGDERKTAAEDTMLAYKAAQDIAAADMAPTHPIRLGLALNFSVFYYEILNSSDKACNMAKQAFEEAIAE
       Mu  (137) EFKSGNERKEAADQSLKAYEIATTAAEAKLPPTHPIRLGLALNFSVFYYEIMNAPERACHLAKQAFDEAISE
       Nu  (137) EFKTGAERKEAAESTLVAYKSAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDRACSLAKQAFDEAISE
  Upsilon  (139) EFKTGAERKEAAESTLVAYKSAQDIALADLAPTHPIRLGLALNFSVFYYEILNSSDRACSLAKQAFDEAISE
      Phi  (143) EFKTGQERKDAAEHTLTAYKAAQDIANAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAIAE
      Chi  (142) EFKSGQERKDAAEHTLTAYKAAQDIANSELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAIAE
      Psi  (136) EFKAGAERKEAAESTLVAYKSASDIATAELAPTHPIRLGLALNFSVFYYEILNSPDRACSLAKQAFDDAIAE
    Omega  (137) EFKTGQERKDAAEHTLAAYKSAQDIANAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAIAE
  Omicron  (135) EFKSGADREEAADLSLKAYEAATSSASTELSTTHPIRLGLALNFSVFYYEILNSPERACHLAKRAFDEAIAE
      Rho  (140) EFKTEQERKEAAEQSLKGYEAATQAASTELPSTHPIRLGLALNFSVFYYEIMNSPERACHLAKQAFDEAIAE
       Pi  (132) EFGSDAERKENADNSLDAYKVAMEMAENSLAPTNMVRLGLALNFSIFNYEIHKSIESACKLVKKAYDEAITE
Consensus  (145) EFKSG ERKEAAE TL AYKAAQDIA AELAPTHPIRLGLALNFSVFYYEILNSPDRAC LAKQAFDEAIAE

  Epsilon  (207) LDSLNEESYKDSTLIMQLLRDNLTLWTSDLNEEG---DERTKGADEPQDEN----------------------
    Kappa  (212) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMQEQMDEA-----------------------------------
   Lambda  (212) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMQEQMDEA-----------------------------------
       Mu  (209) LDTLSEESYKDSTLIMQLLRDNLTLWTSDISEEGGDDAHKTNGSAKPGAGGDDAE-----------------
       Nu  (209) LDTLGEESYKDSTLIMQLLRDNLTLWNSDINDEAGGDEIKEASKHEPEEGKPAETGQ---------------
  Upsilon  (211) LDTLGEESYKDSTLIMQLLRDNLTLWTSDLNDEAG-DDIKEAPKEVQKVDEQAQPPPSQ-------------
      Phi  (215) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMQDESP-EEIKEAA--APKPAEEQKEI----------------
      Chi  (214) LDTLGEESYKDSTLIMQLLRDNLTLWASDMQDDVA-DDIKEAAPAAAKPADEQQS-----------------
      Psi  (208) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMTDEAG-DEIKEASKPDGAE----------------------
    Omega  (209) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMQDDAA-DEIKEAA--APKPTEEQQ-----------------
  Omicron  (207) LDSLNEDSYKDSTLIMQLLRDNLTLWTSDLEEGGK-----------------------------------
      Rho  (212) LDTLSEESYKDSTLIMQLLRDNLTLWTSDLPEDGGEDNIKTEESKQEQAKPADATEVVIHFKMRTDQRAWKL
       Pi  (204) LDGLDKNICEESMYIIEMLKYNLSTWTSGDGNGNKTDG-----------------------------------
Consensus  (217) LDTLGEESYKDSTLIMQLLRDNLTLWTSDMQDEAG DEIK

  Epsilon  (255) ---
    Kappa  (249) ---
   Lambda  (249) ---
       Mu  (264) ---
       Nu  (266) ---
  Upsilon  (269) ---
      Phi  (268) ---
      Chi  (268) ---
      Psi  (256) ---
    Omega  (260) ---
  Omicron  (242) ---
      Rho  (284) NEI
       Pi  (242) ---
Consensus  (289)
```

Figure 3-3. Rooted tree of the Arabidopsis thaliana 14-3-3 family. This rectangular cladogram summarizes the relationships among the 14-3-3 isoforms. A human 14-3-3 was included as an outgroup, and essentially roots the tree between the Epsilon and Non-Epsilon groupings. The basic branch topology presented in this neighbor-joining tree is essentially supported by bootstrapped parsimony analysis, however the confidence of the branching patterns within the Epsilon group is less well supported than the branching within the highly supported Non-Epsilon group. The major branches of the tree are also clearly supported by the gene structures, which is reflected by the numbers of exons and introns as indicated on the branches. Bolded isoforms are present in the chloroplast as well as the cytoplasm. The underlined isoform is putative in that, as yet, they have not been isolated as cDNAs or ESTs.

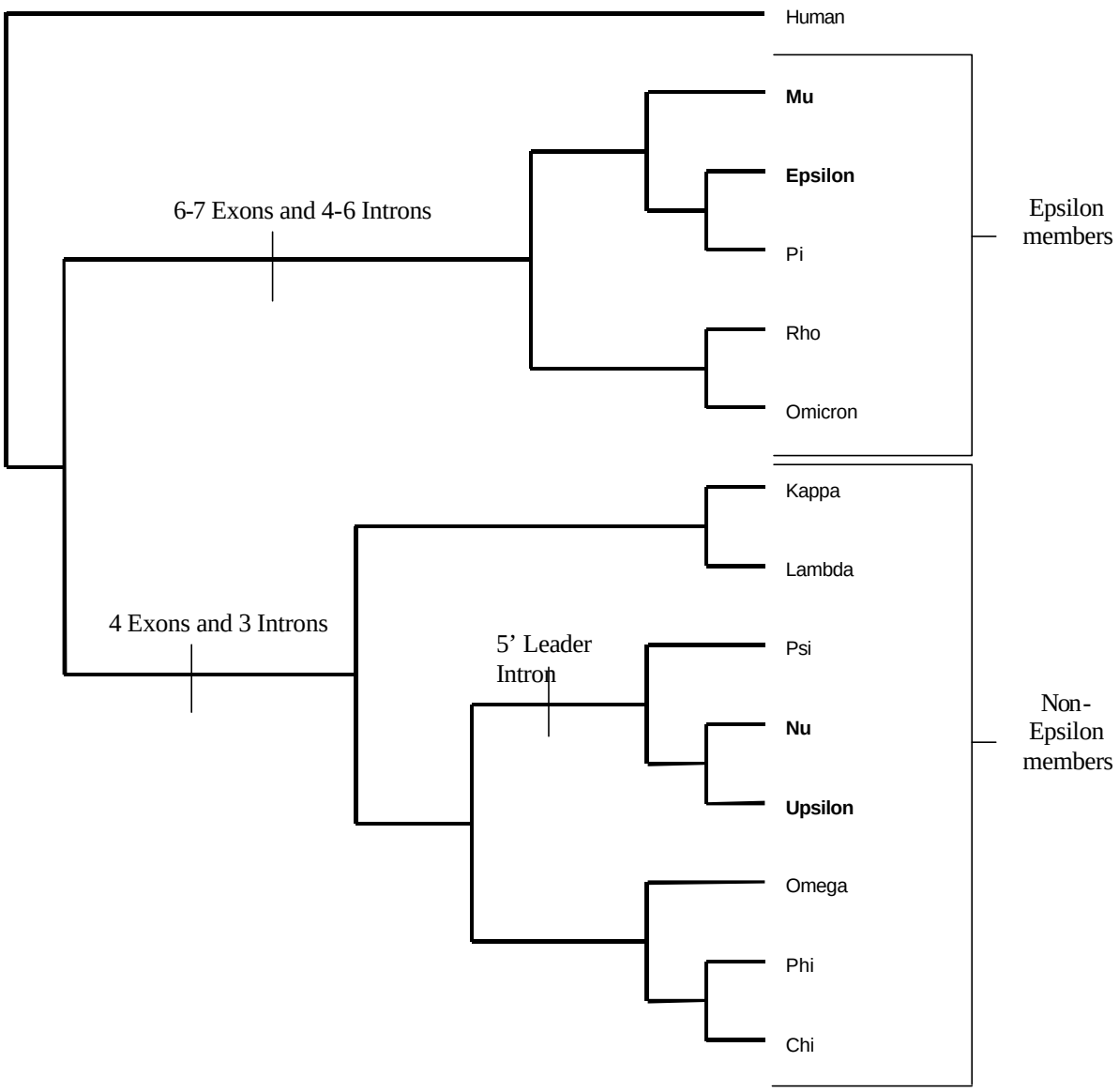

Human
Mu
Epsilon
Pi
Rho
Omicron
Epsilon members
6-7 Exons and 4-6 Introns
Kappa
Lambda
4 Exons and 3 Introns
5' Leader Intron
Psi
Nu
Upsilon
Omega
Phi
Chi
Non-Epsilon members

Figure 3-3.

CHAPTER 4
WHOLE-MOUNT IMMUNOFLUORESCENCE STUDIES

Introduction

The 14-3-3s are a family of proteins that were initially discovered during an extensive study of bovine brain proteins by Moore and Perez in 1967 (Moore and Perez, 1967). The 14-3-3s are named based on fractionation and electrophoretic mobility in starch gel electrophoresis. It was long thought that 14-3-3s were limited to mammalian brain tissue, however studies over the past fifteen years have proven 14-3-3s to be ubiquitous. The 14-3-3s are found in greatly diverse organisms from drosophilia and plants to frogs and humans (Finnie et al., 1999). This family of proteins has been found in almost every eukaryotic organism that scientists have studied (Robinson et al., 1994).

The 14-3-3s are usually found as multi-isoform families within a single organism. For example, there are nine isoforms found in mammalian tissue and are designated as Greek letters (alpha, beta, gamma, delta, epsilon, zeta, eta, tau, and sigma) (Ichimura et al., 1988; Patel et al., 1994). The Arabidopsis thaliana 14-3-3 members are designated in a similar fashion starting at the end of the Greek alphabet and working forward. There are currently thirteen isoforms found in Arabidopsis thaliana and are designated as omega, psi, chi, phi, upsilon, rho, pi, omicron, nu, mu, kappa, lambda, and epsilon (Lu et al., 1992; Lu et al. 1994b; Wu et al., 1997; Rosenquist et al., 2001; DeLille et al., 2001).

The 14-3-3s are involved in a number of diverse functions within the cell. They are known to regulate key enzymes in metabolic processes such as nitrogen metabolism

and carbon metabolism (Chung et al., 1999), and to associate with the plasma membrane H^+-ATPase (Sehnke and Ferl, 2000) and transcription factors in plants (Lu et al., 1992). The 14-3-3s are also involved in protein kinase activities (Ferl, 1996). In most of these associations, 14-3-3s participate by binding to phosphorylated proteins in order to consummate signal transduction events.

To fully understand the functions and interaction partners of the Arabidopsis thaliana 14-3-3 family of proteins it is essential to know where the specific isoforms are localized at the subcellular level. The biological question that this thesis attempts to answer is this: Do the 14-3-3s have functional specificity? As previously mentioned, the 14-3-3s usually exist as multi-isoform families within a particular organism. Do multiple isoforms exist simply to insure their presence in the proper tissues or does each isoform play an essential and necessary role? Studies have been performed that give clues as to specificity. For example, in Arabidopsis thaliana only isoforms epsilon, mu, nu, and upsilon are present in chloroplasts (Sehnke et al., 2000). Why those four isoforms? There is nothing obviously inherent about these isoforms that would make them chloroplast localized. Mu and epsilon are the only two well-characterized isoforms making up the epsilon sub-group. Nu and upsilon are closely related members of the non-epsilon group. There are no obvious signal or targeting sequences in any of the arabidopsis 14-3-3s. Therefore, they must be localizing to the chloroplast via interaction with other proteins. It is the unpredictable nature of the 14-3-3s that makes localization studies so important. We must know where the isoforms are localized.

Using laser scanning confocal microscopy, Bihn et al. reported the localization of 14-3-3s in the nucleus and cytoplasm using a mixture of ascites from three GF14

monoclonal antibody cell lines (Bihn et al., 1997). These cell lines combined recognize at least ten of the thirteen 14-3-3 isoforms in Arabidopsis thaliana. However, the authors did not localize individual 14-3-3 isoforms within the cell.

In the present study, we use laser scanning confocal microscopy along with isoform specific polyclonal antibodies to study the subcellular localization of 14-3-3s within the root tip of Arabidopsis thaliana. We find that individual isoform localization occurs in two distinct groups, a nuclear localized group and a non-nuclear group.

Materials and Methods

Whole Mount Procedure

Arabidopsis thaliana seeds, ecotype Wassileweskija (WS), were surface sterilized by washing with 70% ethanol for five minutes, further rinsed with 50% bleach/water solution with a drop of Tween-20 for eight to ten minutes. The seeds were then rinsed with sterile water five to eight times in order to remove detergent and bleach and plated on germination media consisting of 4.4 g/L MS salts, 0.5 g/L MES, 10.0 g/L sucrose, 1.0 mL/L Gamborg's vitamin solution (1000x), pH 5.7, 4.0 g/L phytagel. After 48 hours at 4°C in the dark, the plates were taken to the Apopka room and grown vertically under continuous illumination for 3-10 days at 23°C. The plants were removed from the plate and fixed according to Shaw and Bourdonck protocol (Bihn et al., 1997). All manipulations of the plant tissue starting with fixation were performed in a 6 or 12 well microtiter plate. Solutions were added and removed to the dish and the plants were not removed until mounted on a microscope slide. The plants were placed in freshly made 4% paraformaldehyde (prepared by dissolving 8% (w/v) solid paraformaldehyde in water and then diluted with an equal volume of 2X PEM/0.4% Nonidet P-40 (NP-40) buffer

(PEM: 50 mM PIPES/KOH pH 6.9; 5 mM EGTA; 5 mM $MgSO_4$/500 mL)) for 1 hour at room temperature. The plants were then washed twice with 1X PEM/0.2% NP-40 and then washed twice with PEM alone. The tissue was digested with 0.05% cellulase/0.025% Y23 Pectolyase/1.0% Driselase in PEM for ten minutes and then washed with PEM/0.2% NP-40 for ten minutes. The plant tissue was blocked with 3% BSA in PEM for 1.5 hours at room temperature. The plants were subsequently incubated with primary antibody in blocking buffer at 4°C overnight. The next day the tissue was washed in blocking buffer for one hour and then incubated with secondary antibody in blocking buffer for two hours at 37°C (dark). The plant tissue was finally washed several times with PEM/0.2% NP-40 and then overnight in PEM alone. The next day, the plant tissue was removed from the solution using a plastic disposable pipette and then placed on the microscope slide. The excess liquid was removed and a small drop of Vectashield mountant (Vector Laboratories Inc., Burlingame, CA 94010, USA) was applied and then coversliped.

Immunofluorescence

Plant tissues were incubated with a polyclonal antibody recognizing a specific 14-3-3 isoform, monoclonal antibody #65 that recognizes eight out of the 13 isoforms (mu, epsilon, omicron, rho, and pi are excluded) (Bihn et al., 1997), Histone H1; 1:1000. They were secondarily incubated with a FITC conjugated antibody (Jackson Laboratories, Inc.); 1:2500.

Laser Scanning Confocal Microscopy and Image Analysis

Plant tissue samples were examined with an Olympus IX70 inverted microscope mounted to a Bio-Rad MRC 1024ES laser scanning confocal microscope with a

krypton/argon laser. Collected images were Kalman averaged four to five times. The images were collected using the Laser Sharp software package (Bio-Rad). The images were transferred to a PC and cataloged using Thumbs Plus 4 (Cerious software). Confocal Assistant 4.02 (Bio-Rad) was used to view collected Z-series files and convert files from a .pic (Bio-Rad format) to an .avi format (suitable for Windows viewing of Z-series).

Results

Polyclonal antibodies were generated by Dr. Paul Sehnke against specific 14-3-3 isoforms in arabidopsis. The arabidopsis 14-3-3s share a very conserved core region and differ primarily at their amino and carboxyl termini. The polyclonal antibodies were therefore made to peptides derived from either the N- or C-termini. At the time the antibodies were generated, we did not yet know of the existence of isoforms omicron, rho, and pi. Also, the psi isoform lacks enough unique sequence to generate an antibody that would not cross react with the other isoforms. Therefore, nine polyclonal antibodies were generated with each one recognizing a specific 14-3-3 isoform; phi, kappa, chi, lambda, epsilon, upsilon, mu, nu, and omega.

Initial studies were performed to optimize three factors: antibody concentration, age of plant tissue, and type of microscope to use. The proper antibody concentration was determined empirically during several experiments. It was determined that a primary antibody concentration of 1:1000 was sufficient for the 14-3-3 polyclonal antibodies. The proper concentration of FITC conjugated secondary antibody was determined to be 1:2500. The combination of these two antibody dilutions provided enough fluorescence

to be seen, but not show a high fluorescence background. These studies were performed using an epifluorescence microscope without image capture capabilities.

The 14-3-3 localization studies were performed on plants subjected to 48 hours at 4°C in the dark and then grown vertically for 3 or 10 days. The goal was to determine which tissues would work the best and if the localization of the isoforms within the tissue changed over time. It was determined that the root tip provided the best tissue to work with and there were no visible changes in localization among any of the 14-3-3 isoforms (Figure 4-1). There was no difference in localization between the two ages. In fact, as the plants progressed in age, they became more difficult to work with. Therefore, it was determined that three day-old seedlings were the best candidates for further study.

The type of microscope to use was also an initial area of concern. The choice was between an epifluorescence microscope with a mounted camera and a laser scanning confocal microscope. We wanted to balance quality of information with cost. The fluorescence microscope had a problem in that we could not get the light source low enough in power to eliminate autofluorescence of the plant tissue. The laser scanning confocal microscope eliminated this issue because it uses a laser and any light not in the focal plane is discarded at image formation. Although the cost of the confocal microscope is relatively high, it was clearly the best choice for our purposes.

Confocal microscopy allows several types of image collection. A single image or optical slice can be captured and viewed. Also, a series of images through the sample can also be captured and viewed as a movie. Both of these types of data are presented in this work.

Upon examination of nine 14-3-3 isoforms in 4-5 day old Arabidopsis thaliana root tips using the polyclonal antibodies and laser scanning confocal microscopy, we were able to identify two distinct patterns of localization, a nuclear group and non-nuclear group. Figure 4-2 is thumbnail images of nine 14-3-3 isoforms and control antibodies for comparison, and individual enlargements are presented in Figures 4-3 and 4-4.

Isoforms phi, kappa, chi, and lambda make up the nuclear group (Figure 4-2 and Figures 4-3a-d, respectively). The fluorescent signal is predominately localized to the nucleus in these isoforms. A significant amount of signal can also be seen throughout the cytoplasm. No other subcellular organelles are visible in this group of isoforms. Also, there are no compartments in the cell devoid of fluorescence, such as vacuoles or other structures. The intensity of the fluorescent signal cannot be directly compared between images, but the signal can be compared in the same image, i.e. the nucleus is more intense than the cytoplasm.

Isoforms epsilon, upsilon, mu, nu, and omega make up the non-nuclear group of isoforms (Figure 4-2 and Figures 4-4e, respectively). The fluorescent signal is present throughout the cell and no organelle shows a predominance of signal. Again, there are no compartments within the cell devoid of fluorescence. However, in all the isoforms, gaps are visible between the cells. This is possibly a product of the fixation process, rather than an absence of signal in the cell wall. The plasma membrane could have pulled away from the cell wall, leaving the gaps. However, the reason for the gaps remains unknown.

Several antibodies are used as controls to verify subcellular location (Figure 4-5). Antibody #65 (Figure 4-5a) is a monoclonal antibody that recognizes eight out of the

thirteen well-characterized 14-3-3 isoforms (mu and epsilon are excluded and the antibody has not been tested against the omicron, rho, or pi isoforms). Antibody #65 is predominately found in the nucleus, but some signal can also be seen in the cytoplasm.

An antibody to Histone H1 (Figure 4-2 and Figure 4-5b) that associates with nuclear material also serves as a control for nuclear localization. This antibody is clearly localized to the nucleus. It provides a pattern of localization very similar to that of antibody #65 and the nuclear 14-3-3 group.

Wildtype (WS) arabidopsis with no antibody treatment is used as a negative control for antibody and as a control for autofluorescence. An image could not be acquired for wildtype plants with no antibody treatment although both the iris and gain were maximized during image capture (Figure 4-5c). Finally, plants treated with only the secondary antibody serve as a control for non-specific antibody binding. Again, no image could be acquired although the iris and gain were maximized at the time of image capture (Figure 4-5d).

Discussion

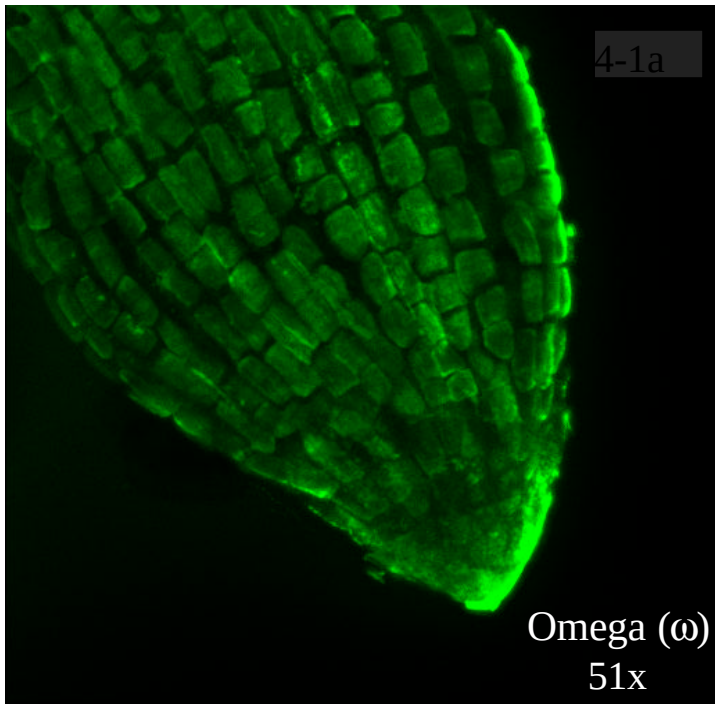
The localization studies reveal two distinct patterns, nuclear and non-nuclear. The isoforms phi, kappa, chi, and lambda make up the nuclear group. The isoforms epsilon, upsilon, mu, nu, and omega make up the non-nuclear group. Both groups are also localized to the cytoplasm. The nuclear vs. non-nuclear localization could be an indication of isoform specificity. However, the polyclonal antibodies themselves pose several problems. First, the way in which the antibodies were made posses a problem. The antibodies to isoforms phi, kappa, chi, and lambda were all generated to the N-terminus. The antibodies to isoforms epsilon, upsilon, mu, nu, and omega were all

generated to the C-terminus. All of the N-terminal antibodies make up the nuclear group and all the C-terminal antibodies make up the non-nuclear group. It is possible that the groupings are purely coincidental, although highly unlikely. Second, the process is lethal to the plant. To use the antibodies, the plant must be fixed in 4% paraformaldehyde. This process limits what can be done to the plant, i.e. time course experiments with the same plant. Third, a series of enzymes must be used to degrade the cell wall. There is no way to tell how much of the cell wall has or has not been degraded or what other unforeseen effect the enzymes may have on the plant tissue.

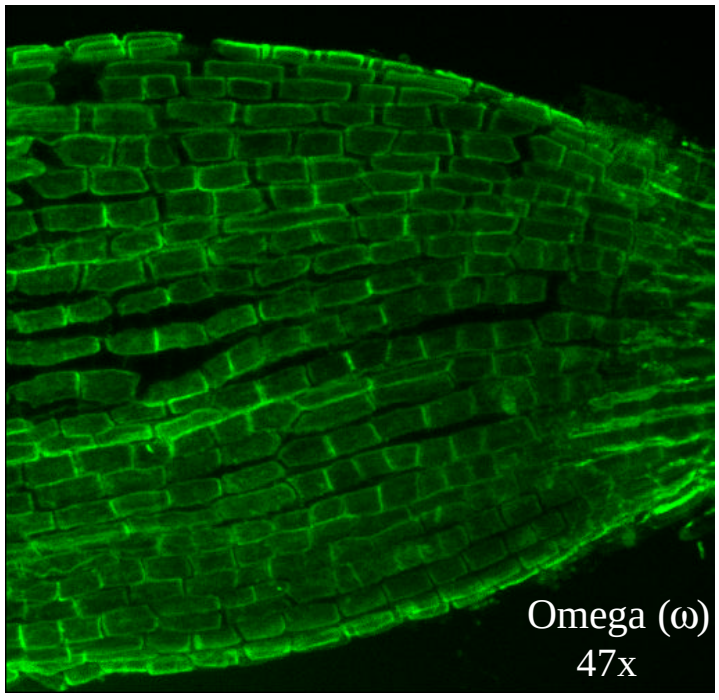
The clean groups that the 14-3-3 antibodies fall into according to termini is highly suspicious. I suspect that there is some unknown interaction that is blocking either the N- or C-terminus of the 14-3-3 and causing the antibodies to localize in this particular manner. The 14-3-3s commonly form homo- and hetero-dimers via their N-termini forming a clamp like structure. The inner groove of the clamp forms the interaction site for other proteins. If the 14-3-3s were dimerized, this might cause enough hindrance, so that the antibodies would not be able to recognize the isoform, thus giving the false appearance of distinct nuclear and non-nuclear groups. The individual isoforms might in fact have a very distinct pattern of localization, but the antibodies are unable to recognize the isoforms.

Penetration of the 14-3-3 antibodies into the deeper cell layers is also an important issue. Figure 4-6 is a graphical representation of an Arabidopsis thaliana root tip. The cell layers from outermost to innermost are lateral root cap, epidermis, cortex, endodermis, pericycle, and vascular bundle. The 14-3-3 localizations in this study are limited to the lateral root cap and possibly the epidermis. Figure 4-7 shows a series of

four images through an Arabidopsis thaliana root tip treated with monoclonal antibody #65. These images were taken from a more detailed series, but the overall meaning is clear. Image 7a shows clear fluorescent labeling of the nuclei, however as you progress through the root tip (4-7b and 4-7c), no nuclei are labeled. Yet, in 4-7b and 4-7c, nuclei in the outer cell layers are still visible. Finally, in 4-7d, the nuclei reappear on the opposite side of the root tip. This effect can be seen with all of the 14-3-3 isoforms to some degree. It is easier to see with the nuclear localized isoforms rather than the non-nuclear isoforms. It is my hypothesis that the antibody cannot get into these inner layers of the root tip, and thus no labeling is occurring. Perhaps with longer incubation times in the digestion mix, it would be possible to visualize the inner cell layers.



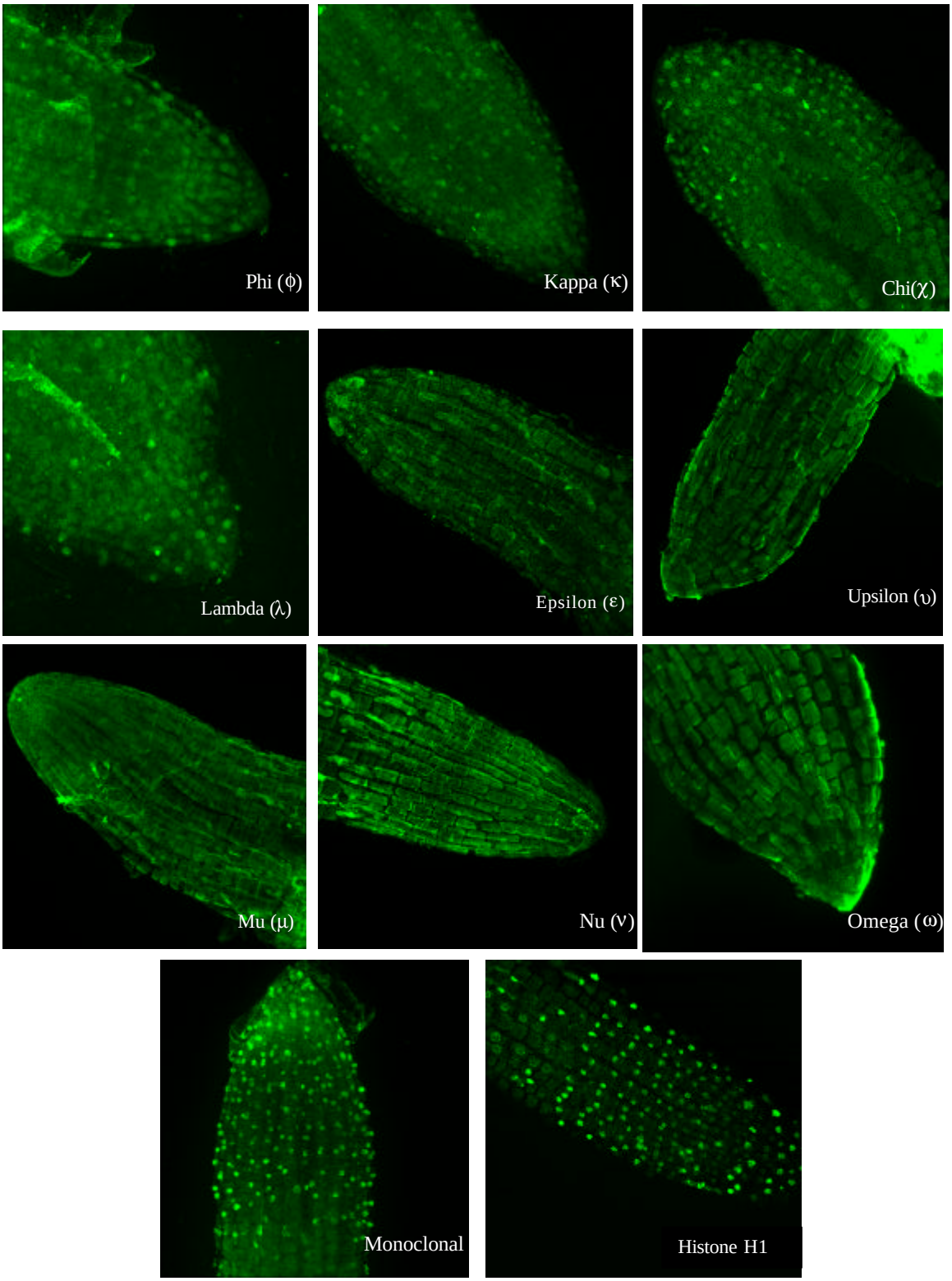
4-1a



4-1b

Figure 4-1. Arabidopsis thaliana root tips treated with a 14-3-3 polyclonal antibody. The images were collected using laser scanning confocal microscopy. (a) Three day-oroot tip treated with a polyclonal antibody to the omega 14-3-3 isoform. The magnification is 51x. (b) Ten day-old root tip treated with a polyclonal antibody to the omega 14-3-3 isoform. The magnification is 47x.

Figure 4-2. Thumbnail images of Arabidopsis thaliana root tips treated with nine 14-3-3 polyclonal antibodies and control antibodies. The thumbnails are labeled as to the specific isoform used. The 14-3-3s fall into two distinct groups, a nuclear and non-nuclear group. Phi, kappa, chi and lambda make up the nuclear group. Epsilon, upsilon, mu, nu, and omega make up the non-nuclear group. Two controls are also shown. The image labeled "monoclonal" is a single monoclonal antibody that recognizes eight out of the ten well-characterized isoforms (mu and epsilon are excluded). Histone H1 is a monoclonal antibody that is included as a control to identify nuclei.


Phi (ϕ)
Kappa (κ)
Chi(χ)
Lambda (λ)
Epsilon (ε)
Upsilon (υ)
Mu (μ)
Nu (ν)
Omega (ω)
Monoclonal
Histone H1

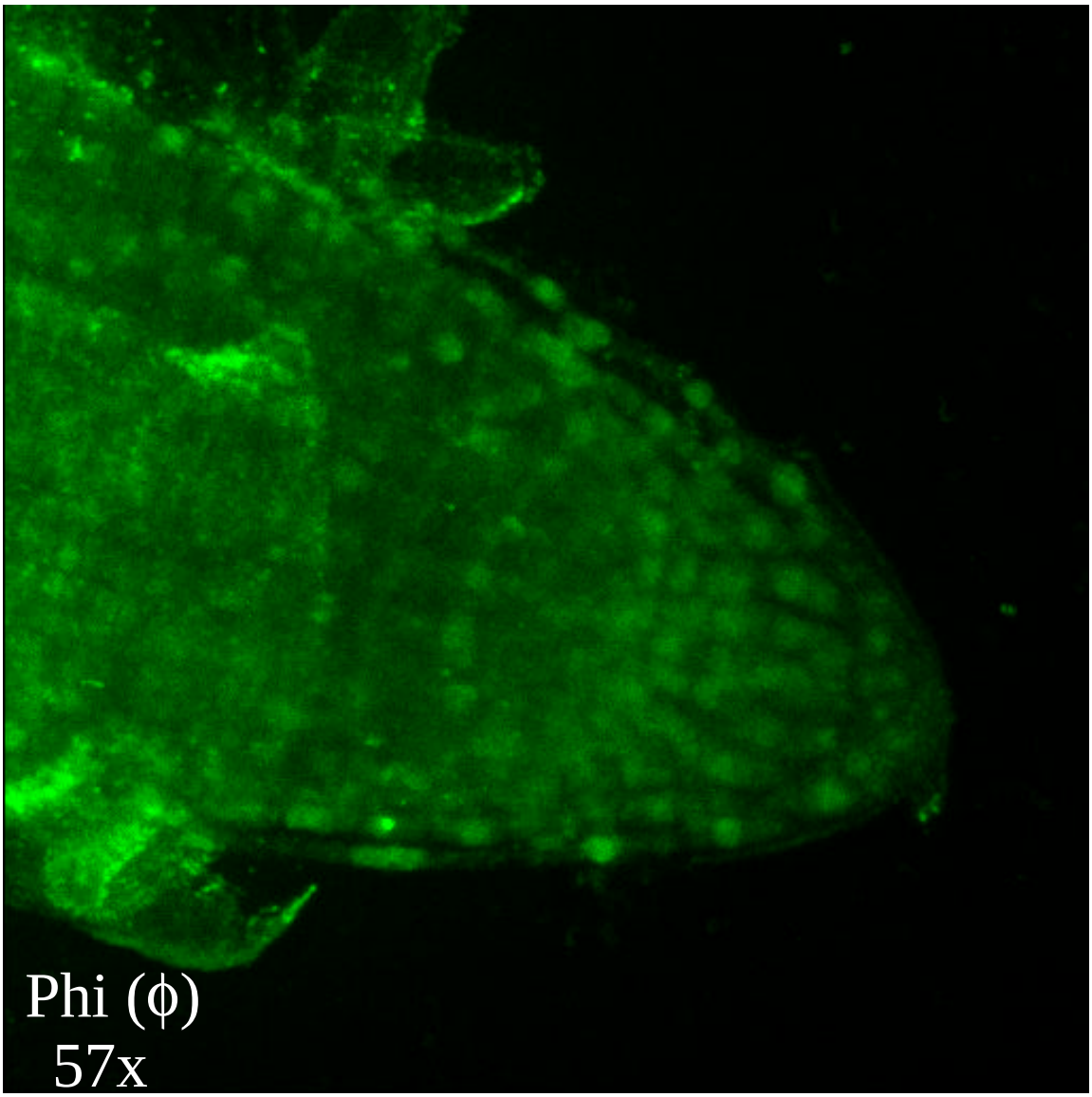


Figure 4-3a. Arabidopsis thaliana root tip treated with a polyclonal antibody to the phi 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 57x. The localization pattern is predominately nuclear, although some signal can be seen in the cytoplasm.

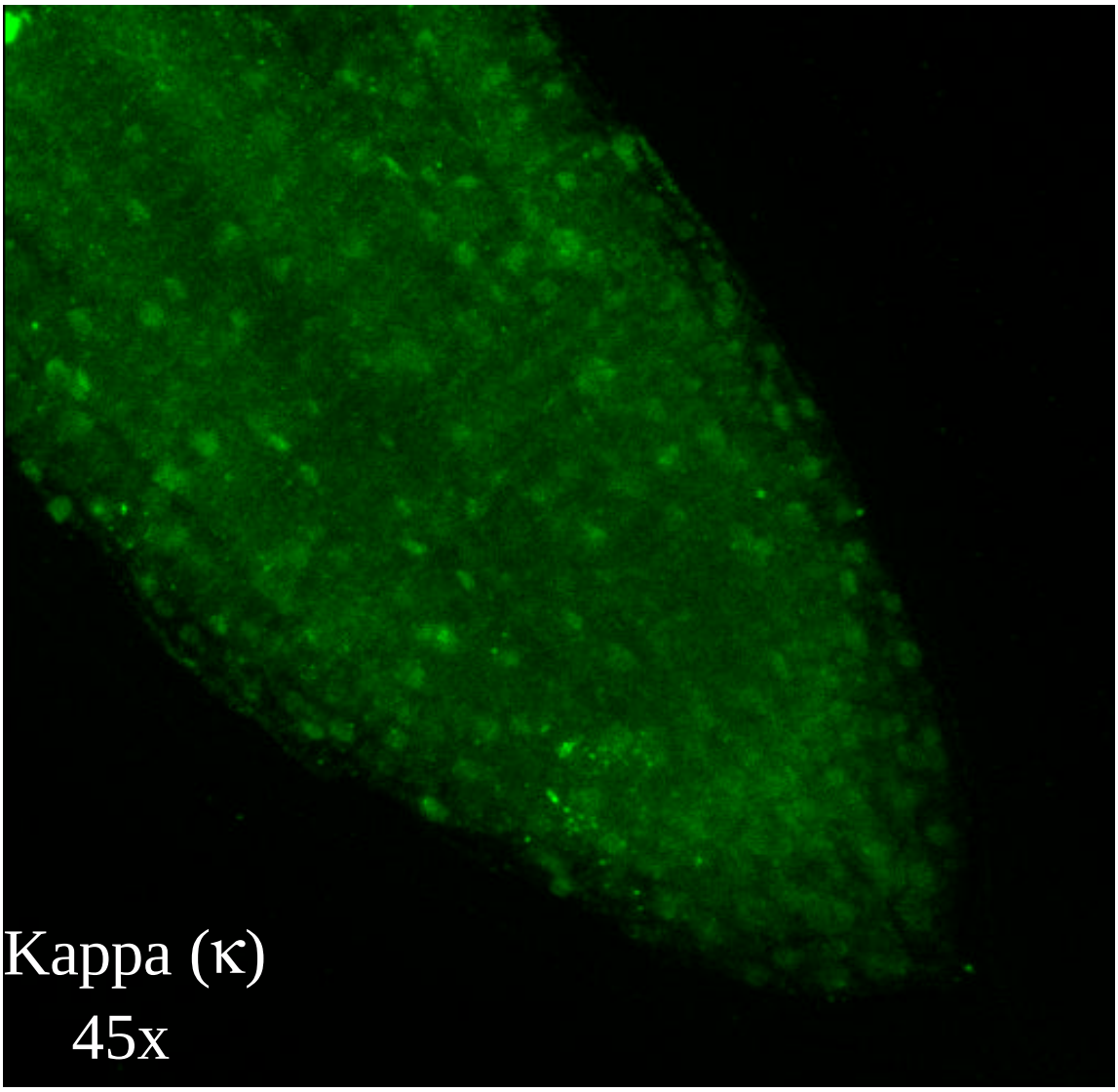


Figure 4-3b. Arabidopsis thaliana root tip treated with a polyclonal antibody to the kappa 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 45x. The localization pattern is predominately nuclear, although some signal can be seen in the cytoplasm.

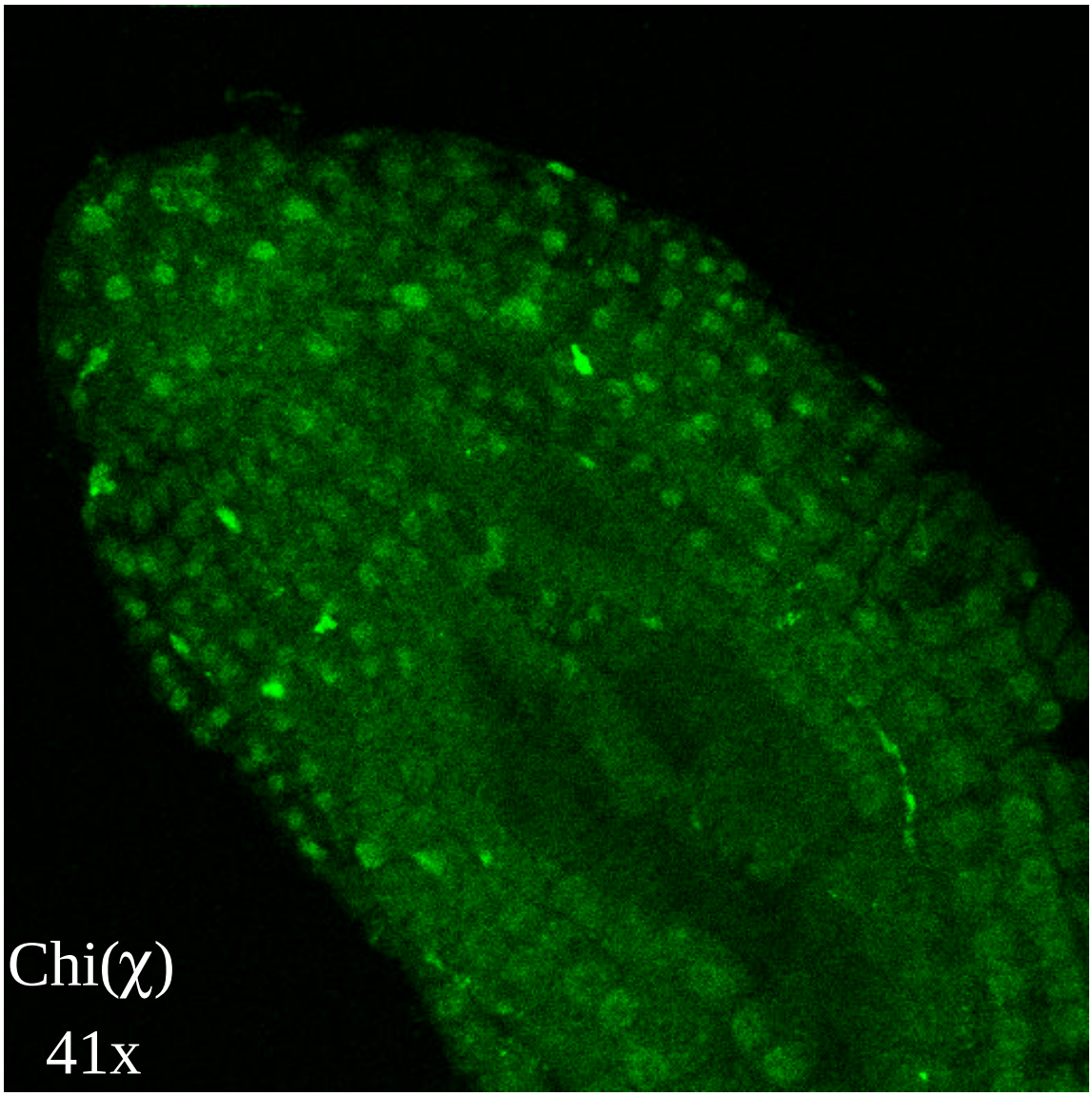


Figure 4-3c. Arabidopsis thaliana root tip treated with a polyclonal antibody to the chi 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 41x. The localization pattern is predominately nuclear, although some signal can be seen in the cytoplasm.

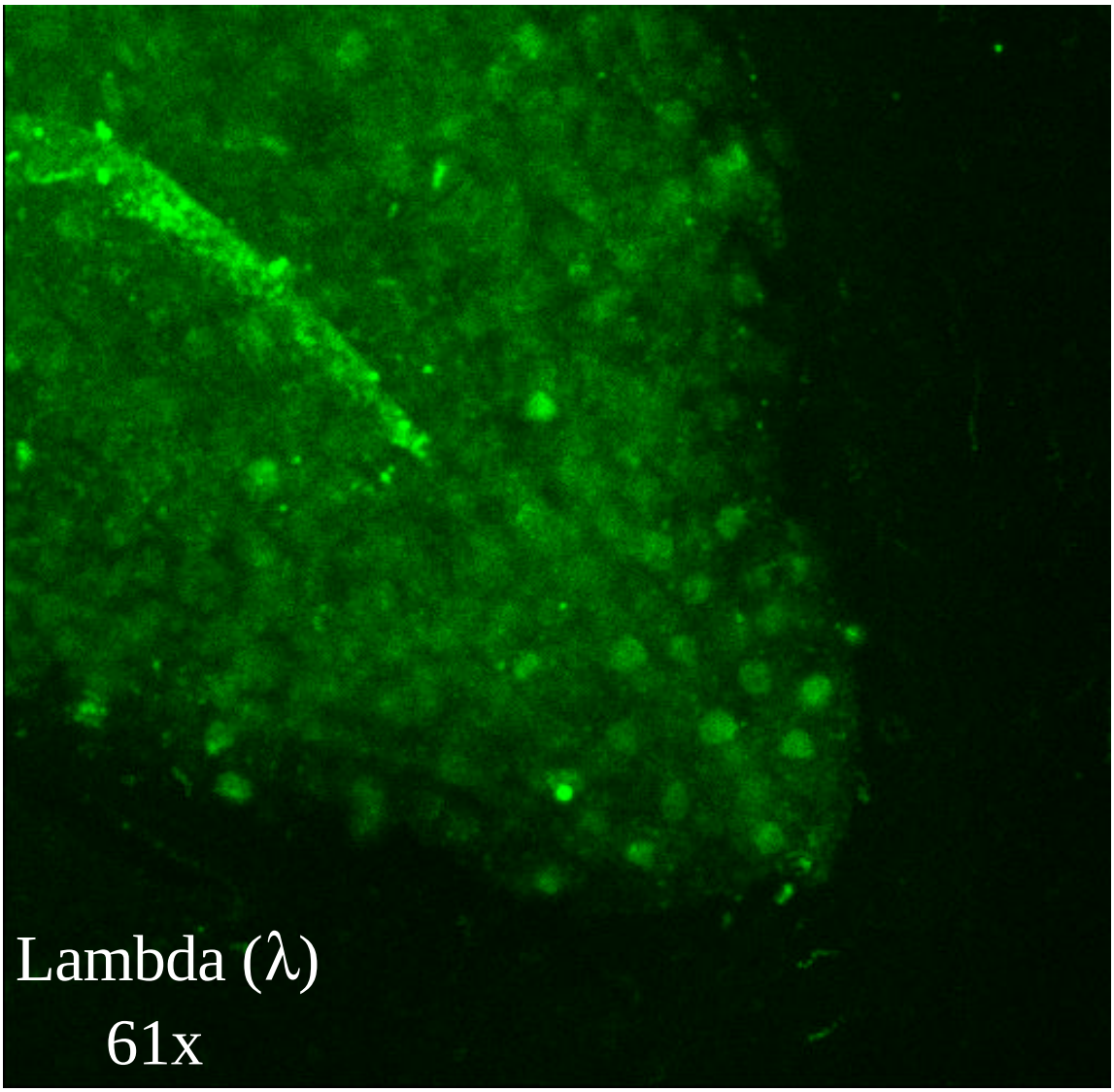


Figure 4-3d. Arabidopsis thaliana root tip treated with a polyclonal antibody to the lambda 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 61x. The localization pattern is predominately nuclear, although some signal can be seen in the cytoplasm.

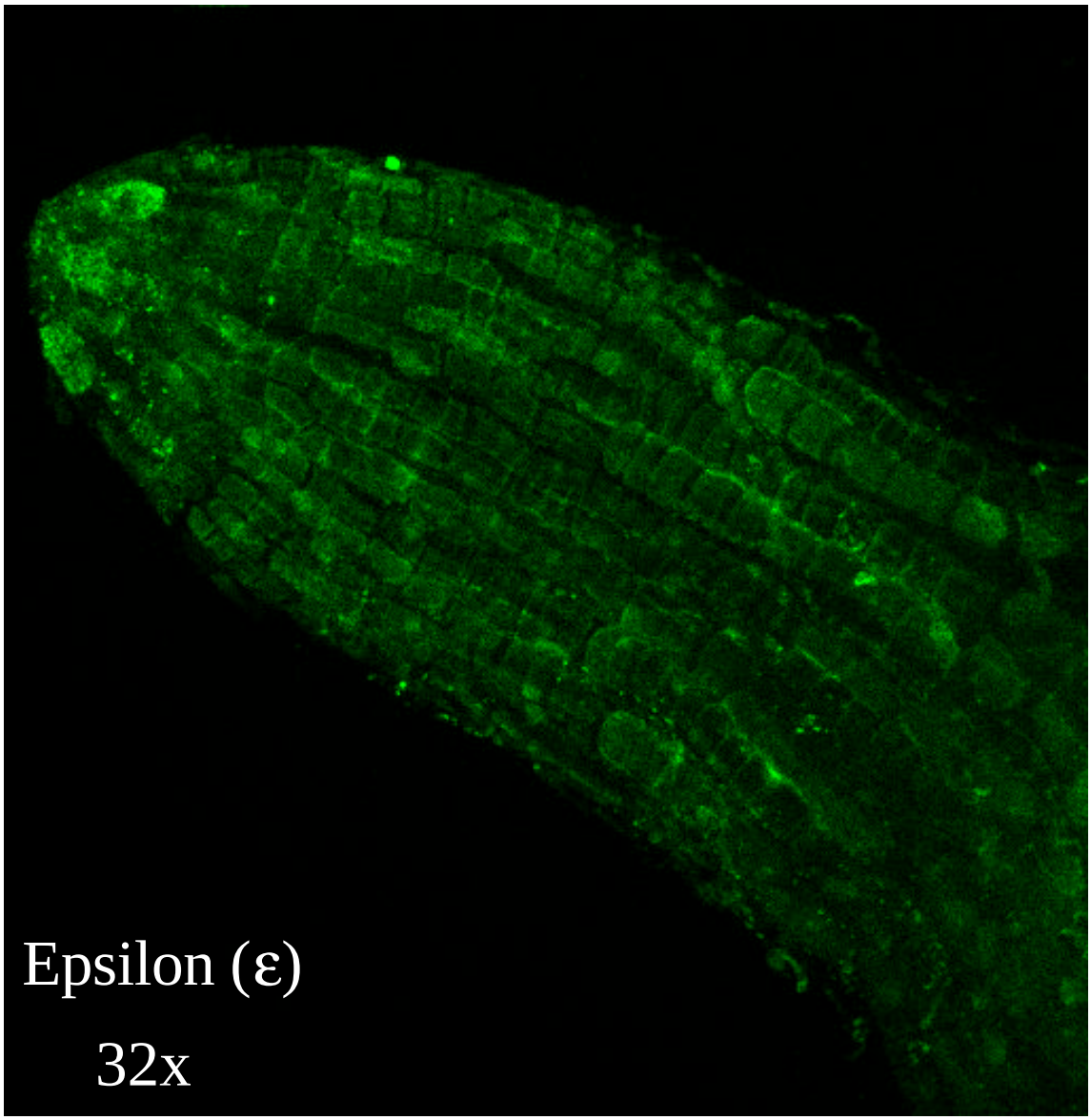


Figure 4-4a. Arabidopsis thaliana root tip treated with a polyclonal antibody to the epsilon 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 32x. The localization pattern is predominately cytoplasmic. No subcellular organelles are visible.

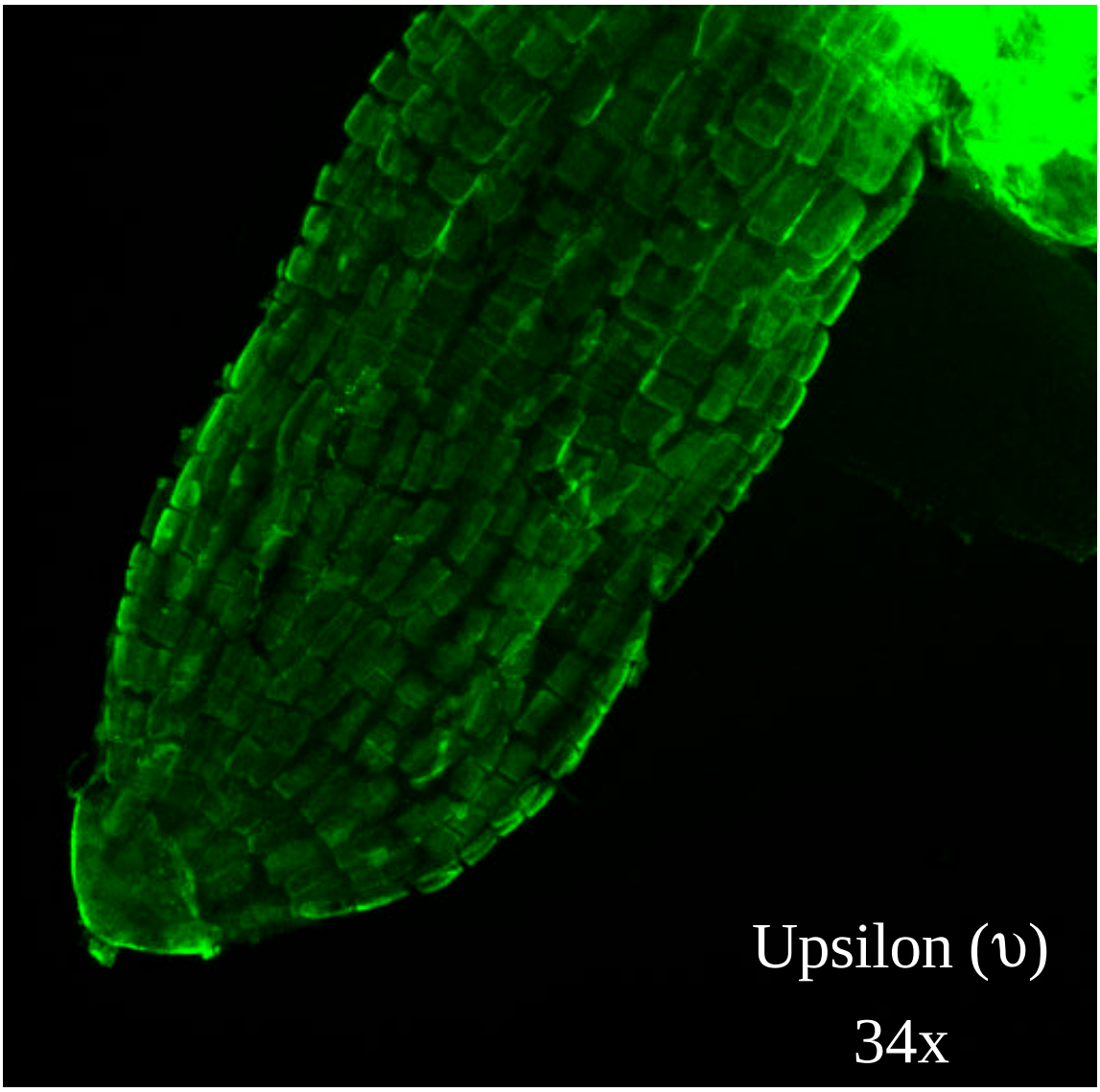


Figure 4-4b. Arabidopsis thaliana root tip treated with a polyclonal antibody to the upsilon isoform. The image was collected using laser scanning confocal microscopy at a magnification of 34x. The localization pattern is predominately cytoplasmic. No subcellular organelles are visible.

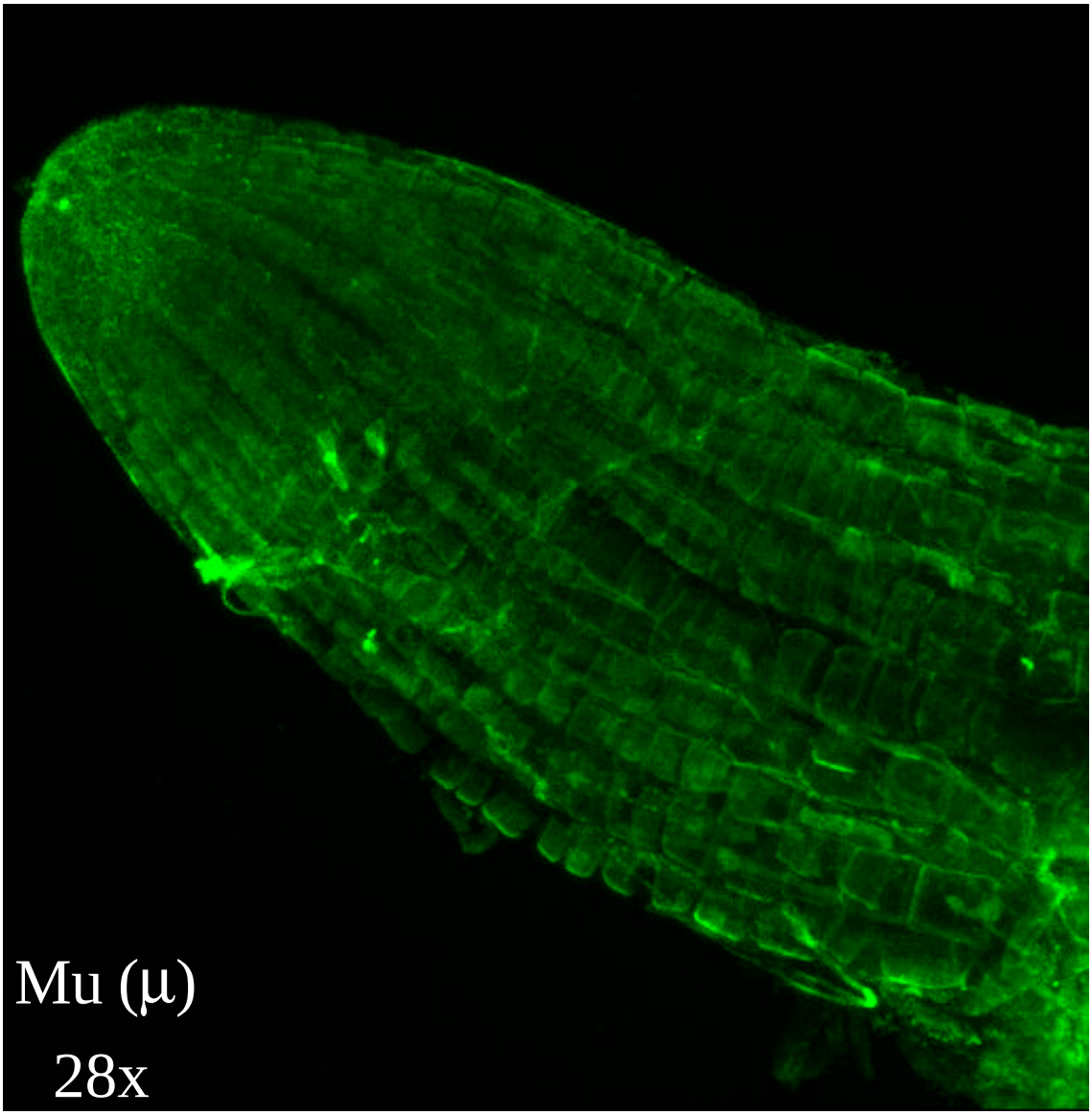


Figure 4-4c. Arabidopsis thaliana root tip treated with a polyclonal antibody to the mu 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 28x. The localization pattern is predominately cytoplasmic. No subcellular organelles are visible.

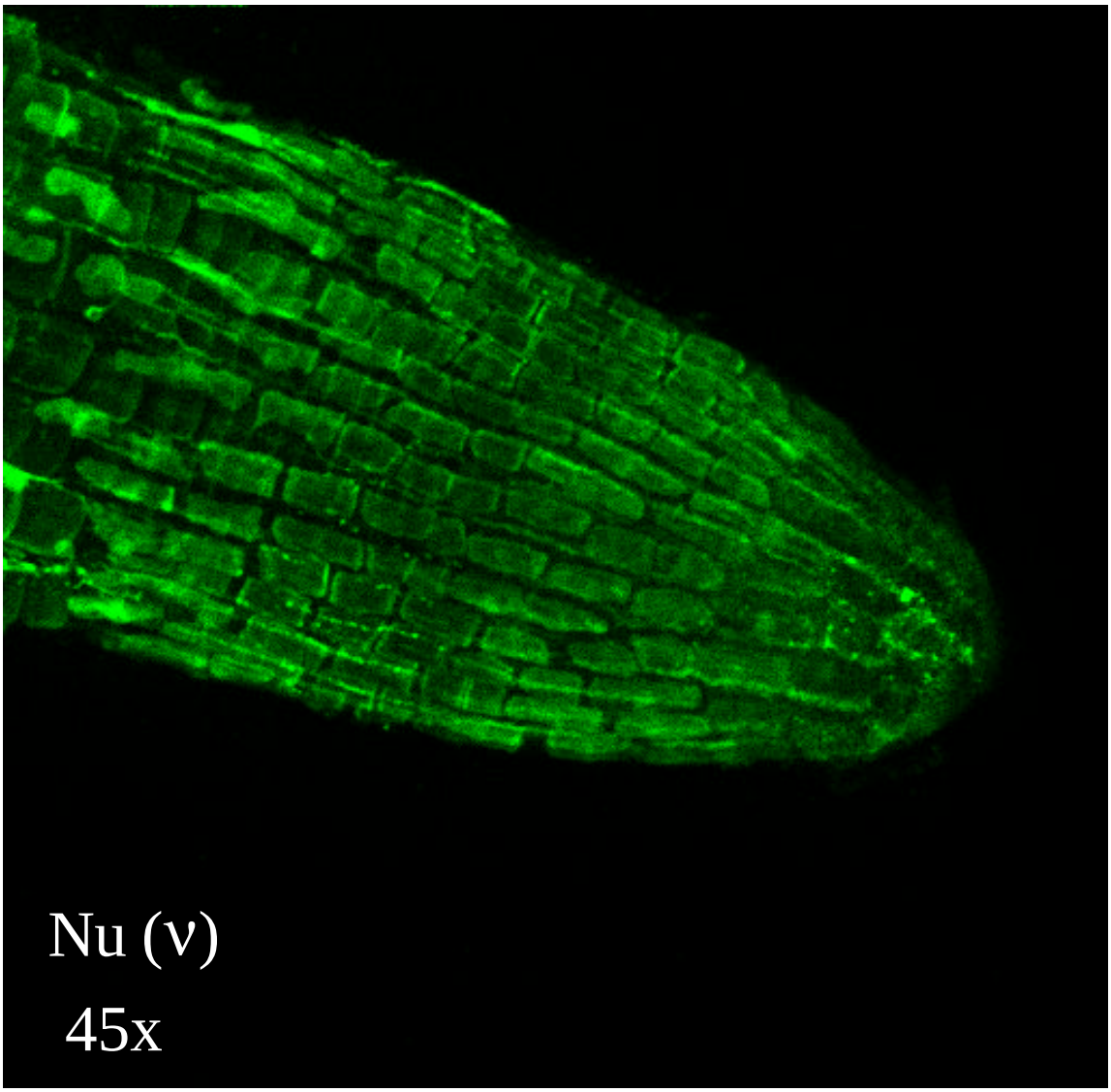


Figure 4-4d. Arabidopsis thaliana root tip treated with a polyclonal antibody to the nu 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 45x. The localization pattern is predominately cytoplasmic. No subcellular organelles are visible.

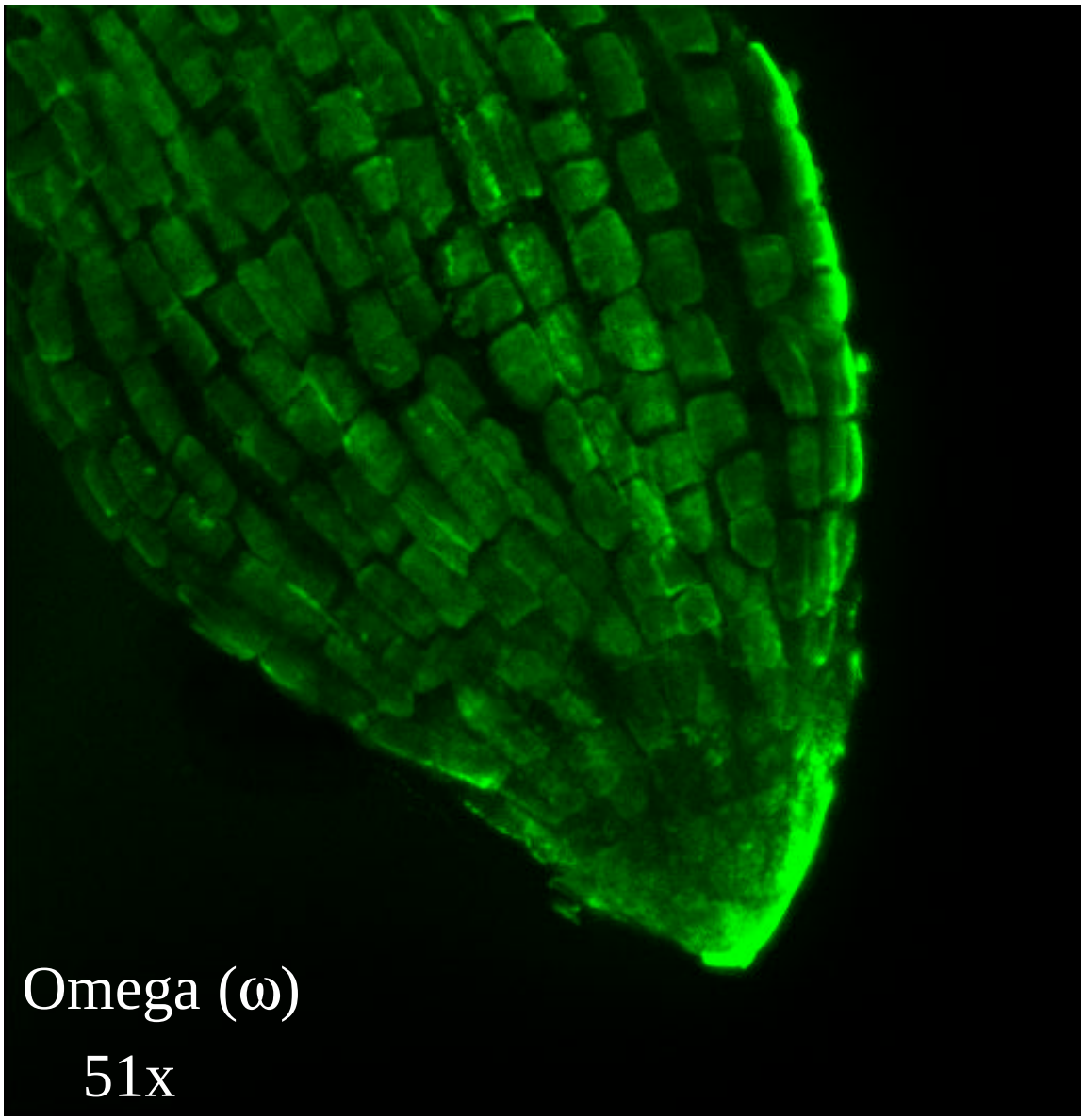


Figure 4-4e. Arabidopsis thaliana root tip treated with a polyclonal antibody to the omega 14-3-3 isoform. The image was collected using laser scanning confocal microscopy at a magnification of 51x. The localization pattern is predominately cytoplasmic. No subcellular organelles are visible.

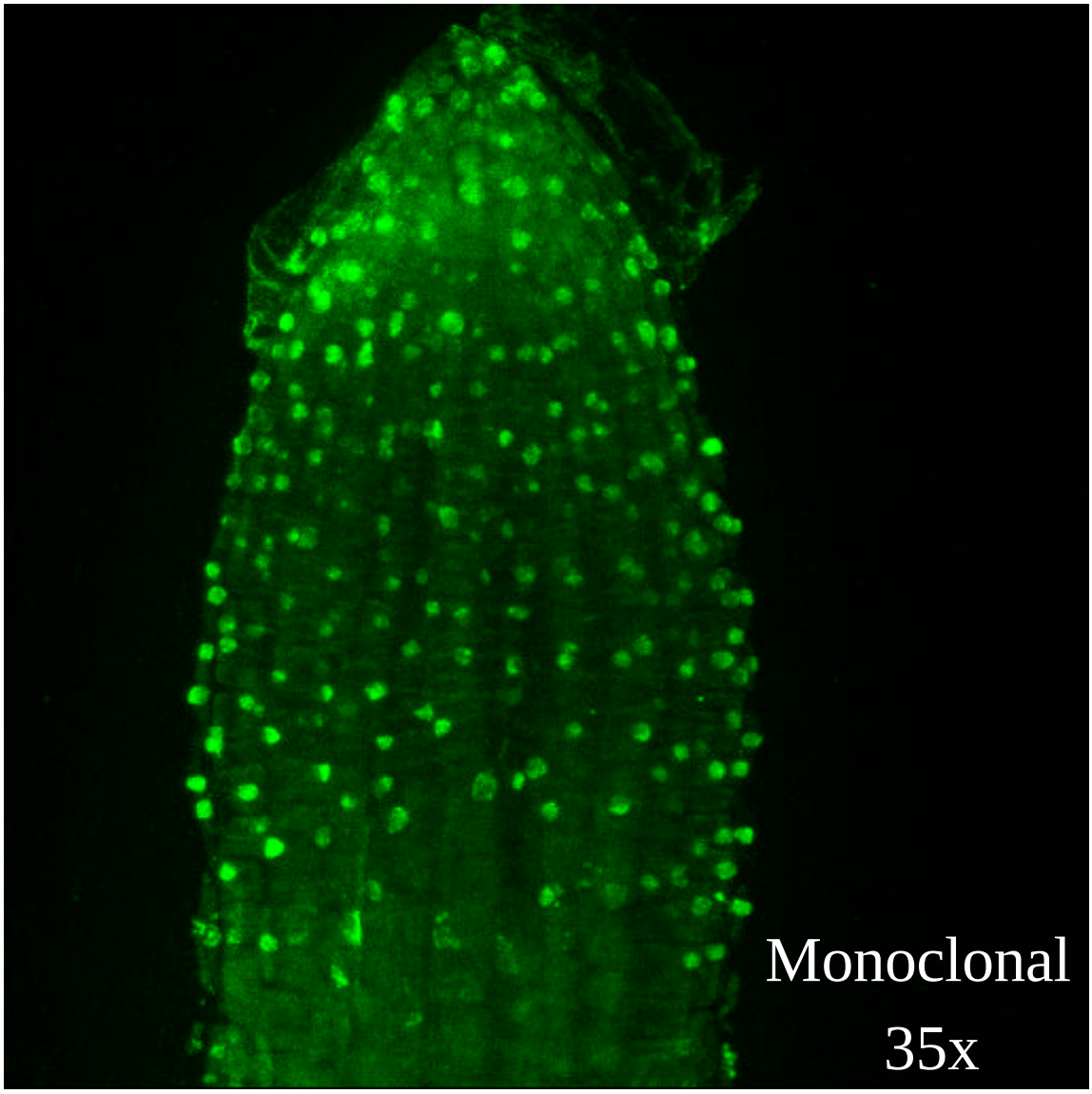


Figure 4-5a. Arabidopsis thaliana root tip treated with a monoclonal antibody recognizing eight out of the ten well-characterized 14-3-3 isoforms (mu and epsilon excluded). The image was collected using laser scanning confocal microscopy at a magnification of 35x. This is a compression image of a larger series of images from a z-series. The localization pattern is predominately nuclear, although some signal is present in the cytoplasm.

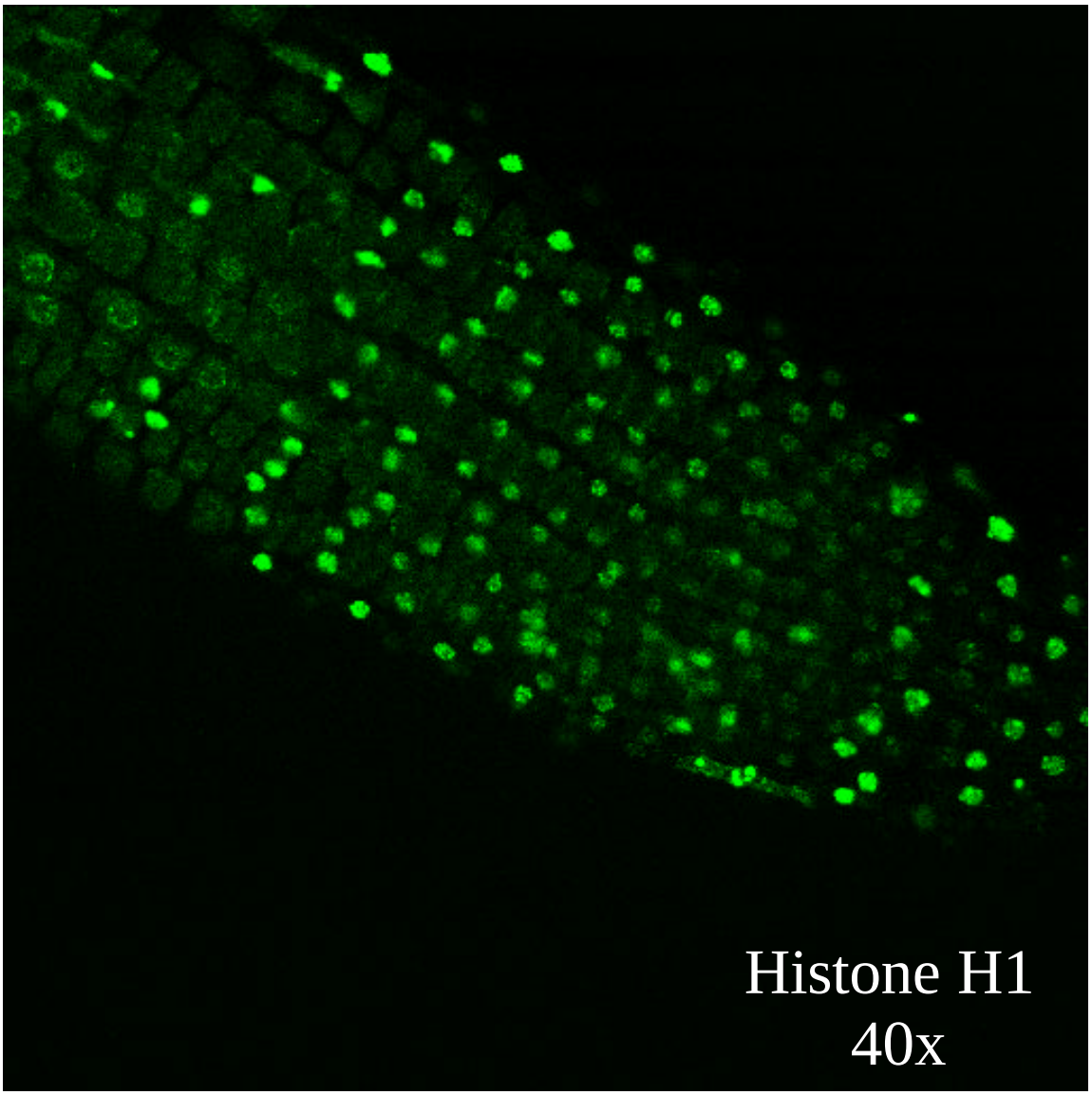


Figure 4-5b. Arabidopsis thaliana root tip treated with a monoclonal antibody to Histone H1. The image was collected using laser scanning confocal microscopy at a magnification of 40x. Histone H1 is located in the nucleus. As expected, the localization pattern is predominately nuclear.

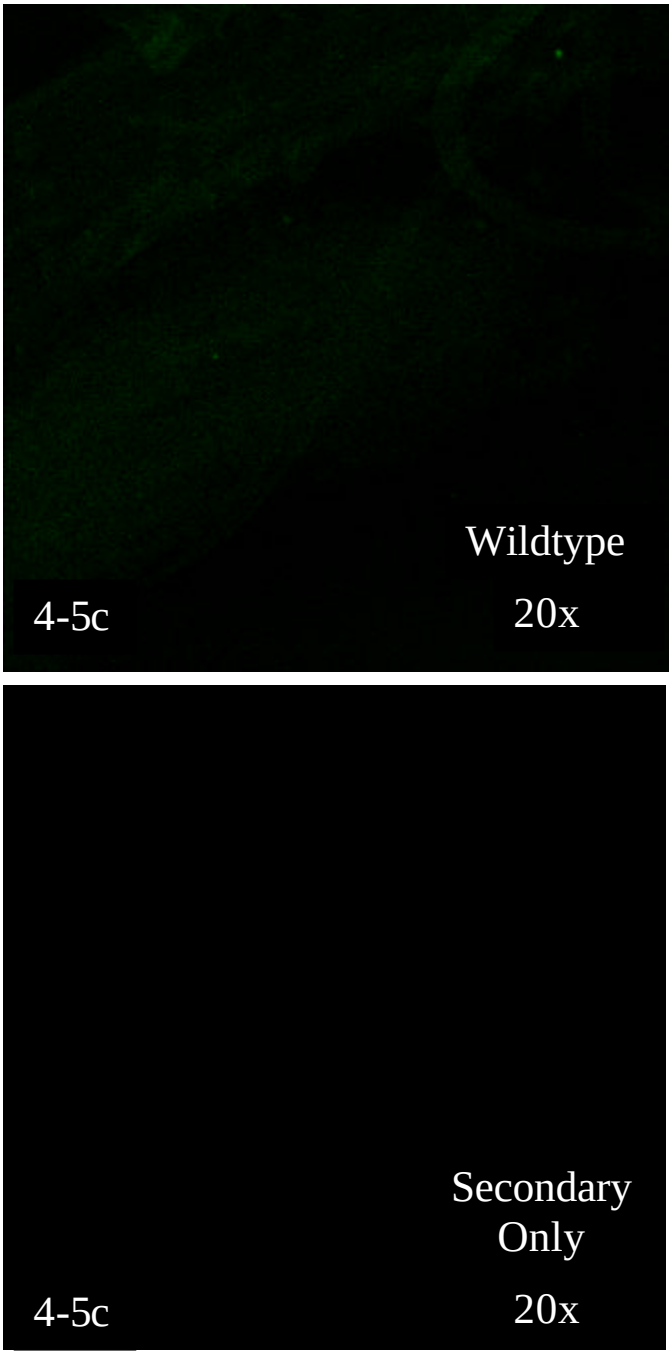


Figure 4-5. Arabidopsis thaliana root tips treated with either no antibody or FITC conjugated secondary antibody only. The images were collected using laser scanning confocal microscopy a magnification of 20x. (a) Wildtype plants with no antibody treatment. (b) Wildtype plants treated with FITC conjugated secondary antibody alone.

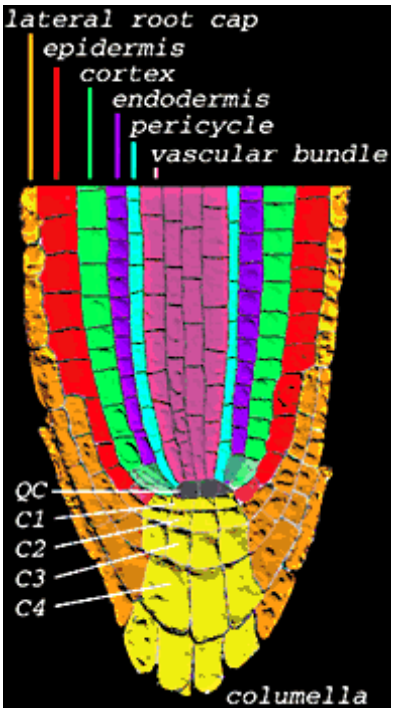


Figure 4-6. Cell layers of an Arabidopsis thaliana root tip. Six cell layers make up the root tip: lateral root cap, epidermis, cortex, endodermis, pericycle, and vascular bundle, form outermost to innermost. Additionally, there are four layers of columella cells immediately in front of the root meristem. This diagram was taken directly from the website of Dr. Jim Haseloff, http://www.plantsci.cam.ac.uk/Haleloff/Arabidopsis/architecture.

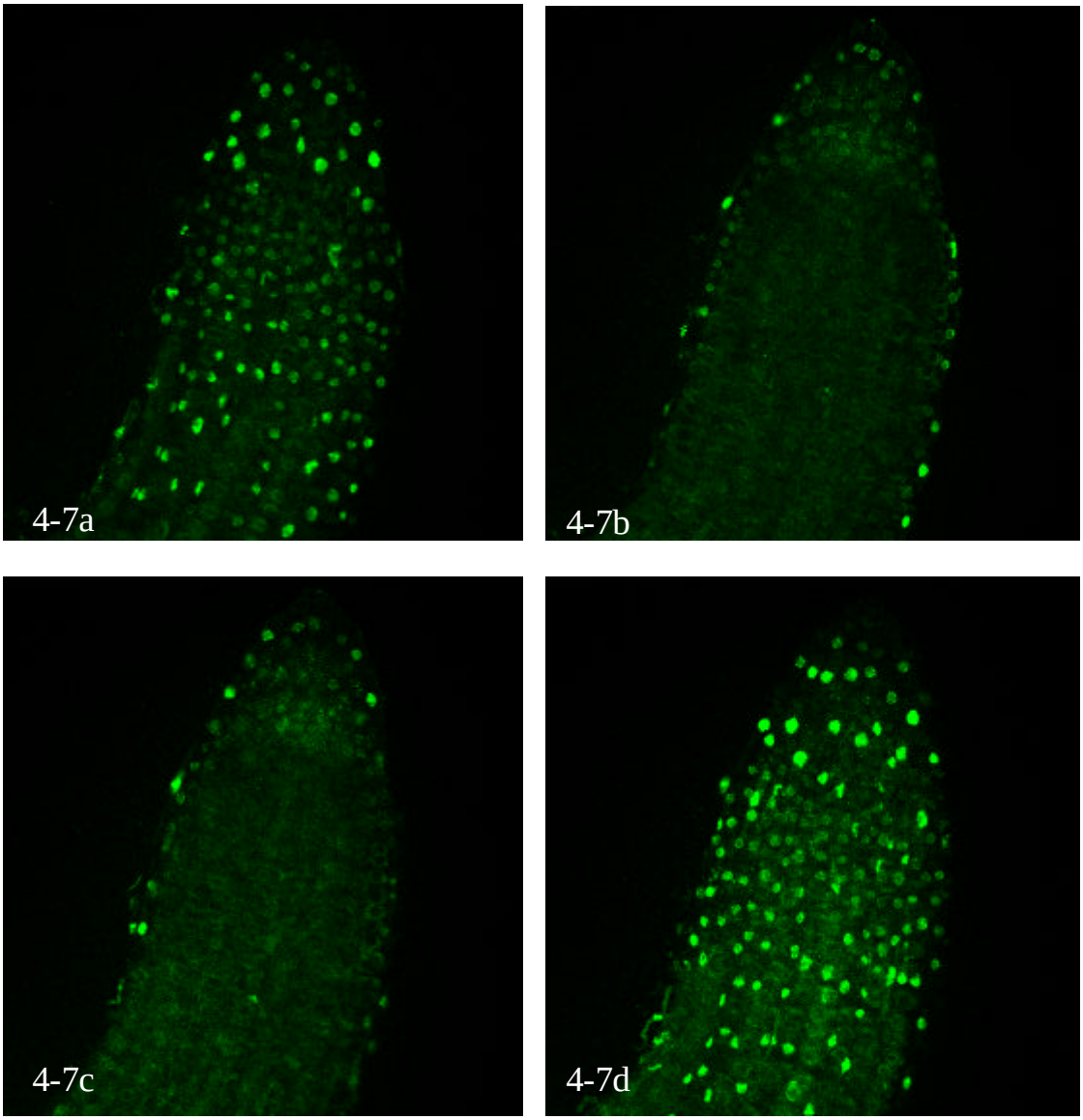


Figure 4-7. Arabidopsis thaliana root tip treated with a monoclonal antibody recognizing eight of the ten well-characterized 14-3-3 isoforms (mu and epsilon excluded). The image was collected using laser scanning confocal microscopy at a magnification of 20x. This is a set of four image taken from a larger z-series through the root tip. The nuclear signal is only present in the first or second cell layers. The lack of signal in the interior cell layers maybe due to inaccessibility of the antibody.

CHAPTER 5
USING GREEN FLUORESCENT PROTEIN FOR 14-3-3 LOCALIZATION

Introduction

The 14-3-3s are a family of acidic, soluble proteins that were initially discovered in 1967 during a study of cytosolic bovine brain proteins (Moore and Perez, 1967). Once thought to be limited to brain tissue, studies over the last fifteen years have proven 14-3-3s to be ubiquitous. The 14-3-3s usually consist of multiple isoforms per organism and have been found in virtually every eukaryotic organism where scientists have looked for them (Robinson et al., 1994).

Once the importance of 14-3-3s was established in the late 1980s, the original mammalian isoforms were re-characterized. There are nine isoforms in mammals and they are designated as Greek letters starting at the beginning of the alphabet (Ichimura et al., 1988). In keeping with that trend, the Arabidopsis thaliana 14-3-3s were named starting at the end of the Greek alphabet as they were discovered. The arabidopsis 14-3-3 family consists of ten well-characterized isoforms and three newly recognized members. The established 14-3-3 isoforms are omega, psi, chi, phi, upsilon, lambda, kappa, mu, nu and epsilon (Lu et al., 1992; Lu et al., 1994b; Wu et al., 1997). The newly recognized members are omicron, rho, and pi (Rosenquist et al., 2000; DeLille et al., 2001).

The functions of 14-3-3s are many. The general theme of protein-protein interaction seems to fit well. 14-3-3s are known to regulate key enzymes in metabolic pathways such as nitrate reductase and sucrose phosphate synthase (Finnie et al., 1999).

Specifically in plants, 14-3-3s are also involved in transcription complexes (Lu et al., 1992). These are just a few of the many known functions of 14-3-3s.

The reason for the large number of 14-3-3 isoforms per organism has always been a key question. Arabidopsis has the largest number of isoforms with 13 and tomato is a close second with 10 members. Is there a specific function for each isoform? Or do the isoforms serve redundant functions and exist in multiple isoforms to insure their presence in every tissue? The question remains largely unanswered.

To try to answer the multiple isoform question, we must know where all the isoforms are located on the subcellular level as well as whole tissue. This chapter attempts to localize seven of the arabidopsis 14-3-3 on the subcellular level using in-frame C-terminal green fluorescent fusions.

Materials and Methods

Oligonucleotide Design

Oligonucleotides were designed to produce a full-length 14-3-3 isoform with the stop codon removed and either an SpeI for the omega isoform or an XbaI restriction site for all other isoforms on both the 5' and 3' ends (Gibco BRL and Integrated DNA Technologies, Inc). The omega isoform used SpeI restriction sites due to the presence of internal XbaI sites in the omega coding region.

Cloning of C-terminal Green Fluorescent Protein Fusions

The coding sequence for the 14-3-3 isoforms phi, psi, kappa, chi, lambda, epsilon, upsilon, omega, and nu were amplified in a polymerase chain reaction (PCR) (1x polymerase buffer, 0.1 mM dNTPs, ~500 ng of DNA template, 3.8 µg/mL primer, and 2.5 U Vent polymerase. The templates for the PCR reactions were the individual 14-3-3

isoform cDNAs in the expression vector, pET-15b, with the exception of omega, which was in pUC18. The PCR product was gel purified using the QIAquick gel extraction kit (Qiagen). The PCR products were then cloned into the pCR-blunt vector (Invitrogen) as an XbaI/XbaI or SpeI/SpeI fragment lacking the stop codon, and transformed into DH10B competent cells for large-scale plasmid preparations. The presence of the isoform in pCR-blunt was confirmed by restriction analysis. Each pCR-blunt vector containing an individual 14-3-3 isoform was restricted using XbaI or SpeI, releasing the entire isoform from the pCR-blunt vector. The proper size fragment was gel purified and further subcloned into pBI12135sGFP (S65T) and transformed into DH10B competent cells. This puts the entire construct under the control of the CaMV 35S promoter and GFP is fused to the C-terminus of the 14-3-3. The pBI12135sGFP (S65T) construct was made by Dr. Paul Sehnke and Michael Manak. The 14-3-3/GFP fusions were subjected to restriction analysis to confirm orientation of the isoforms, and sequence analysis to verify that they were truly fusions. The University of Florida ICBR sequencing core performed all sequence analysis. The 14-3-3/GFP fusions in pBI12135SGFP (S65T) were then transformed into agrobacterium (GV 3101). An additional construct made by Michael Manak includes pBI101GFP (S65T), which is a promoterless construct and serves as a negative control.

Vacuum Infiltration Protocol

This protocol was adapted from the Masters thesis of Elizabeth Bihn, formally of the Ferl lab. Arabidopsis thaliana, ecotype Wassilevskija (WS), seeds were planted in a plastic four pack with soil, covered with screen door mesh and secured with two rubber bands. The pots were placed at 4°C for two days in the dark. The muffins were then

moved to the Apopka room, where they received constant light at 23°C. Once the plants started to germinate, they were thinned down to ~20 plants per muffin. The plants continued to grow until they started to bolt. The bolts were cut everyday for the next 3-4 days with scissors. On the fifth day, the bolts were not cut and vacuum infiltration was performed.

For infiltration, one 500 mL culture of agrobacterium strain GV 3101 per clone (nine total) containing the 14-3-3/GFP fusion were grown in 2YT media (10 g/L yeast extract, 16 g/L tryptone, 5 g/L NaCl, pH 7.3) for several days at 28°C. Cultures were spun down in a centrifuge at 7,000 rpm for ten minutes and re-suspended in ~ 500 mL of infiltration media (2.15 g/L Murashige and Skoog salts, 1mL/L Gamborg's B5 vitamins, 50 g/L sucrose, 10 µL/L of benzylamino purine (1 mg/mL stock), 50 µL Silwet L-77). The suspension was placed in a 600 mL beaker, which was placed inside a vacuum chamber. Plants were placed upside down in the infiltration media suspension and the lid was placed on the chamber. A vacuum was drawn for seven minutes. Plants were removed from the suspension, placed on their sides, covered with saran wrap, and allowed to recover overnight under constant light at 23°C. The next day, the plants were righted, allowed to grow normally and set seed. The seed was plated onto MS media plates (4.4 g/L Murashige and Skoog salts, 0.5 g/L MES, 1 mL 1000X Gamborg's B5 vitamins, pH 5.7 with KOH, 4.0 g/L phytagel) containing carbenicillin (250 mg/L) and kanamyacin sulfate (50mg/L) to kill agrobacteria and select for transformants, respectively.

Protein Extraction and Western Blot Analysis

Fresh tissue (100-200 mg) was used in protein extractions. The tissue was placed into a pre-chilled 1.5 mL tube with equal volume of cold extraction buffer (50 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20, 25 mM NaF, 0.1 mM EDTA). Tissue was ground with a polytron grinder at a setting of forty for two minutes and immediately placed back on ice. Samples were centrifuged at 14,000 rpm for five minutes at 4°C. 60 µL of supernatant was removed and an equal volume of pre-warmed 2x SDS sample buffer was added. All samples were heated in boiling water for two minutes. 30 µL of sample was loaded per lane and subject to 10% SDS-PAGE. The proteins were electro-transferred to a nitrocellulose membrane and taken through a standard Western-blotting procedure. Rabbit anti-GFP (Santa Cruz Biotechnology) was used as a primary antibody at a dilution of 1:400 and polyclonal 14-3-3 antibodies were used at either 1:1000 or 1:2000. Goat anti-rabbit horseradish peroxidase was used as secondary antibody at a dilution of 1:1500.

Whole Mount Sample Preparation

Transgenic seeds were surface sterilized and plated onto MS media. After two days at 4°C in the dark, the seedlings were placed under constant light at 23°C and grown vertically for 3-7 days. The entire plant was removed from the media plate and placed on a regular microscope slide with a drop of water and coversliped. Some samples were treated with propidium iodide (10 µg/mL) for ten minutes to stain cell walls.

Laser Scanning Confocal Microscopy and Image Analysis

Plant tissue samples were examined with an Olympus IX70 inverted microscope mounted to a Bio-Rad MRC 1024ES laser scanning confocal microscope with a

krypton/argon laser. Collected images were Kalman averaged four to five times. The images were collected using the Laser Sharp software package (Bio-Rad). The images were transferred to a PC and cataloged using Thumbs Plus 4 (Cerious software). Confocal Assistant 4.02 (Bio-Rad) was used to view collected z-series files and convert files from a .pic (Bio-Rad format) to an .avi format suitable for Windows viewing of z-series.

Results

The individual 14-3-3 isoform coding sequences for omega, chi, psi, phi, upsilon, lambda, kappa, epsilon, and nu were amplified using PCR based methods. All of the primers were designed to remove the stop codons from each isoform and to put an XbaI restriction site on the 5' and 3' ends. Omega used a SpeI restriction site due to internal XbaI sites within the omega coding region (Table 5-1). The nine isoforms were blunt end cloned into the pCR-blunt vector (Invitrogen). The isoforms were then restricted using either XbaI or SpeI, thus releasing the entire isoform. The 14-3-3s were then cloned into the vector pBI12135SGFP (S65T). The result is an individual 14-3-3 isoform C-terminally fused in-frame with GFP. The constitutive CaMV 35S promoter drives the expression of the fusion construct. A graphical representation of the fusion construct is presented in Figure 5-1. All nine isoforms were successfully fused in this manner as confirmed by sequence analysis of the 14-3-3/GFP junction.

The pBI12135S14-3-3GFP (S65T) fusion constructs were transformed into agrobacterium for plant transformation. Arabidopsis thaliana, ecotype Wassilevskija (WS), was transformed with each of the nine 14-3-3 constructs. The transgenic plants were allowed to set seed and were ultimately taken through at least two generations.

Two of the constructs, chi and psi, did not express GFP in any of the R_0 seed. The 14-3-3/GFP fusions themselves were verified by sequence analysis. Therefore, it is my opinion that the transformation process itself was not successful. The other remote possibility is that they are undergoing gene silencing. However, this is unlikely given the nature of the 35S promoter. Re-transformation of these constructs is planned in future experiments.

Western blot analysis was performed on the remaining seven 14-3-3/GFP fusions. Whole plant tissue was used in a protein extraction protocol found in materials and methods. The protein was subjected to a 10% SDS-PAGE and further subjected to electro-transfer to a nitrocellulose membrane. The membrane was incubated with a rabbit anti-GFP antibody, and a goat-anti-rabbit secondary antibody conjugated to horseradish peroxidase. The results of the western can be seen in Figure 5-2a. All of the isoforms have a band present at ~55 kDa. GFP is ~27 kDa and the 14-3-3 proteins are between 27-30 kDa. Therefore, you would expect a protein of ~55 kDa if they were true fusions. In a separate experiment, the same procedure was followed and the membrane was incubated with 14-3-3 polyclonal antibodies, kappa, omega, lambda, phi, epsilon, upsilon, and nu. The membrane was secondarily incubated with goat-anti-rabbit horseradish peroxidase antibody. The results of the Western can be seen in Figure 5-2b. Two distinct bands are visible for omega, epsilon, upsilon, and nu isoforms. The lower band is endogenous 14-3-3 and is ~27-30 kDa. The upper band at ~55-60 kDa is the 14-3-3/GFP fusion. A single band at ~55 kDa is present for the lambda isoform and a faint band is visible for the kappa isoform. There are no visible bands present for the phi isoform. Figure 5-2c is a Western blot analysis of wildtype plants, plants transformed

with pBI12135SGFP (S65T) only and plants transformed with epsilon/GFP and kappa/GFP fusion constructs. Rabbit anti-GFP was used as the primary antibody. No bands are visible for the wildtype lane. A band at ~27 kDa is visible for the GFP only lane and a band at ~55 kDa is visible for both the epsilon/GFP and kappa/GFP lanes. The amount of tissue used was not consistent between constructs and no effort was made to load the samples equally in either experiment. The Western blots were used simply to demonstrate that the plants were transformed with GFP and that they were fused to a 14-3-3.

Three to four lines per construct were examined to confirm that there were no major differences in localization due to position effects. Normally many more lines per construct would be used, however GFP and kanamyacin selection allow fewer lines to be examined. False-positives can be eliminated by visual inspection of GFP expression in a large number of plants. Transgenic plants were grown vertically on MS media under constant light for 3-7 days. The plants were removed from the media plates and mounted in water and viewed with a laser scanning confocal microscope. The seven 14-3-3/GFP fusions demonstrate variable locations within the cell.

Epsilon/GFP is localized predominately to the nuclear envelope, plasma membrane and cytoplasm. Figure 5-3a shows cells at the root tip. A small ring of brighter fluorescence can be seen wrapped around the nucleus and is thought to be the nuclear membrane. No signal can be seen in the nucleolus and only a slight signal, compared to the rest of the cell, can be seen in the nucleus. Figure 5-3b is a magnified view of cells not at the true root tip, but further up the root. In these cells, a clear ring around the nucleus can be seen and also structures streaming from the nucleus can be

seen. These streaming structures could be endoplasmic reticulum or golgi apparatus, however the data is difficult to interpret. Again, no signal is present in the nucleolus. Figure 5-4 shows single images taken from a larger series of images through a root tip expressing the epsilon/GFP fusion. A contrast can be seen between the cells of the true root tip versus cells higher up the root. The cells higher up are elongated and appear to be less cytoplasmic and more vacuolated. The cells at the true root tip appear to be less vacuolated and more cytoplasmic in nature. However, the location of the epsilon/GFP signal within the cell does not change.

Kappa/GFP is predominately localized to the nucleus, plasma membrane and cell wall. A small amount of fluorescence can be seen in the cytoplasm as well. Figure 5-5a is a compressed image of a complete z-series through the root sample. In short, the Laser Sharp software takes all of the individual images (65 images in this series) and combines them into one complete image. The result is a three-dimensional looking image. Figure 5-5b is a higher magnification of a root tip expressing kappa/GFP. There is no signal in the nucleolus and no signal in what appear to be vacuoles. Figure 5-6a is a root tip expressing kappa/GFP using the FITC filter set. Figure 5-6b is the same root tip that has been counter stained with propidium iodide and viewed using the Texas Red filter set. This stain predominately labels the cell walls, however longer incubation times allow the nuclei to become labeled. Figure 5-6c is a dual image of the green filter and the red filter combined. The result is overlap of the fluorescence of GFP and the propidium iodide in what appears to be the cell wall, which creates a yellow color. Figure 5-7 is a series of single images taken from a larger z-series that shows the progression through the root tip. The fluorescence in the nucleus, plasma membrane and cell wall do not change.

Figure 5-8a is of lambda/GFP showing localization in the nucleus, plasma membrane and cytoplasm. Figure 5-8b is a higher magnification of the same sample. The nucleoli as well as the vacuoles appear to be absent of any fluorescence. There are not any subcellular structures that are more intensely labeled than another with this isoform. The fluorescent signal appears to be evenly distributed among the structures. Figure 5-9 is a series of four images taken from a more detailed z-series. These images are from a different sample than those of Figure 5-8. The overall fluorescence is higher in Figure 5-9, however the ratio of fluorescence among subcellular structures remains the same.

Figure 5-10a is of omega/GFP demonstrating localization in the cytoplasm, plasma membrane, and nucleus. Again, signal is absent from the nucleolus and what appear to be vacuoles. Figure 5-10b is a higher magnification of a different sample expressing omega/GFP. This figure shows what could be endoplasmic reticulum or the golgi apparatus expressing GFP as well. Figure 5-10c represents the true root tip extremely well. The columella root cap and meristem are clearly visible.

Phi/GFP is predominately localized to the cytoplasm, nuclear membrane and plasma membrane (Figure 5-11a). However, slight fluorescence can also be seen in the nucleus. Structures that appear to be vacuoles and the nucleolus lack fluorescence in Figure 5-11a. Figure 5-11b is a higher magnification image of a different sample expressing phi/GFP. Other unknown structures are also labeled and visible in Figure 5-11b. However, without a cellular marker, it is difficult to identify them with any level of confidence. Figure 5-12 is a series of five images taken from a larger, more detailed z-series through the root tip. The first image, Figure 5-12a, starts high and the last image,

Figure 5-12g, ends at the true root tip. The common theme of elongated, less cytoplasmic cells higher up the root tip, proceeding to heavily cytoplasmic cells at the true root tip is demonstrated well here.

Upsilon/GFP is also predominately localized to the cytoplasm, nuclear membrane and plasma membrane (Figure 5-13a). A higher magnification of a different sample expressing upsilon/GFP can be seen in Figure 5-13b. Again, fluorescent signal is absent from the nucleolus and what appear to be vacuoles. A slight fluorescent signal can be seen in the nucleus, but it is not as intense as the signal in the cytoplasm. Figure 5-14 is a series of four images taken from a larger z-series. These images show the progression through a root tip. The GFP signal does not change location in the cytoplasm, nuclear membrane, or plasma membrane. As the images progress through the root tip, many unknown cellular structures show fluorescence.

Nu/GFP is predominately localized to the cytoplasm, plasma membrane and nuclear membrane (Figure 5-15a). Figure 5-15b represents a higher magnification of Nu/GFP. The nucleolus is void of any signal and the nucleus is only slightly fluorescing. Several unknown structures are also visible with this isoform as well. Figure 5-16 is a series of four images taken from a larger series of images through the root tip. As the images progress, no major changes in localization can be seen.

Plants were also transformed with the pBI12135SGFP (S65T) vector with no 14-3-3 present as a control. These plants demonstrate localization largely in the nucleus and some signal is present in the cytoplasm (Figure 5-17a). Signal is absent from the nucleolus and appears to be absent from the vacuoles. A vector was also transformed into arabidopsis that contains GFP (S65T), but lacks a promoter, pBI101GFP. An image

could not be acquired although the gain and iris were maximized at the time of image capture (Figure 5-17b). Finally, wildtype arabidopsis is used as a control for autofluorescence (Figure 5-17c). Again, the iris and gain were maximized, but no image was visible.

Discussion

Nine constructs were made C-terminally fusing an individual Arabidopsis 14-3-3 member to green fluorescent protein. The successfully fused 14-3-3s are kappa, omega, lambda, phi, epsilon, upsilon, nu, chi, and psi. All fusion constructs were verified by sequence analysis before being transformed into agrobacterium. All nine constructs were subsequently transformed into arabidopsis. Upon screening the R_0 seed, no visible fluorescence was seen for the chi and psi isoforms, therefore they were dropped from further study. Chi and psi will be re-transformed into arabidopsis at a later date.

Western analysis of the remaining seven constructs revealed some interesting results. All seven isoforms reveal a proper size band at ~55 kDa when treated with anti-GFP antibody. In the blot treated with the 14-3-3 antibodies, the results of omega, epsilon, upsilon, and nu are exactly as expected. The specific 14-3-3 antibody recognizes two different size bands, the endogenous 14-3-3 and the 14-3-3/GFP fusion. The lambda, kappa, and phi 14-3-3 antibodies provide a much different result. A single band is visible for the kappa and lambda isoforms at ~55 kDa. However, no endogenous 14-3-3 is visible. This result could be an indication that the endogenous kappa and lambda isoforms are in low abundance. There is not an endogenous or fusion band visible for the phi isoform. Multiple attempts to show this isoforms presence, using the 14-3-3 antibody, have failed. Varying concentration of antibody and tissue amount has had no

effect. This is curious considering a clear band is visible at the proper size when treating with the anti-GFP antibody. The lack of both an endogenous and fusion band lead me to believe there is a problem with the antibody recognizing the 14-3-3, rather than a problem with the 14-3-3/GFP fusion. Finally, it is important to show that the GFP antibody is specific. No bands are visible for wildtype plants using anti-GFP antibody. Additionally, a size shift can be seen between plants transformed with pBI12135SGFP (S65T) only and those fused to a 14-3-3 as in Figure 5-2c.

The 14-3-3/GFP fusions demonstrate a dynamic localization pattern in the root tip of Arabidopsis. Table 5-2 is a summary of the predominate localization patterns for seven of the 14-3-3/GFP isoform fusions. The location is ordered from strongest to weakest. The isoforms appear to localize along evolutionary lines in some instances. For example, the kappa and lambda isoforms make up one sub-branch of the non-epsilon isoforms in a neighbor-joining parsimony tree. Furthermore, both the kappa and lambda isoforms appear to be present strongly in the nucleus and plasma membrane/cell wall. No other isoforms have this particular localization pattern. This could point to a specific and evolutionarily conserved role for these two isoforms. Also, the presence of the kappa isoform in the cell wall is a unique finding, in that no other arabidopsis 14-3-3 has been localized to the cell wall. This result, however, is not conclusive. An argument could be made that although the signal appears to be yellow, it could because the green in the plasma membrane is so close to the red of the cell wall. Clearly, this result needs to be investigated further.

The remaining five isoforms have a similar localization pattern among themselves, although there is some variance. For example, the epsilon isoform looks

predominately localized to the nuclear envelope and to a lesser degree in the plasma membrane and cytoplasm. The phi isoform is predominately localized to the cytoplasm, however, it is also localized to the nuclear membrane and plasma membrane to a lesser extent. However, evidence is present of these isoforms falling along evolutionary lines as well. Upsilon and nu are on the same sub-branch in a neighbor- joining parsimony tree and they also have very similar localization patterns. Both are predominately localized to the cytoplasm and to a lesser extent the nuclear membrane and plasma membrane. Although not as dramatic as lambda and kappa, these isoforms could also be localized according to evolutionary relatedness.

The GFP fusions provide a unique look at 14-3-3 localization within the cell. The GFP fusions also provide a valuable tool for future studies. Other tissues within the plants are clearly expressing the 14-3-3/GFP fusion and need to be examined in more detail. These seven constructs can also be used in future environmental stress experiments to examine changes in 14-3-3 localization. Further, native promoters can easily replace the current constitutive promoter for in-depth studies.

Table 5-1. Primers used to amplify the individual 14-3-3 isoforms from expression vectors. Each primer has an XbaI restriction site incorporated into the 5' and 3' ends, with the exception of omega which uses SpeI. Additionally, the primers were designed to remove the stop codon at the 3' end of each isoform.

Epsilon	Forward: 5’ GCTCTAGAACAATGGAGAATGAGAGGGAAAAGCAG 3’
	Reverse: 5’ TCTAGAGTTCTCATCTTGAGGCTCATCAGCACC 3’
Kappa	Forward: 5’ GCTCTAGAACAATGGCGACGACCTTAAGCAGA 3’
	Reverse: 5’ TCTAGAGGCCTCATCCATCTGCTCCTGCATATC 3’
Lambda	Forward: 5’ GCTCTAGAACAATGGCGGCGACATTAGGC 3’
	Reverse: 5’ TCTAGAGGCCTCGTCCATCTGCTCCTGCAT 3’
Omega	Forward: 5’ GCACTAGTACAATGGCGTCTGGGCGTGAA 3’
	Reverse: 5’ ACTAGTCTGCTGTTCCTCGGTCGGTTTTGG 3’
Psi	Forward: 5’ GCTCTAGAACAATGTCGACAAGGGAAGAGAATG 3’
	Reverse: 5’ TCTAGACTCGGCACCATCGGGCTTTGATGC 3’
Nu	Forward: 5’ GCTCTAGAACAATGTCGTCTTCTCGGGAAGAG 3’
	Reverse: 5’ TCTAGACTGCCCTGTCTCAGCTGGTTTCCC 3’
Phi	Forward: 5’ GCTCTAGAACAATGGCGGCACCACCAGCA 3’
	Reverse: 5’ TCTAGAGATCTCCTTCTGTTCTTCAGCAGG 3’
Upsilon	Forward: 5’ GCTCTAGAACAATGTCTTCTGATTCGTCCCGG 3’
	Reverse: 5’ TCTAGACTGCGAAGGTGGTGGTTGGGC 3’
Chi	Forward: 5’ GCTCTAGAACAATGGCGACACCAGGAGCTT 3’
	Reverse: 5’ TCTAGAGGATTGTTGCTCGTCAGCGGGTTT 3’

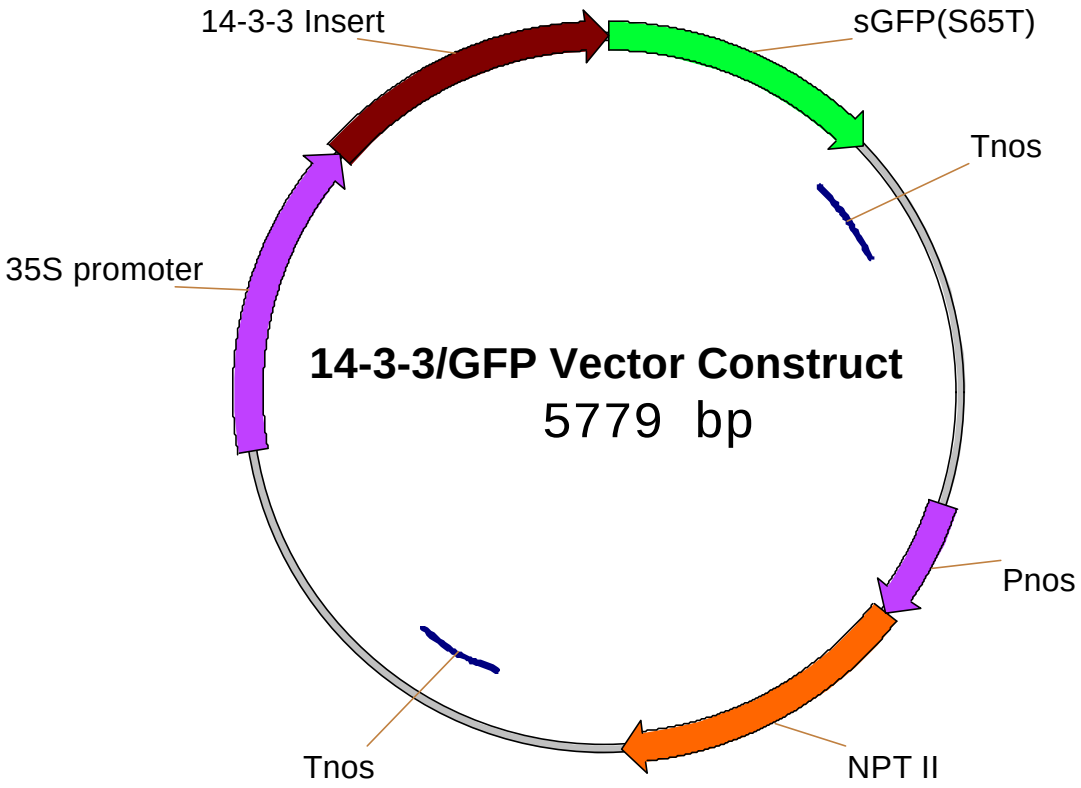


Figure 5-1. General representation of the completed Arabidopsis thaliana 14-3-3/GFP fusion constructs. Nine individual Arabidopsis thaliana 14-3-3 isoforms were fused to green fluorescent protein in this manner. The constitutive Cauliflower Mosaic 35S promoter drives the expression of the fusion constructs. The vector also contains the Kanamyacin resistance gene (NPT II).

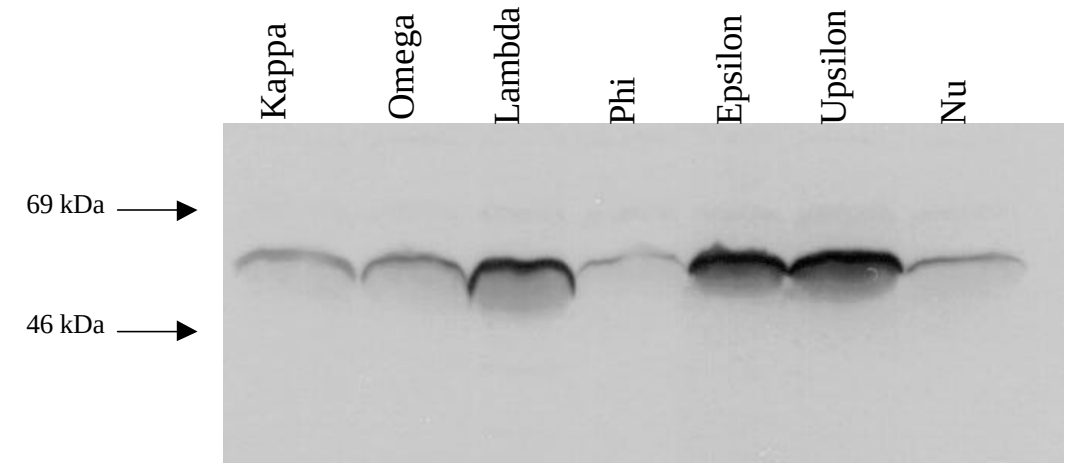


Figure 5-2a

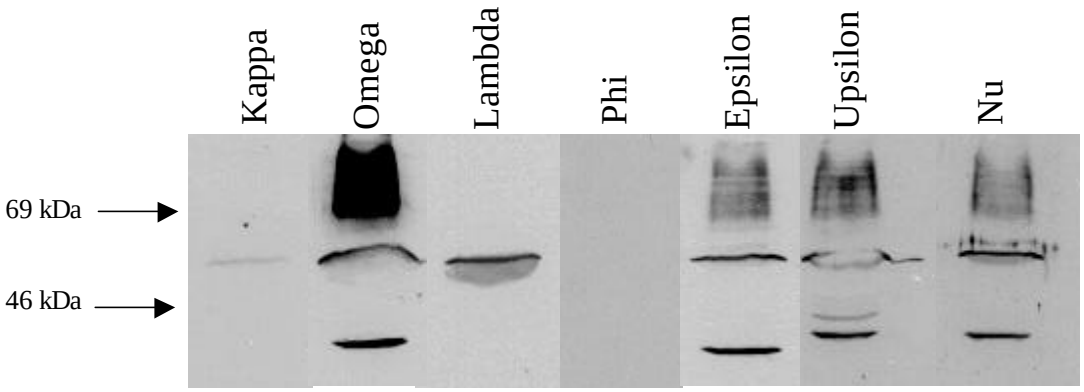


Figure 5-2b

Figure 5-2. Protein extraction and Western blot analysis. (a) Proteins were extracted from whole plant tissue and subjected to SDS-PAGE. Proteins were electro-transferred to a nitrocellulose membrane and treated with anti-GFP antibody. A single band at ~55 kDa is visible for all seven constructs demonstrating that GFP is fused to another protein (14-3-3). (b) A similar procedure was followed as in (a). The nitrocellulose membrane was treated with polyclonal antibodies to the Arabidopsis thaliana 14-3-3s. A band at ~55 kDa can be seen for all constructs except phi. Additionally, the isoforms omega, epsilon, upsilon, and nu have a band at ~30 kDa. This lower band is endogenous 14-3-3. No bands are present for the phi isoform.

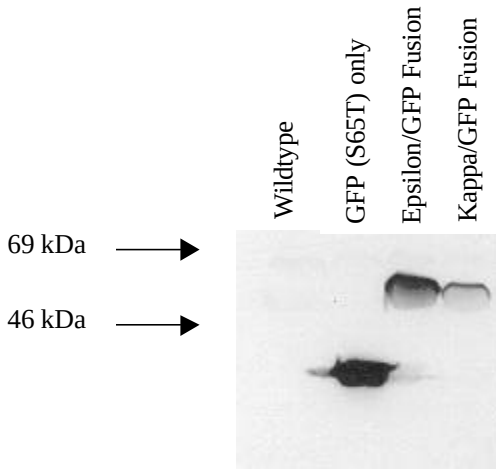
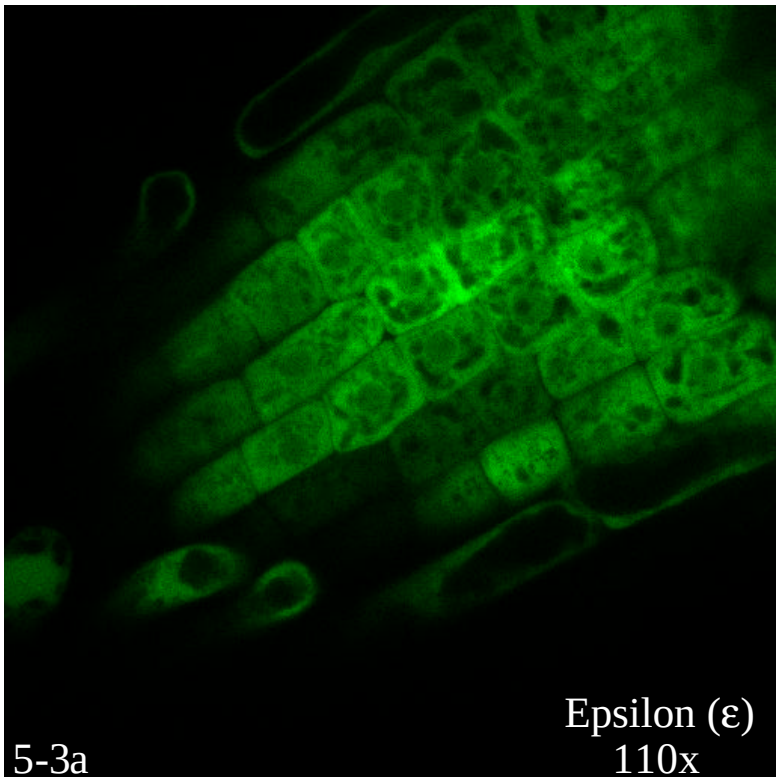
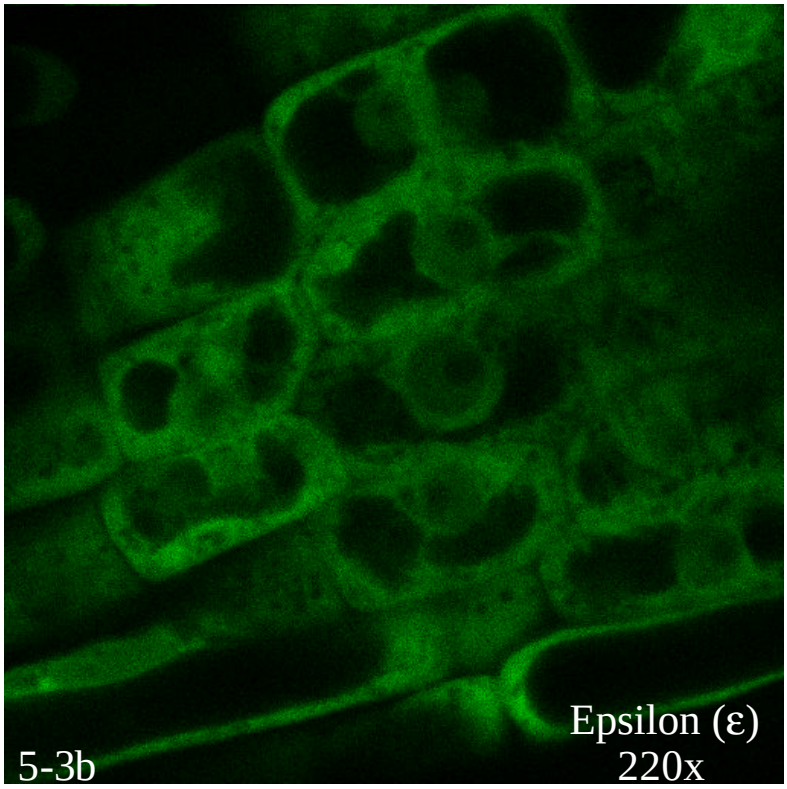


Figure 5-2c. Protein extraction and Western blot analysis. Proteins were extracted from whole plant tissue and subjected to SDS-PAGE. Proteins were electro-transferred to a nitrocellulose membrane and treated with anti-GFP antibody. No bands are present for the lane containing wildtype plants. A single band at ~27 kDa is visible for the lane labeled "GFP (S65T) only." Finally a single band at ~55 kDa can be seen for both epsilon/GFP and kappa/GFP fusion constructs.

Figure 5-3. Epsilon/GFP fusion construct demonstrates localization predominately in the nuclear envelope, plasma membrane and cytoplasm. No signal can be seen in the vacuoles or nucleolus. (a) Image of root tip at 110x magnification. (b) Image of cells further up the root tip at 220x magnification.


Epsilon (ε)
110x
5-3a


Epsilon (ε)
220x
5-3b

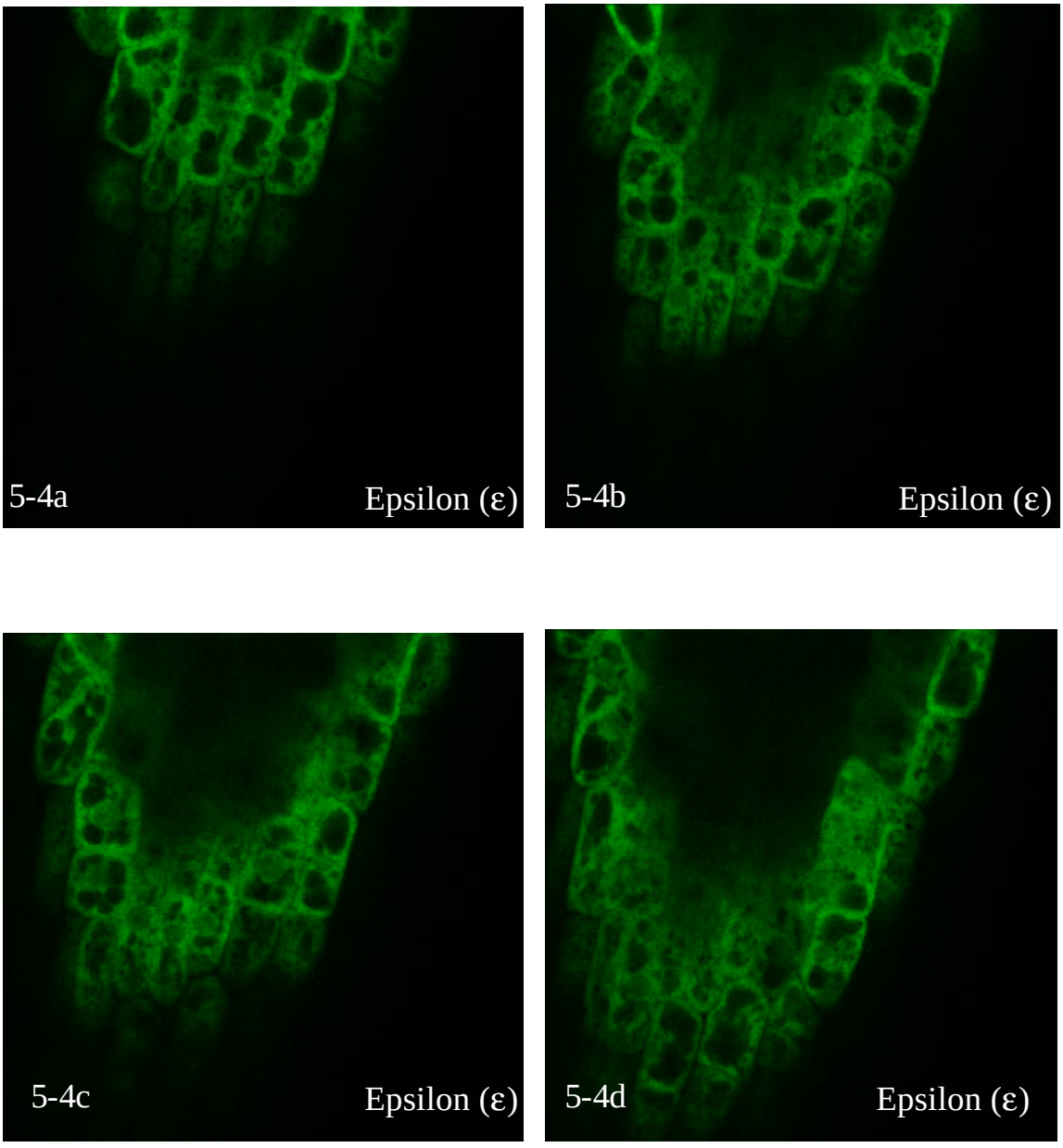
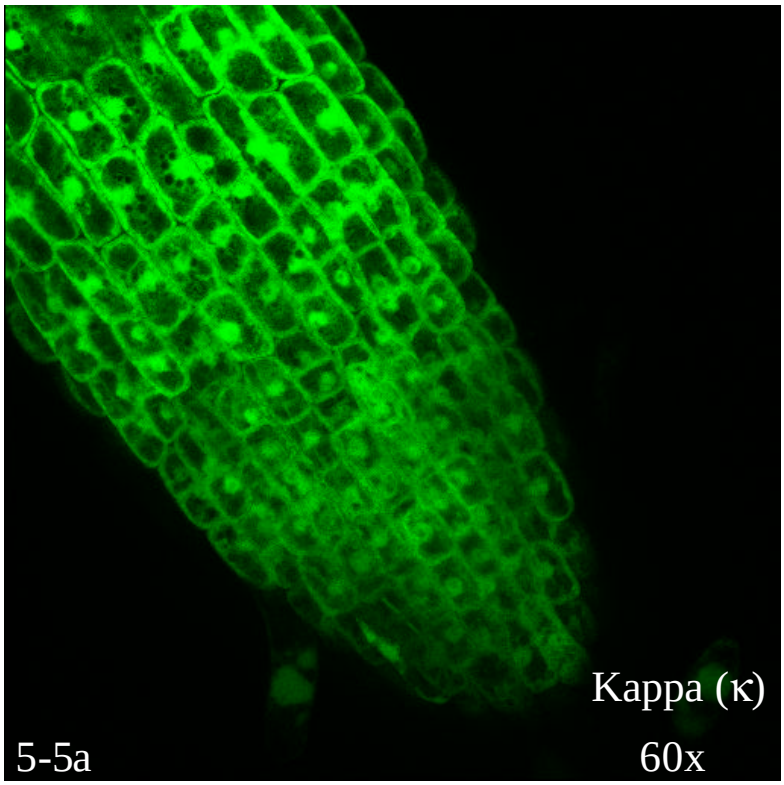
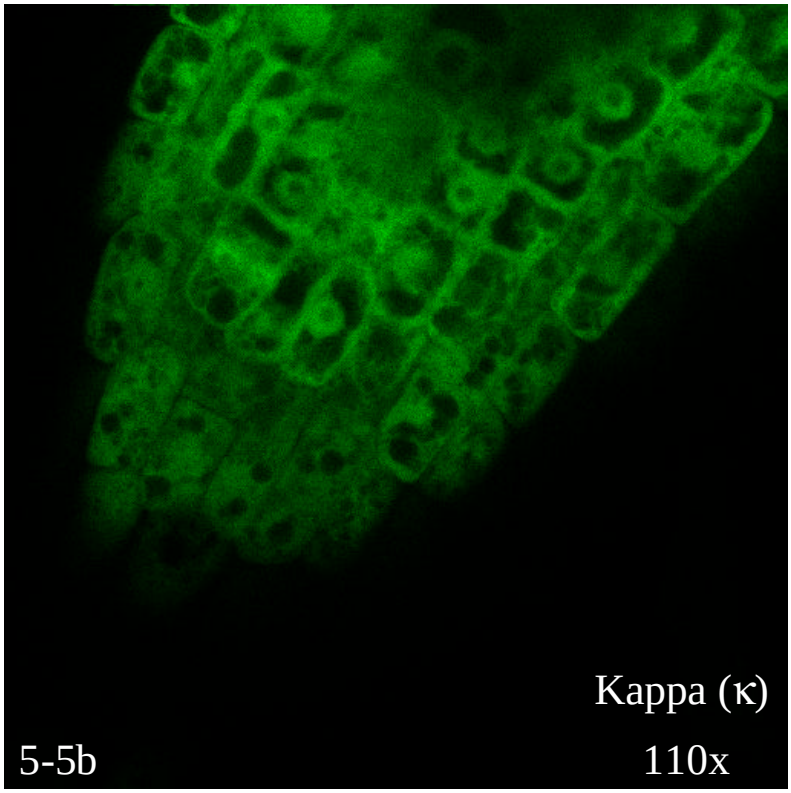


Figure 5-4. Series of four images taken from a larger z-series of epsilon/GFP. The images show that cells distal to the root tip are elongated and less cytoplasmic, however that cells at the true root tip are more cytoplasmic. The images are at 115x magnification.

Figure 5-5. Kappa/GFP fusion construct demonstrates localization predominately in the nucleus, plasma membrane, and cell wall. No signal can be seen in the vacuoles or nucleolus. (a) A compressed image of a complete z-series through the root tip at 60x magnification. (b) A single optical slice of cells in the root tip of the same plant at 110x magnification.


Kappa (κ)
5-5a
60x


Kappa (κ)
5-5b
110x

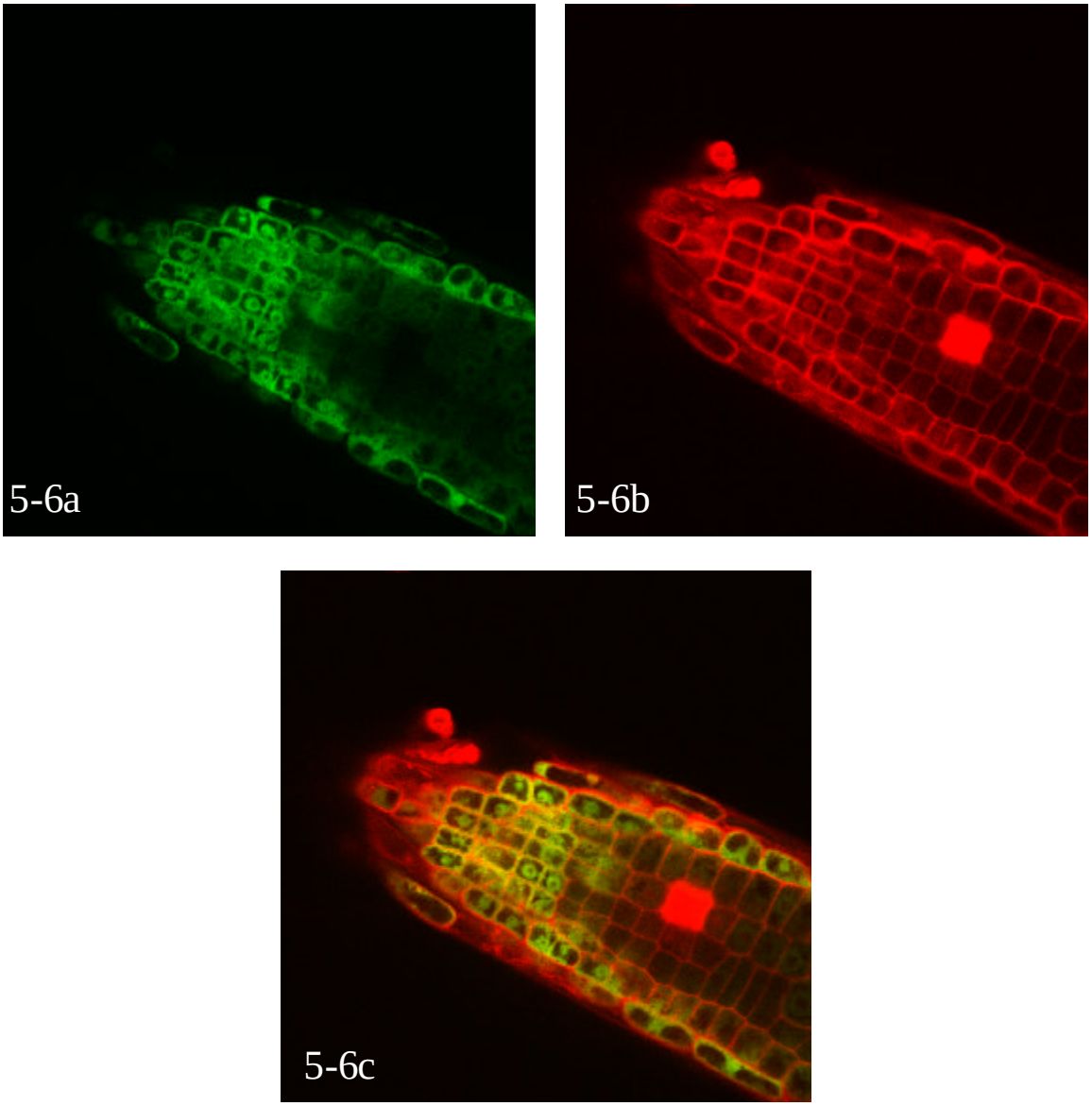


Figure 5-6. Kappa/GFP fusion in the root tip. (a) Image of a root tip expressing kappa/GFP collected using a FITC filter set. (b) Image of the same root tip using a Texas Red filter set after staining with propidium iodide to identify the cell walls. (c) Image of the same root tip using a dual filter set that collects both green and red signals. Any overlap in the two colors results in yellow. There appears to be a yellow signal in the cell walls, indicating 14-3-3/GFP presence. All images are at 60x magnification.

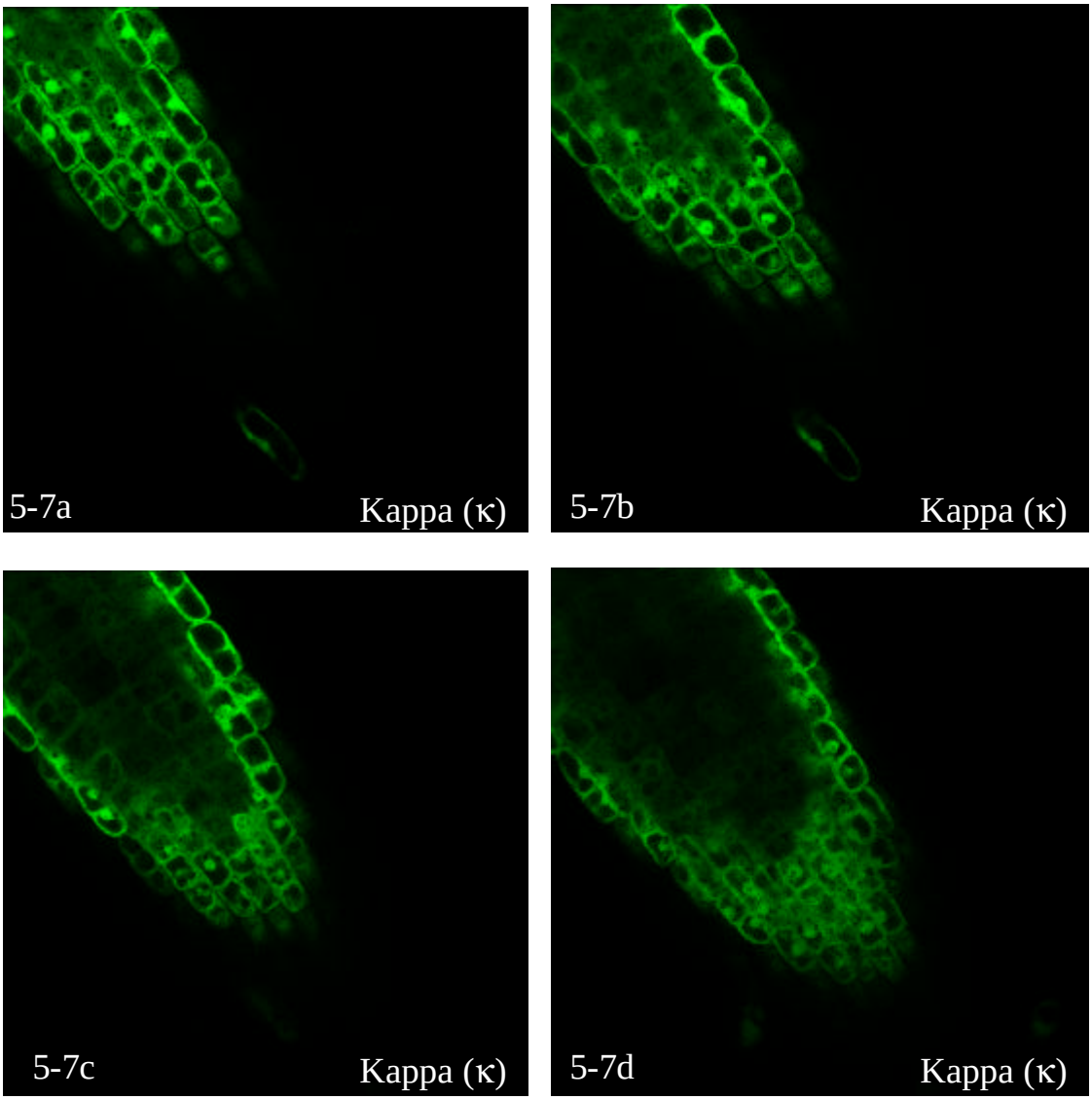
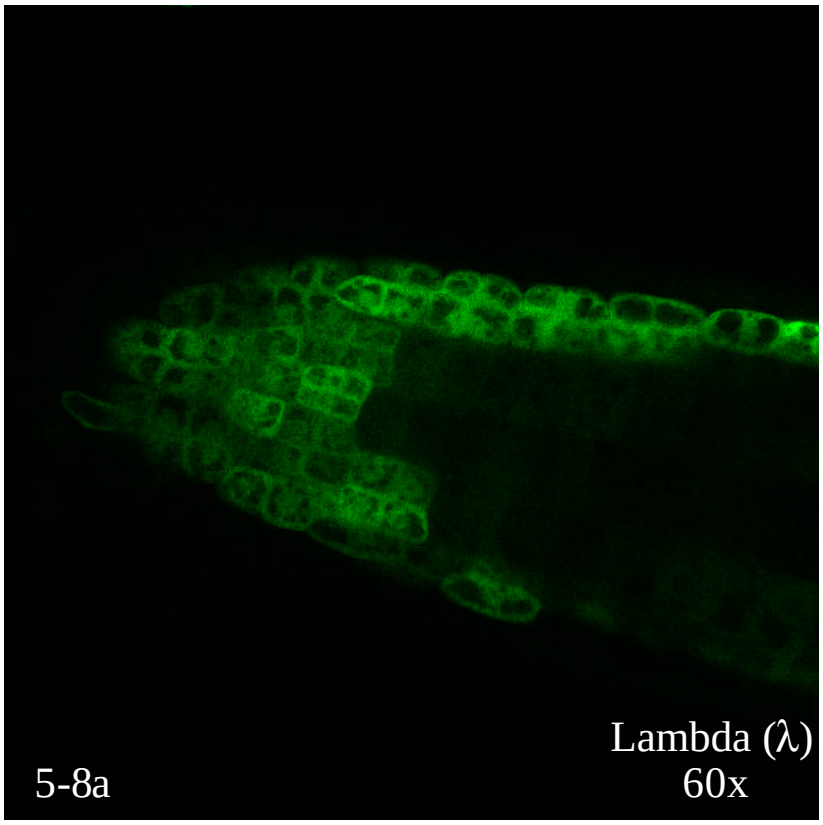
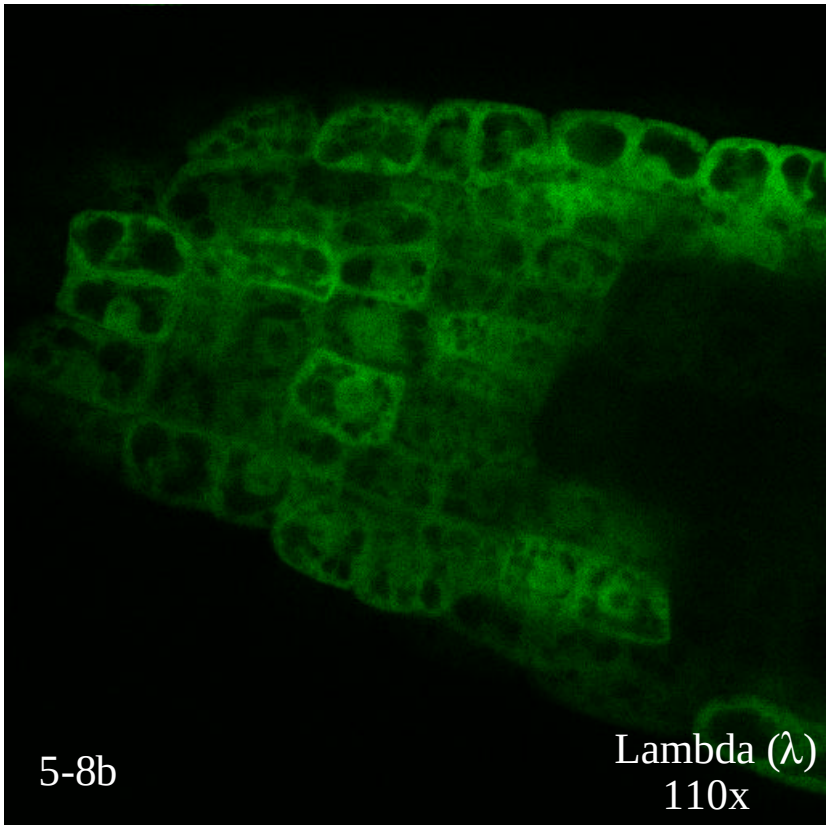


Figure 5-7. Series of four images taken from a larger z-series of kappa/GFP. The images show no change in signal as the z-series progresses. Again, localization is predominately in the nucleus, plasma membrane, and cell wall. These images are at 60x magnification.

Figure 5-8. Lambda/GFP fusion construct demonstrates localization predominately in the nucleus, plasma membrane and cytoplasm. No signal can be seen in the vacuoles or nucleolus. (a) Magnification at 60x. (b) Magnification at 110x.


5-8a
Lambda (λ)
60x


5-8b
Lambda (λ)
110x

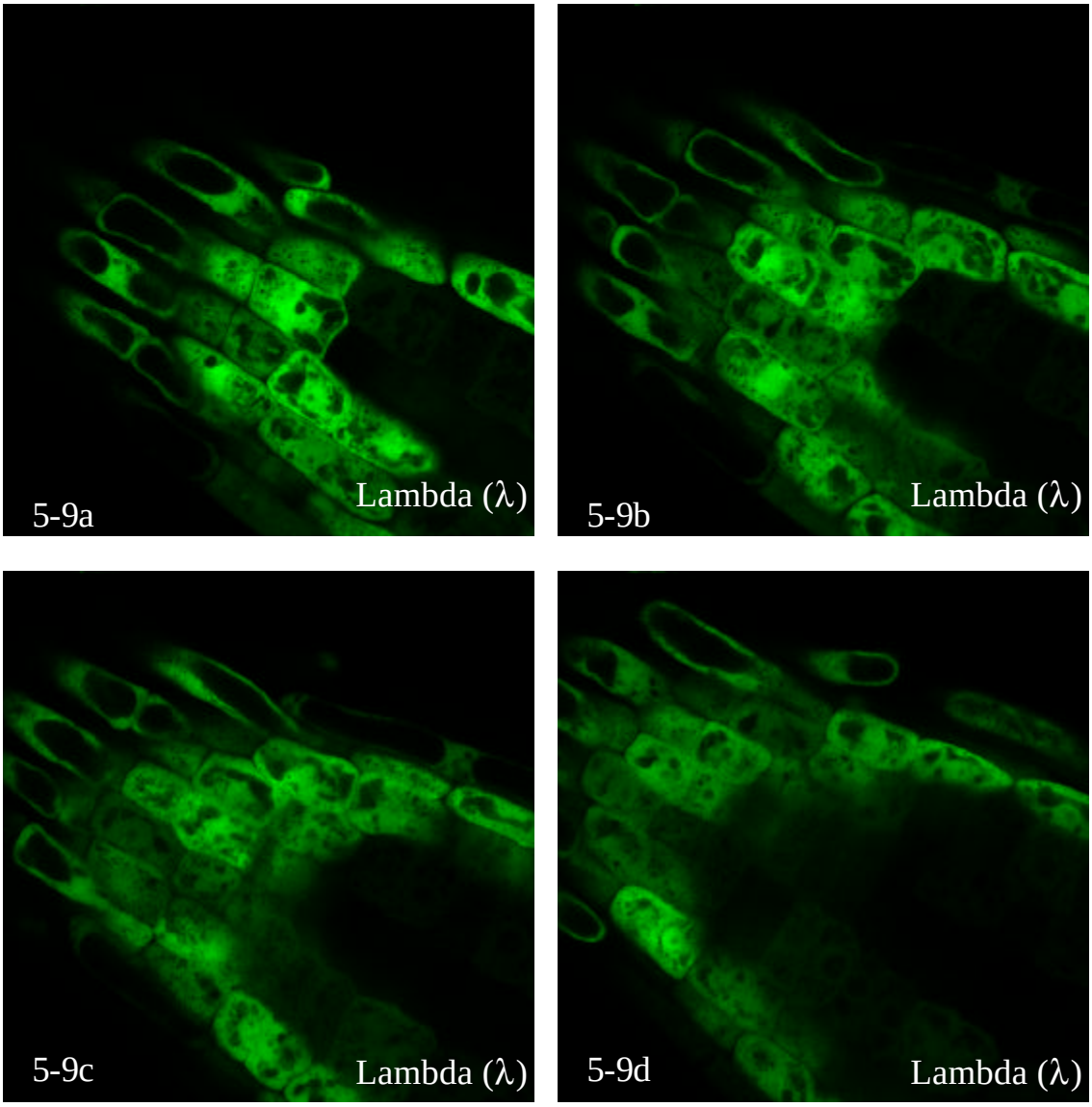


Figure 5-9. Series of four images taken from a larger z-series of lambda/GFP. The overall fluorescence is more intense in these images, but the ratio of intensity within the cell doesn't change from that of (a) and (b). These images were collected at a magnification of 115x. No signal is visible in the vacuoles or nucleolus.

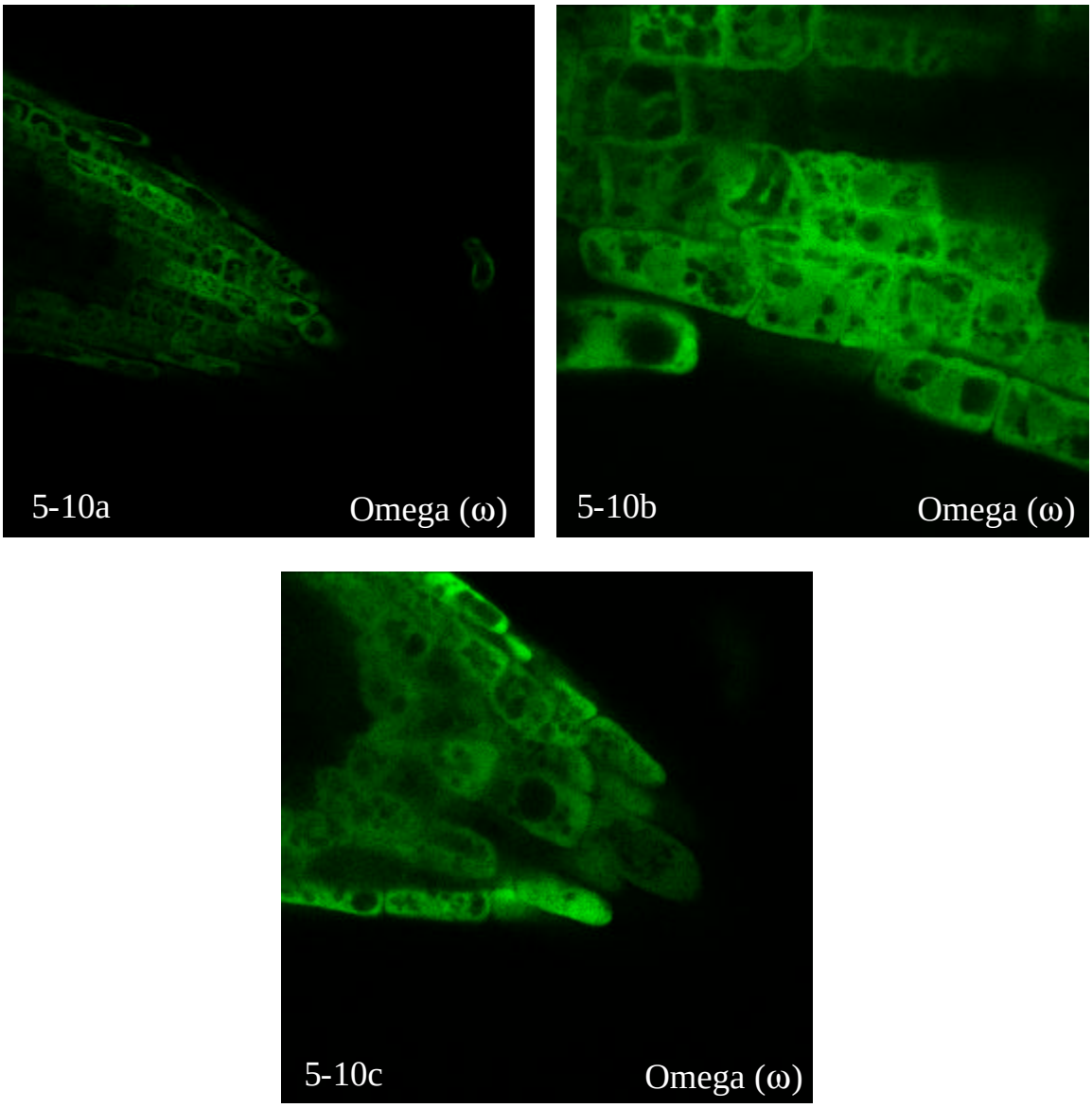
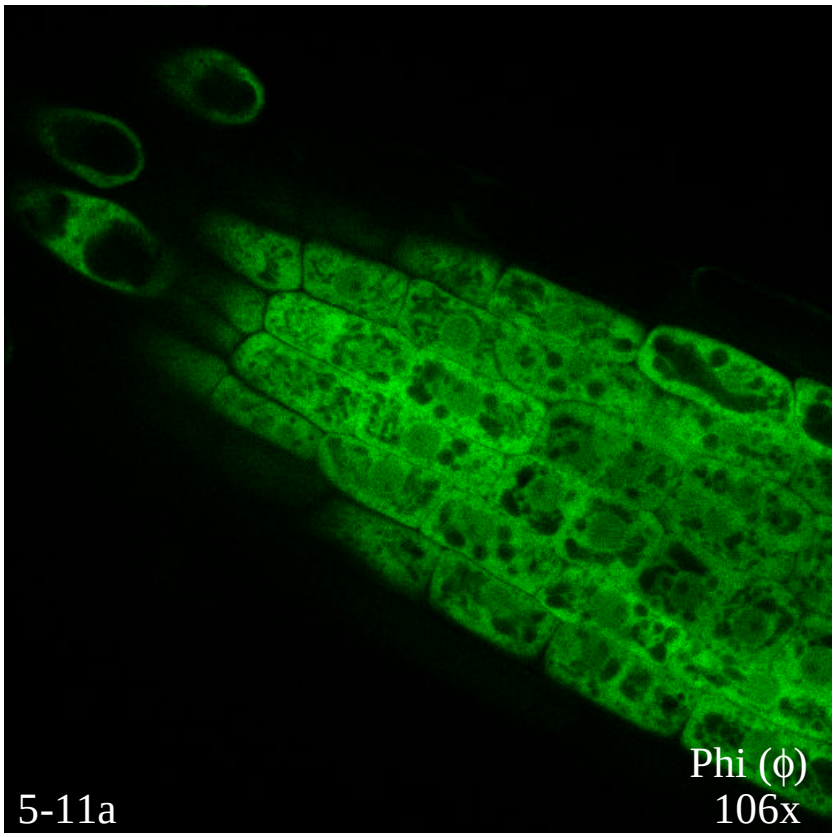
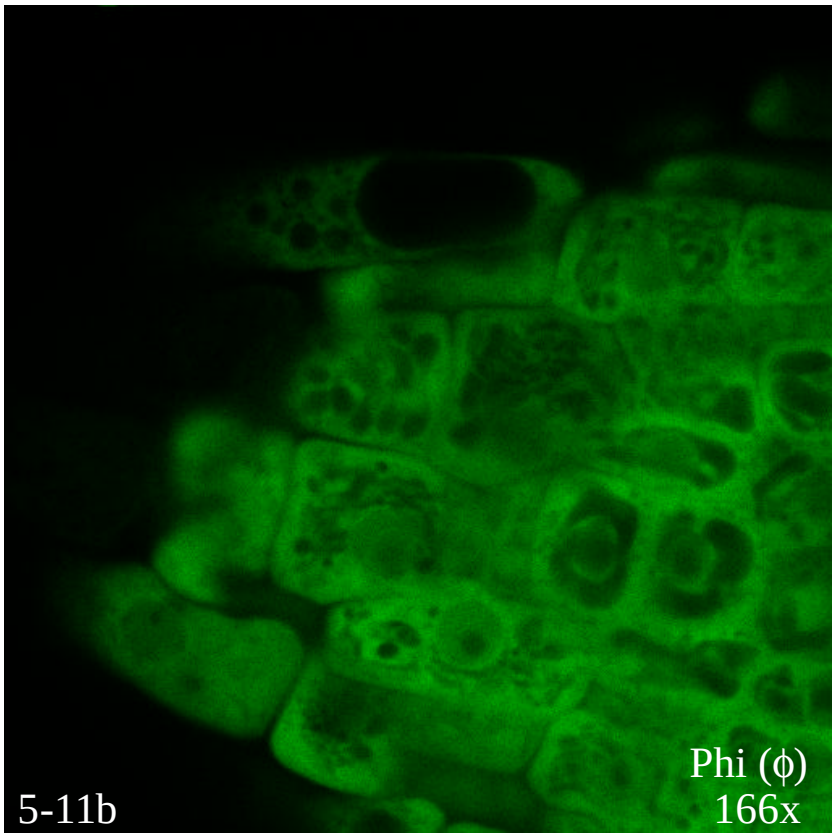


Figure 5-10. Omega/GFP fusion construct demonstrates localization predominately in the cytoplasm, plasma membrane, and nucleus. (a) Image collected at a magnification of 60x. (b) Image collected at a magnification of 180x. (c) Image collected at a magnification of 97x. No signal is visible in the vacuoles or nucleolus in any of these images. The columella root cap and meristem are shown well in (c).

Figure 5-11. Phi/GFP fusion construct demonstrates localization predominately in the cytoplasm, nuclear membrane, and plasma membrane. (a) A single optical slice through the root tip at a magnification of 106x. (b) A single optical slice through the root tip at a magnification of 166x. No signal is visible in the vacuoles or nucleolus in either image.


Phi (ϕ)
106x
5-11a


Phi (ϕ)
166x
5-11b

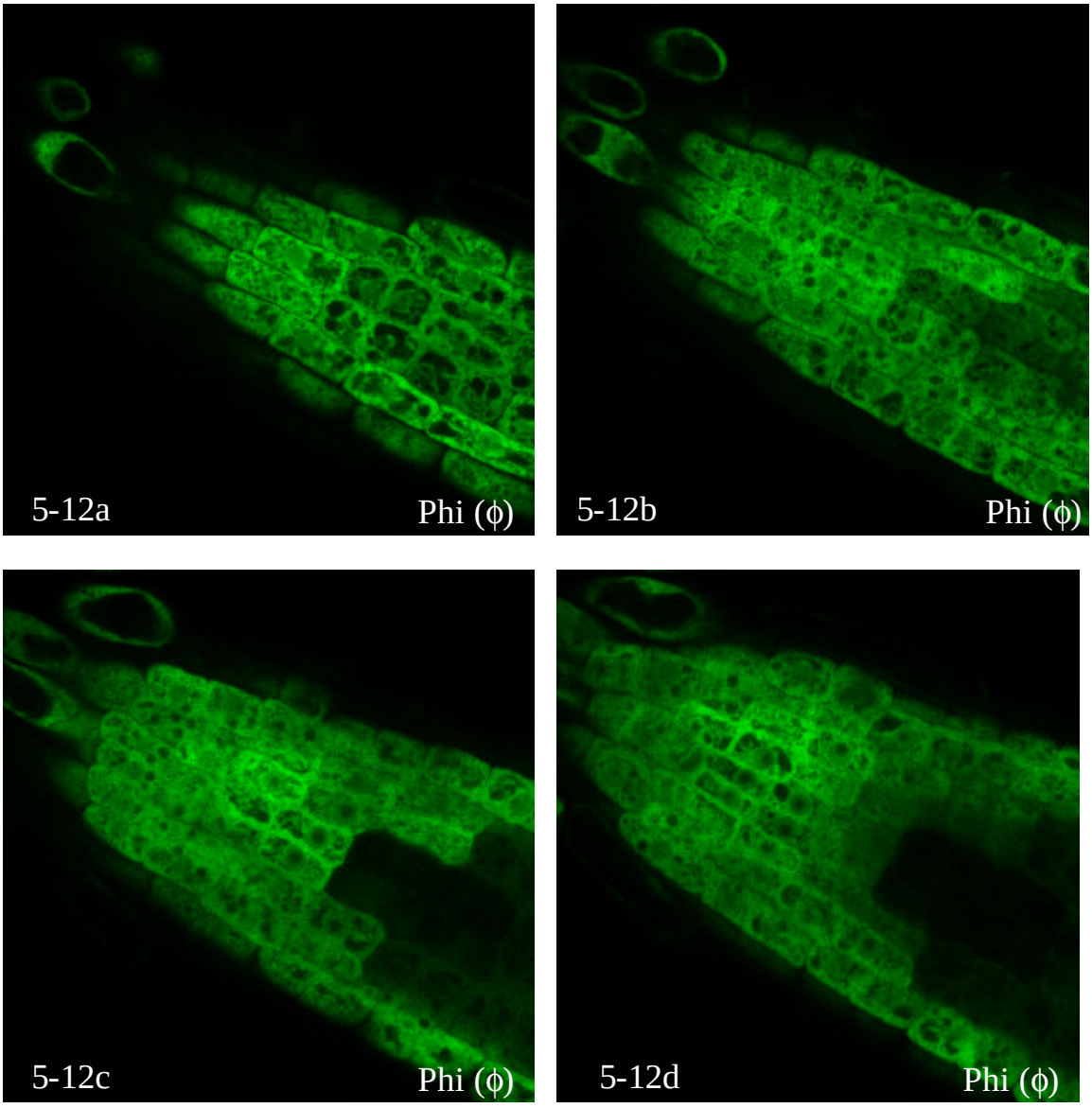
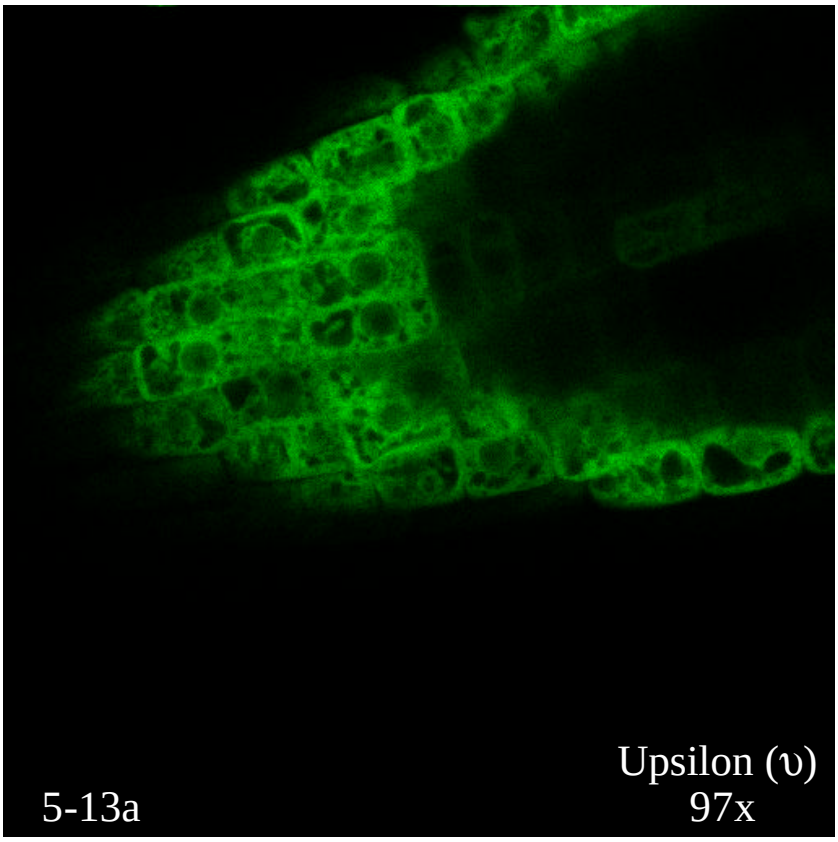
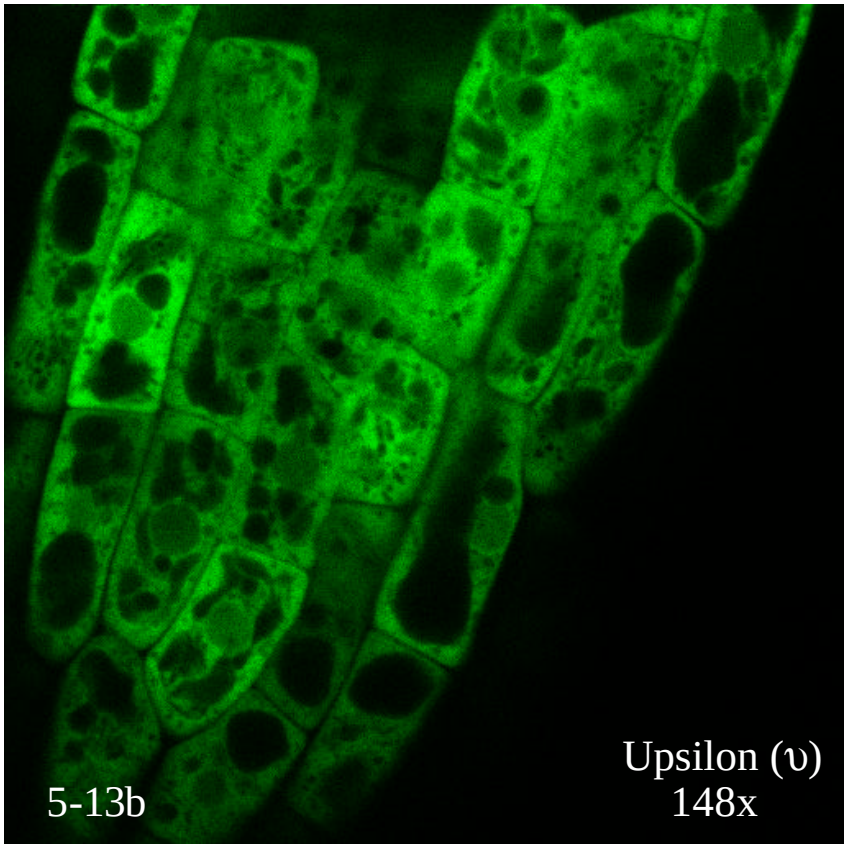


Figure 5-12. Series of four images taken from a larger z-series of phi/GFP. The signal does not change in any of the images as the z-series progresses. These images are the same sample as 5-11 and are at a magnification of 106x. No signal can be seen in the vacuoles or nucleolus in these images.

Figure 5-13. Upsilon/GFP fusion construct demonstrates localization predominately in the cytoplasm, nuclear membrane, and plasma membrane. (a) A single optical slice at a magnification of 97x. (b) A single optical slice at a magnification of 148x. No signal can be seen in the vacuoles or nucleolus in these images.


Upsilon (υ)
97x
5-13a


Upsilon (υ)
148x
5-13b

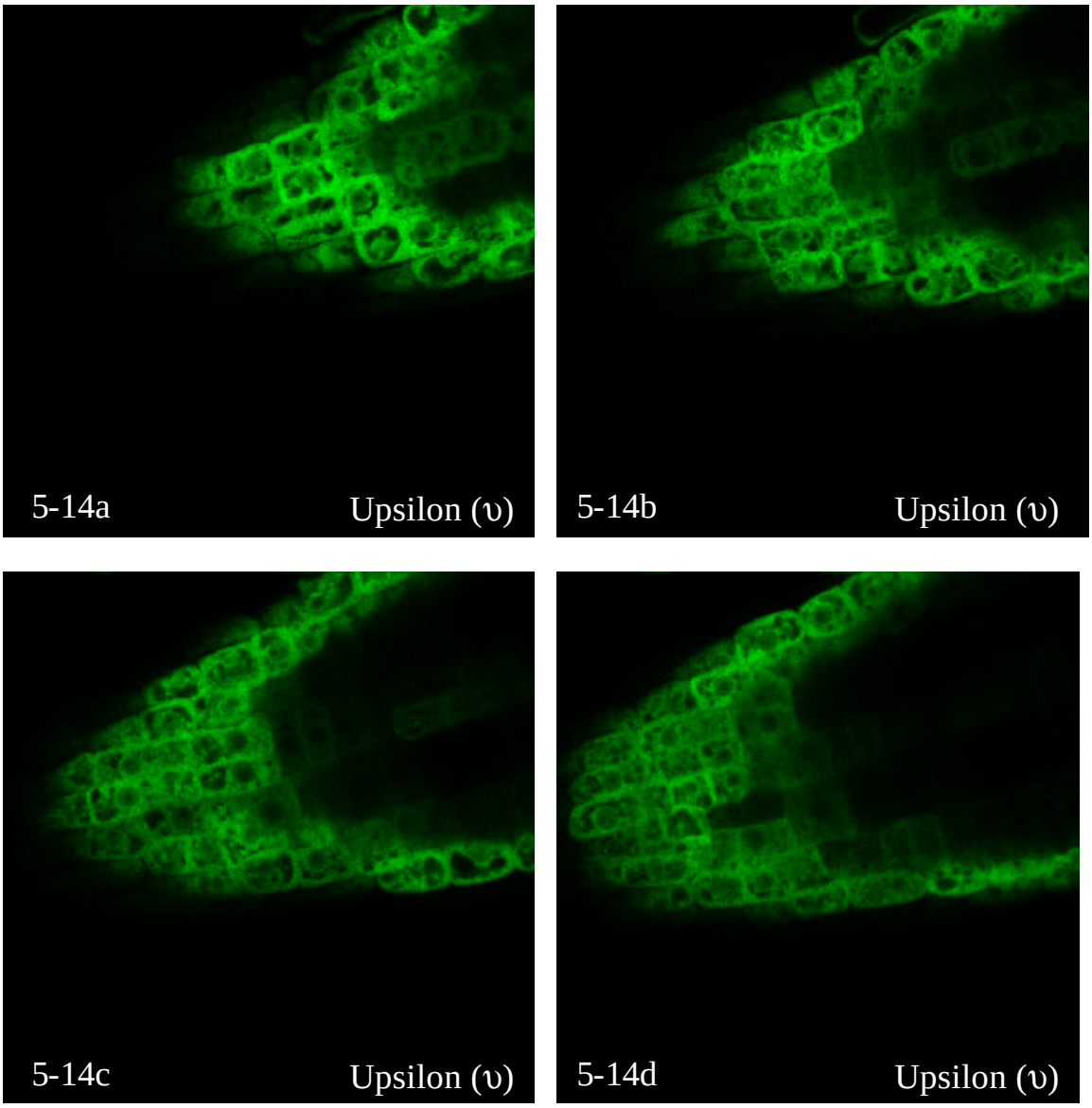
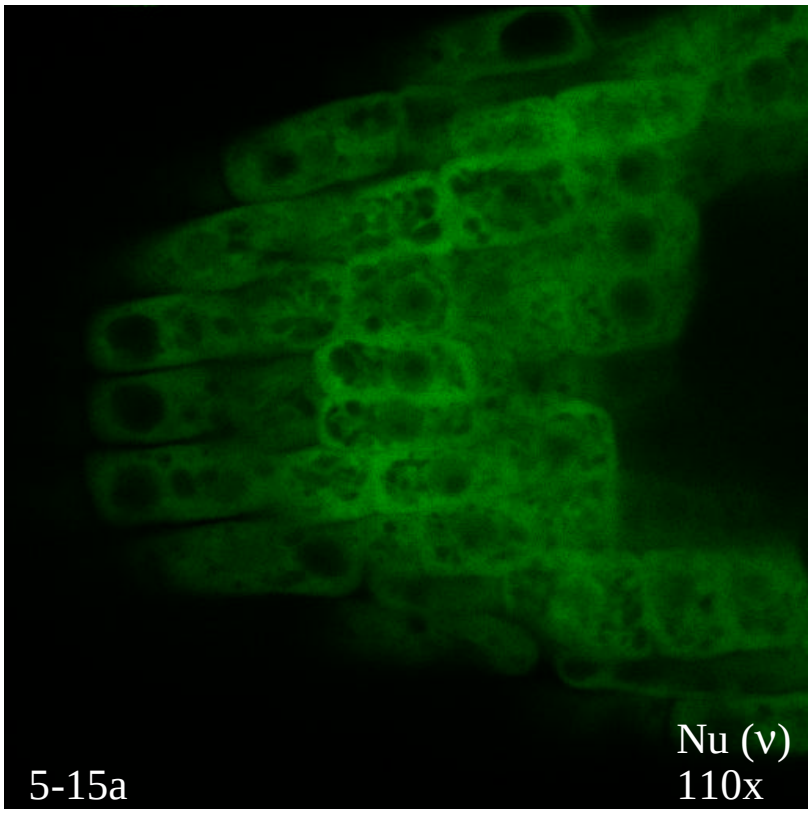
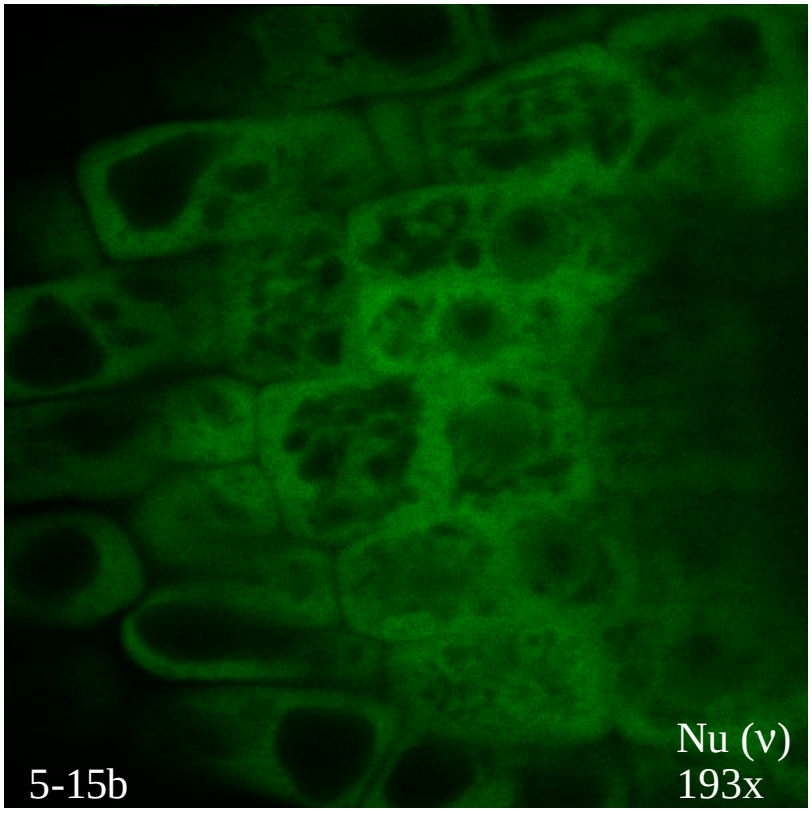


Figure 5-14. Series of four images taken from a larger z-series of upsilon/GFP. The signal does not change in any of the images as the z-series progresses. These images are at a magnification of 97x. No signal can be seen in the vacuoles or nucleolus in these images.

Figure 5-15. Nu/GFP fusion construct demonstrates localization predominately in the cytoplasm, plasma membrane, and nuclear membrane. (a) A single optical slice at a magnification of 110x. (b) A single optical slice at a magnification of 193x. No signal can be seen in the vacuoles or nucleolus in any of these images.


Nu (ν)
5-15a
110x


Nu (ν)
5-15b
193x

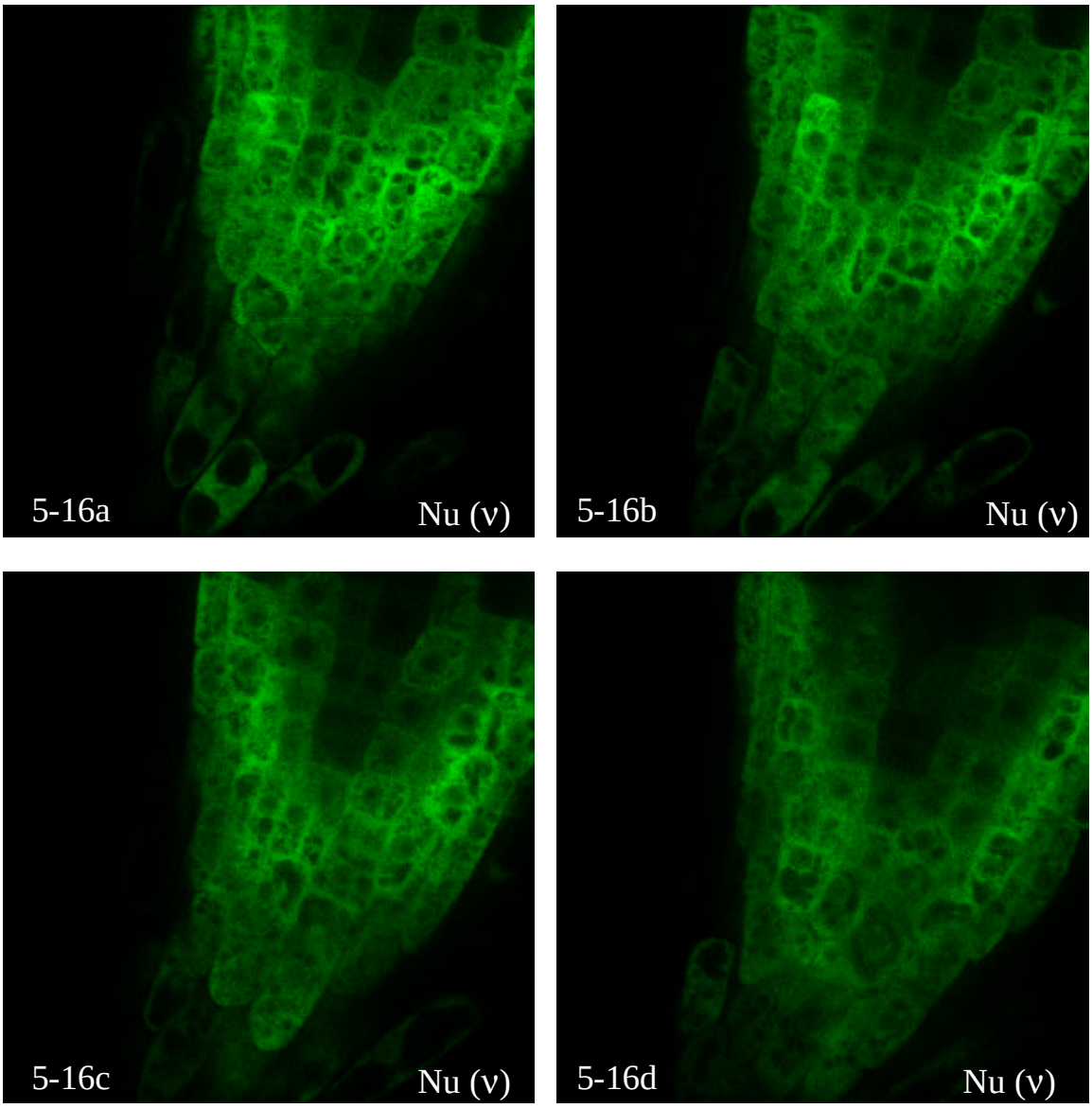


Figure 5-16. Series of four images taken from a larger z-series of nu/GFP. The signal does not change in any of the images as the z-series progresses. These images are at a magnification of 106x. No signal can be seen in the vacuoles or nucleolus in these images.

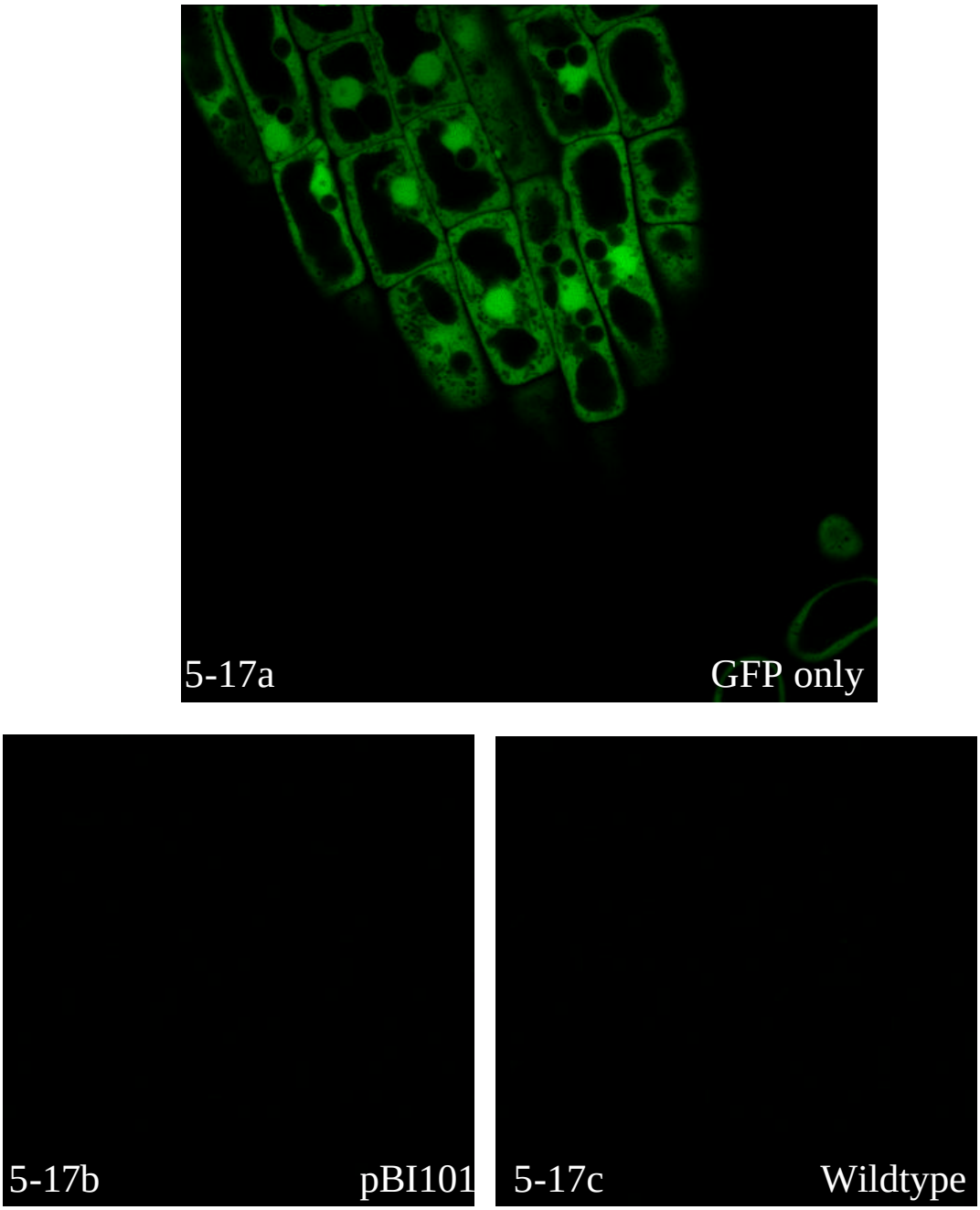


Figure 5-17. Control constructs. (a) pBI12135sGFP (S65T) with no 14-3-3 present. This construct is used as a control for un-fused GFP expression. GFP localizes predominately to the nucleus and cytoplasm. This image is at a magnification of 115x. (b) pBI101 is a construct that contains GFP, but lacks a promoter. No image could be captured although both the iris and gain were maximized. (c) Wildtype arabidopsis is used as a control for autofluorescence. No signal could be captured although both the iris and gain were maximized.

Table 5-2. 14-3-3/GFP fusion locations within the cell.

14-3-3 Location using GFP Fusions	
Isoform	Predominate Location within Cell
Epsilon	Nuclear envelope, plasma membrane and cytoplasm
Kappa	Nucleus, plasma membrane/cell wall, cytoplasm
Lambda	Nucleus, plasma membrane and cytoplasm
Omega	Cytoplasm, plasma membrane and nucleus
Phi	Cytoplasm, nuclear membrane and plasma membrane
Upsilon	Cytoplasm, nuclear membrane and plasma membrane
Nu	Cytoplasm, nuclear membrane and plasma membrane

CHAPTER 6
CONCLUSIONS

14-3-3s commonly exist as multiple isoform families within a particular organism. 14-3-3s are seemingly ubiquitous, having been found in virtually every eukaryotic organism where they have been sought. This family of proteins is involved in numerous functions as well as metabolic pathways. 14-3-3s associate with protein kinases, act as positive and negative regulators, and also associate with transcription factors in plants.

This thesis has dealt primarily with the 14-3-3 family found in Arabidopsis thaliana. In 1992 and 1994, Lu et al. identified five 14-3-3 isoforms in arabidopsis (Lu et al., 1992; Lu et al., 1994b). In 1997, Wu et al. identified five additional isoforms bringing the number of Arabidopsis thaliana 14-3-3 isoforms to ten (Wu et al., 1997). It was widely believed that these ten isoforms were the complete arabidopsis 14-3-3 family. However, the completion of the Arabidopsis thaliana genome project allowed the identification of three additional 14-3-3 isoforms (Rosenquist et al., 2000; DeLille et al., 2001), bringing the total number of 14-3-3 isoforms to thirteen. In fact, the genome project provided information on two additional 14-3-3 genes that appear to produce non-functional protein products (Rosenquist et al., 2000; DeLille et al., 2001).

Given the large number of isoforms within Arabidopsis thaliana, functional specificity among the isoforms emerged as an important issue. Do all the isoforms essentially play the same role within the cell or does each isoform play a specific role?

One way to answer this question is through localization studies. If the isoforms play the same role, then you would expect all the isoforms to be found in the same location within the cell and a general localization pattern. However, if each isoform plays a specific role, then you would expect the isoforms to be found in different locations within the cell and exhibit a unique localization pattern.

Two methods were used to study 14-3-3 localization within 3-7 day old arabidopsis root tips. In the first method, a set of nine polyclonal antibodies was used. Each antibody recognizes a specific Arabidopsis thaliana 14-3-3 isoform. All the Arabidopsis thaliana 14-3-3s share a conserved core region with the N- and C-termini being the most divergent. It is thought that the divergent N- and C-termini could give each isoform functional specificity. Due to the similarity of each isoform, the antibodies were made to peptides derived from either the N- or C-terminus. The antibodies to isoforms phi, kappa, chi, and lambda were all generated to the N-terminus. The antibodies to isoforms epsilon, upsilon, mu, nu, and omega were all generated to the C-terminus. Whole plant tissue was fixed, digested, treated with a single 14-3-3 antibody and secondarily treated with a FITC conjugated antibody. The nine antibodies demonstrated a localization pattern in one of two groups, nuclear or non-nuclear. All of the N-terminal antibodies are found in the nucleus and all the C-terminal antibodies are found in the cytoplasm (non-nuclear).

A clear difference in localization among the 14-3-3 isoforms was apparent using the antibodies. However, the two distinct groups of localization according to termini were unexpected. As previously mentioned, the N- and C-termini are thought to provide functional specificity to each isoform. If these regions are involved in associations with

other proteins, they may not be accessible to the antibody. The lack of accessibility would give a false impression of localization.

In the second method, C-terminal green fluorescent protein (GFP) fusions to nine of the thirteen isoforms were used in localization studies. Isoforms epsilon, kappa, lambda, omega, phi, upsilon, nu, chi, and psi were each fused to GFP and transformed into arabidopsis. The isoforms chi and psi showed no visible fluorescence in the R_0 seed and it is thought that they failed to transform properly. The remaining isoforms were taken through at least two generations.

The 14-3-3/GFP fusions demonstrate a diverse localization pattern both among isoforms and with GFP alone. GFP alone primarily localizes to the nucleus and cytoplasm. No signal can be seen in the nucleolus or vacuoles of GFP alone or any of the 14-3-3/GFP fusion constructs. The seven 14-3-3/GFP fusions show a wide range of localization. Phi, upsilon, and nu are primarily localized to the cytoplasm, nuclear membrane and plasma membrane. Omega is localized to the cytoplasm, plasma membrane and nucleus. Epsilon is predominately localized to the nuclear envelope, plasma membrane and cytoplasm. Kappa and lambda demonstrate the most dramatic localization pattern among the isoforms. These two isoforms are present predominately in the nucleus and plasma membrane and to a lesser extent in the cytoplasm. None of the other isoforms have this particular localization pattern. Additionally, kappa and lambda are closely related evolutionarily and in fact make up one sub-branch of the non-epsilon group members in a phylogenetic tree. Upsilon and nu appear to have the same general localization pattern although it is not as dramatic as kappa and lambda. Upsilon, nu, and psi also make up a sub-branch in a phylogenetic tree. It is interesting to speculate that the

14-3-3 isoforms might localize according to evolutionary relationships. However, that is not clear at this point because we lack localization data for all the thirteen isoforms.

Both the polyclonal antibodies and GFP fusions provide important information concerning 14-3-3 localization. The GFP fusions both confirmed the antibody data and provided new and specific localization information for each 14-3-3 isoform. Figures 6-1a-c are a comparison of plants treated with an individual 14-3-3 polyclonal antibody and those transformed with a 14-3-3/GFP fusion construct. In the case of kappa and lambda, the antibody data and GFP fusion data correlated well. Using both methods of localization, we proved that kappa and lambda are present in the nucleus as well as the cytoplasm. Additionally, the GFP fusions were able to provide information about the plasma membrane and cell wall.

In the case of the omega and epsilon isoforms, the data sets did not correlate as well. Using the polyclonal antibodies, omega and epsilon localized to the cytoplasm. The GFP fusions showed the omega isoform to be primarily in the cytoplasm, but also in the plasma membrane and nucleus. The GFP fusions showed the epsilon isoform to be primarily in the nuclear envelope, plasma membrane and cytoplasm. Both omega and epsilon polyclonal antibodies recognize the C-terminus. Perhaps the C-terminus is involved in an interaction that prevents it from being recognized by the antibody. A logical way around this problem is to simply generate an N-terminal antibody for these two isoforms. The hypothesis is that an N-terminal antibody for omega and epsilon would provide localization data sets that now correlate with the GFP fusion data sets.

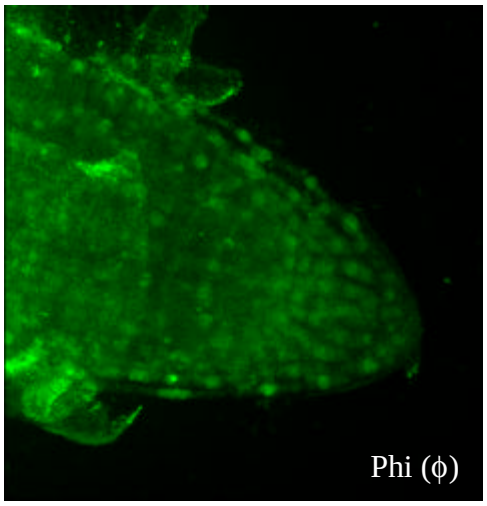
Many additional experiments can be performed with these constructs. Plans are under way to re-transform chi and psi into Arabidopsis thaliana. Also, a mu/GFP fusion

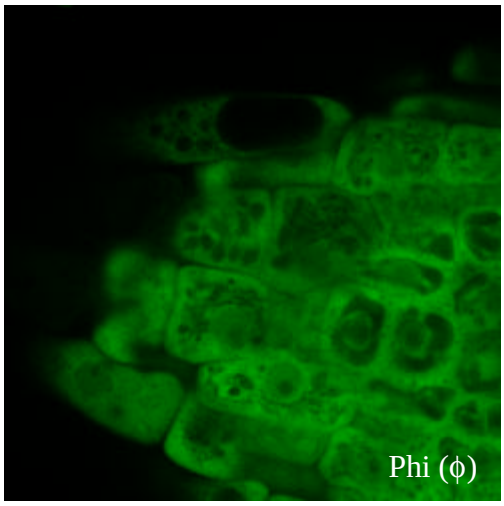
is currently being constructed and will also be transformed into Arabidopsis thaliana. Additionally, 14-3-3s are found in other Arabidopsis thaliana tissues and these tissues need to be examined further. For example, indications of the 14-3-3/GFP fusions being expressed in guard cells are seen, but have not been investigated in any depth.

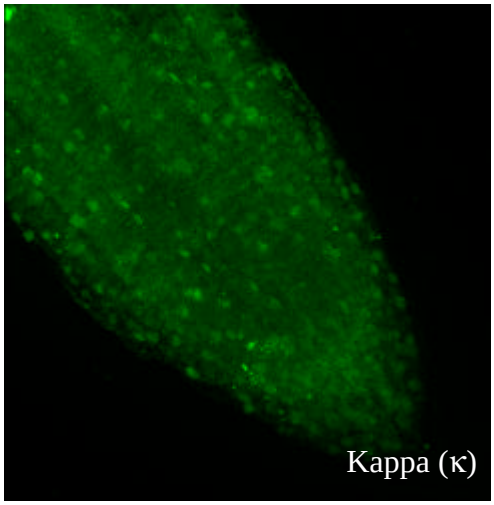
The 14-3-3/GFP fusions should also be tested for environmental stresses such as dehydration and cold tolerance, and chemical stresses such as abscisic acid and fusicoccin. It would be interesting if some, if not all, the 14-3-3/GFP fusions change localization upon being stressed.

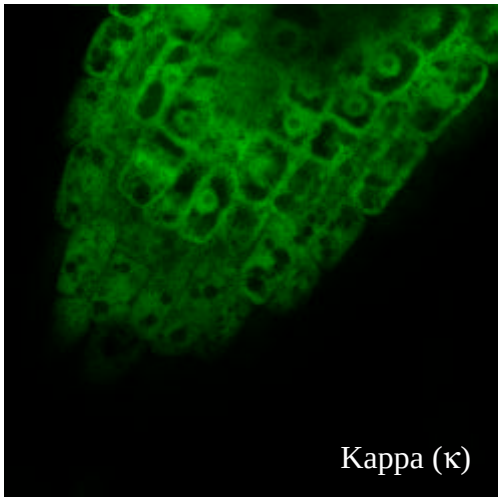
There is still much to be learned about the 14-3-3 family. Arabidopsis thaliana provides the unique opportunity to study a large and complete 14-3-3 family within a single organism. These localization studies provide the first steps into isoform specific functions and should be used as a springboard for future experiments.

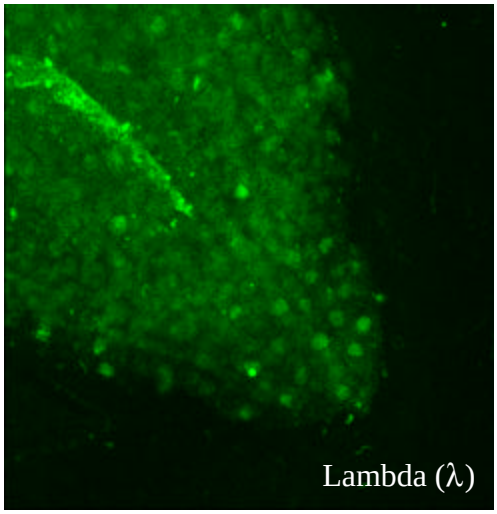
Figure 6-1a. Thumbnail images of Arabidopsis thaliana root tips. The plants in the left hand column were treated with 14-3-3 polyclonal antibodies. The plants in the right hand column were transformed with the respective 14-3-3/GFP fusion construct. These are the same images that are found in chapters 4 and 5.

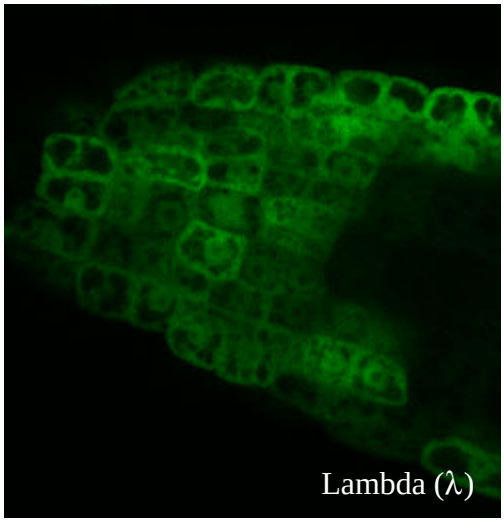

Phi (ϕ)


Phi (ϕ)


Kappa (κ)


Kappa (κ)


Lambda (λ)


Lambda (λ)

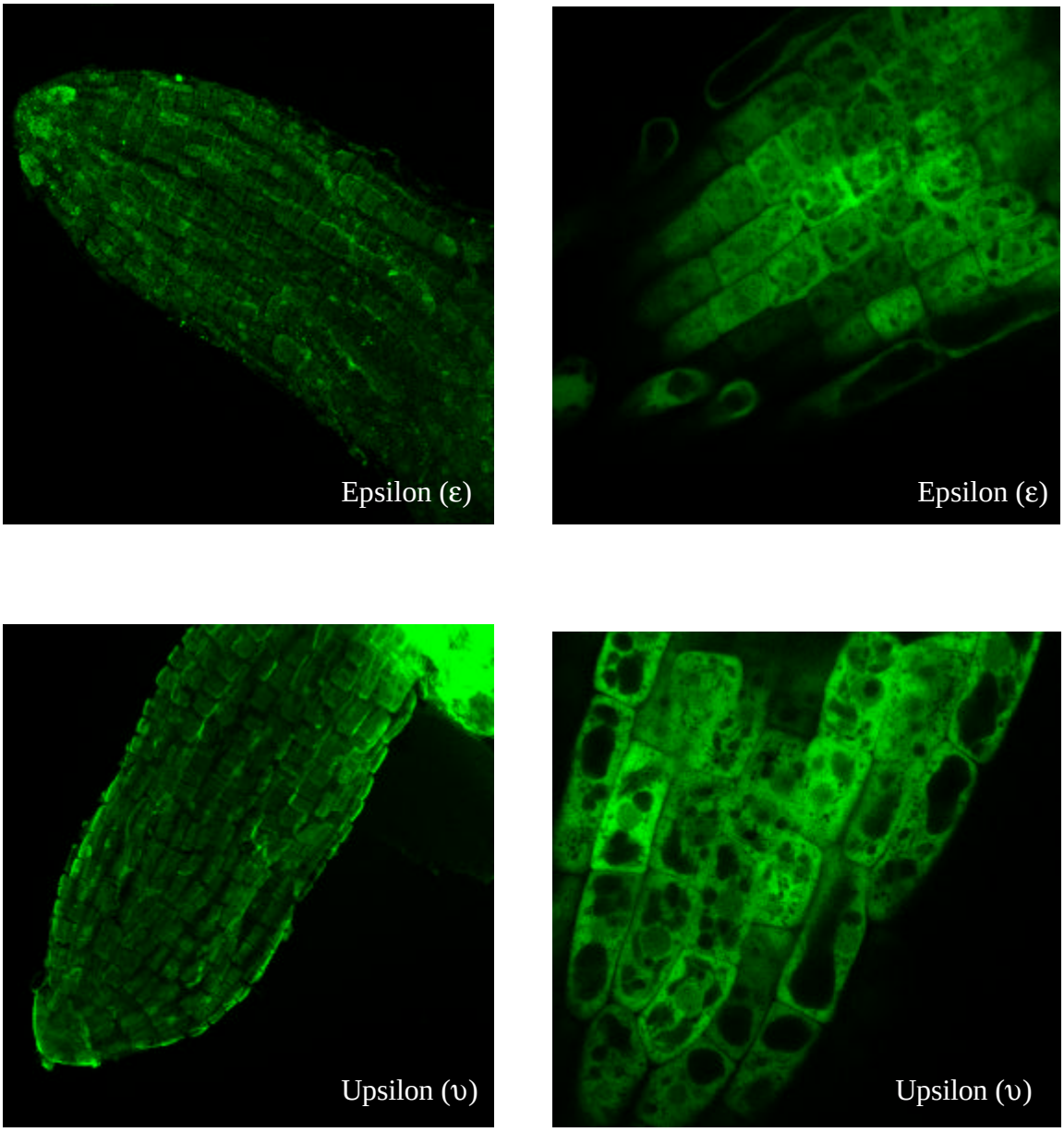


Figure 6-1b. Thumbnail images of Arabidopsis thaliana root tips. The plants in the left hand column were treated with 14-3-3 polyclonal antibodies. The plants in the right hand column were transformed with the respective 14-3-3/GFP fusion construct. These are the same images that are found in chapters 4 and 5.

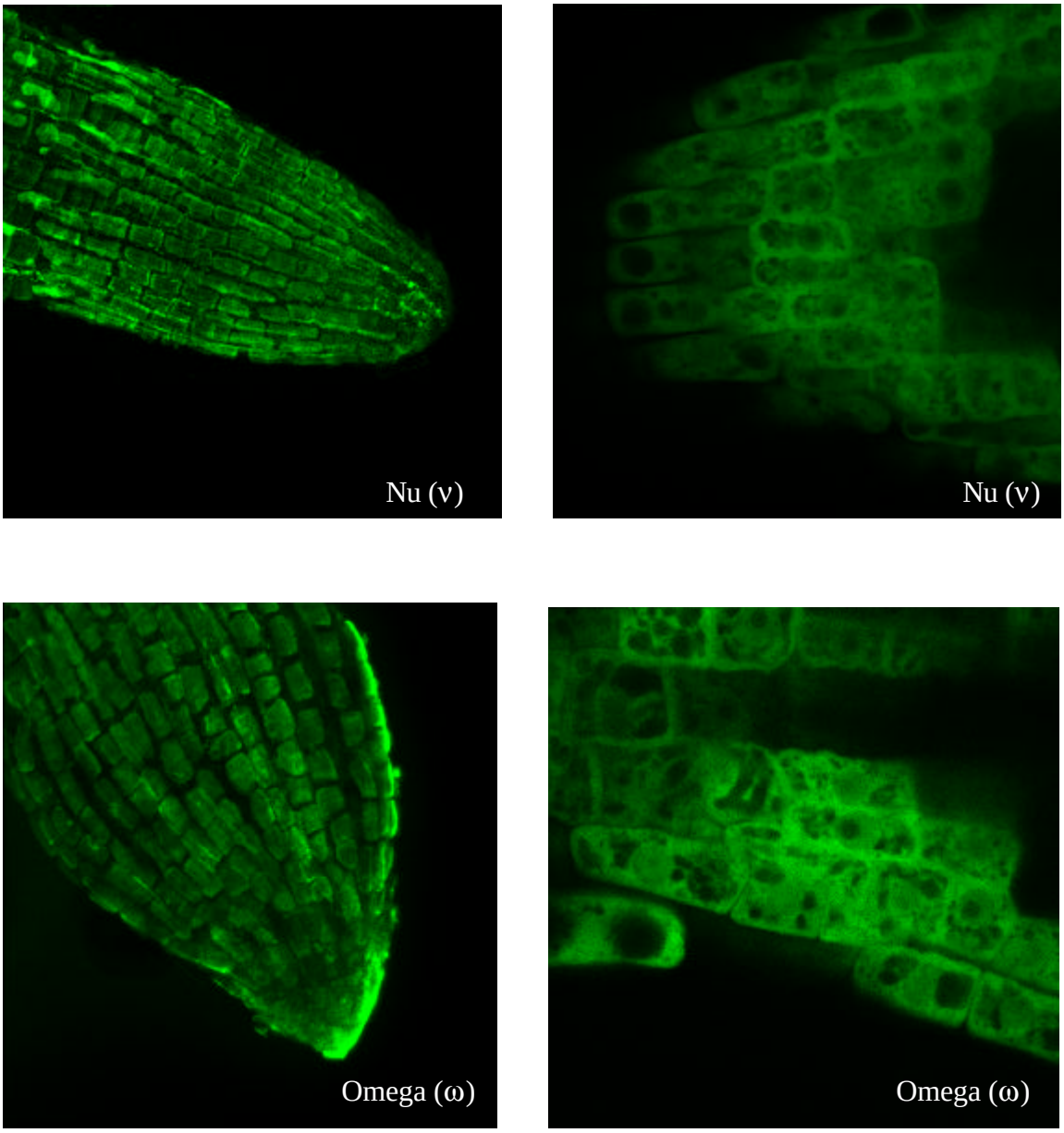


Figure 6-1c. Thumbnail images of Arabidopsis thaliana root tips. The plants in the left hand column were treated with 14-3-3 polyclonal antibodies. The plants in the right hand column were transformed with the respective 14-3-3/GFP fusion construct. These are the same images that are found in chapters 4 and 5.

APPENDIX
SEQUENCES OF 14-3-3/GFP FUSIONS

This appendix contains the sequences of nine 14-3-3/GFP fusions. The 14-3-3 isoforms are epsilon, kappa, lambda, omega, phi, upsilon, nu, chi, and psi. Two sequencing reactions per clone were performed, a forward and reverse. The forward reaction used a primer that recognizes the tail end of the 35S promoter and provides sequence for anything behind the promoter using the sense strand. The reverse reaction used a primer that recognizes the beginning of GFP and provides sequence for anything in front of the GFP, using the anti-sense strand. The goal of using these two primers was two fold. First, the forward primer was used to check that the start site and beginning of the 14-3-3 were correct. Second, the reverse primer was used to verify the 14-3-3/GFP junction. We wanted to insure that there were no single or double base frame shifts and that GFP was properly fused to the 14-3-3. Because the 35S promoter and the GFP in each clone were identical, we could use the same two primers for all nine constructs. The sequence of the forward primer is (5'CGCACAATCCCACTATCCTT3'). The sequence of the reverse primer is (5'CGGACACGCTGAACTTG3').

The following pages contain the sequences for all nine 14-3-3 constructs. There are two pages per clone. The first page contains the ideal and correct sequence showing both sense and anti-sense strands. The second page contains the actual forward and reverse sequences for that particular clone. Further, the text has been color coded to make it easier to interpret. Black text is the sequence of the 35S promoter. Blue text is

sequence of the particular 14-3-3. Green text is the sequence of GFP. The name of each isoform is identified at the tip of each page.

Additionally, the sequences have not been edited in any way. Therefore, there are bases that are not identified and are shown as "n." Also, the further into the clone that the sequence goes, the less accurate it becomes. Both junctions, 35S/14-3-3 and 14-3-3/GFP, are correct in every clone.

Figure A-1 is a short summary of all nine actual 14-3-3/GFP fusions. The first five and last five 14-3-3 amino acids are shown as well the first five amino acids of GFP. Again, the amino acids are color coded as to their identity. The point is to illustrate that there are no frame shifts or dropped amino acids and that they are true fusions.

	14-3-3						14-3-3					GFP				
Epsilon -	M	E	N	E	R	.....	D	E	N	S	R	M	V	S	K	G
Kappa -	M	A	T	T	L	.....	D	E	A	S	R	M	V	S	K	G
Lambda -	M	A	A	T	L	.....	D	E	A	S	R	M	V	S	K	G
Omega -	M	A	S	G	R	.....	E	Q	Q	T	R	M	V	S	K	G
Phi -	M	A	A	P	P	.....	Q	E	I	S	R	M	V	S	K	G
Upsilon -	M	S	S	D	S	.....	P	S	Q	S	R	M	V	S	K	G
Nu -	M	S	S	S	R	.....	T	G	Q	S	R	M	V	S	K	G
Chi -	M	A	T	P	G	.....	Q	Q	S	S	R	M	V	S	K	G
Psi -	M	S	T	R	E	.....	G	A	E	S	R	M	V	S	K	G

Figure A-1. A summary of the actual amino acid sequence for the 14-3-3/GFP constructs. The first five and the last five amino acids of each 14-3-3 are shown, as well as the first five amino acids of GFP. The 14-3-3 is identified as blue and GFP as green. There are no frame shifts or dropped amino acids in any of the constructs. Phi has a single amino acid change, denoted by the red Q (should be a K). However, this error could be attributed to the unedited sequence.

Epsilon Isoform

```
4951  AGACCCTTCC TCTATATAAG GAAGTTCATT TCATTTGGAG AGAACACGGG
      TCTGGGAAGG AGATATATTC CTTCAAGTAA AGTAAACCTC TCTTGTGCCC

5001  GGACTCTAGA ACAATGGAGA ATGAGAGGGA AAAGCAGGTT TACTTGGCTA
      CCTGAGATCT TGTTACCTCT TACTCTCCCT TTTCGTCCAA ATGAACCGAT

5051  AGCTCTCCGA GCAAACCGAA AGATACGATG AAATGGTGGA GGCGATGAAG
      TCGAGAGGCT CGTTTGGCTT TCTATGCTAC TTTACCACCT CCGCTACTTC

5101  AAAGTTGCTC AGCTTGATGT GGAGCTAACT GTGGAAGAGA GGAATCTTGT
      TTTCAACGAG TCGAACTACA CCTCGATTGA CACCTTCTCT CCTTAGAACA

5151  ATCTGTAGGG TACAAGAATG TGATTGGTGC AAGGAGAGCA TCATGGAGAA
      TAGACATCCC ATGTTCTTAC ACTAACCACG TTCCTCTCGT AGTACCTCTT

5201  TACTATCTTC CATTGAGCAG AAGGAAGAGT CCAAGGGAAA TGATGAAAAT
      ATGATAGAAG GTAACTCGTC TTCCTTCTCA GGTTCCCTTT ACTACTTTTA

5251  GTCAAGAGGC TTAAGAATTA TCGTAAGAGA GTTGAAGATG AGCTTGCTAA
      CAGTTCTCCG AATTCTTAAT AGCATTCTCT CAACTTCTAC TCGAACGATT

5301  AGTTTGTAAT GACATCTTGT CTGTCATTGA TAAGCATCTC ATTCCATCGT
      TCAAACATTA CTGTAGAACA GACAGTAACT ATTCGTAGAG TAAGGTAGCA

5351  CTAACGCTGT GGAGTCAACT GTCTTTTTCT ACAAAATGAA AGGAGATTAC
      GATTGCGACA CCTCAGTTGA CAGAAAAAGA TGTTTTACTT TCCTCTAATG

5401  TATCGCTATC TTGCGGAGTT CAGTTCTGGT GCTGAACGCA AGGAAGCTGC
      ATAGCGATAG AACGCCTCAA GTCAAGACCA CGACTTGCGT TCCTTCGACG

5451  AGATCAGTCT CTTGAAGCAT ATAAGGCTGC TGTTGCTGCT GCAGAGAATG
      TCTAGTCAGA GAACTTCGTA TATTCCGACG ACAACGACGA CGTCTCTTAC

5501  GTTTGGCACC CACACATCCA GTTAGACTTG GCTTGGCGTT GAACTTTTCA
      CAAACCGTGG GTGTGTAGGT CAATCTGAAC CGAACCGCAA CTTGAAAAGT

5551  GTTTTCTACT ATGAGATCTT GAACTCTCCC GAAAGCGCAT GCCAATTGGC
      CAAAAGATGA TACTCTAGAA CTTGAGAGGG CTTTCGCGTA CGGTTAACCG

5601  TAAGCAAGCA TTCGATGATG CAATTGCTGA ACTTGACAGC CTCAACGAGG
      ATTCGTTCGT AAGCTACTAC GTTAACGACT TGAACTGTCG GAGTTGCTCC

5651  AATCATACAA AGACAGCACT CTTATTATGC AGCTACTTAG AGACAATCTC
      TTAGTATGTT TCTGTCGTGA GAATAATACG TCGATGAATC TCTGTTAGAG

5701  ACCTTGTGGA CTTCAGACCT TAATGAGGAA GGAGATGAGA GAACCAAAGG
      TGGAACACCT GAAGTCTGGA ATTACTCCTT CCTCTACTCT CTTGGTTTCC

5751  TGCTGATGAG CCTCAAGATG AGAACTCTAGAATGGTGAGCAAGGGCGAGGTG
      ACGACTACTC GGAGTTCTAC TCTTGAGATCTTACCACTCGTTCCCGCTCCAC
```

Epsilon Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

TTTCANTTCCNTTACCTNCCTCTATATAAGGAAGTTNTTTCCTTTGGAGAGAACACGGGGGACTCTAGAAC
AATGGAGAATGAGAGGNAATATCNTGTTTACTTGGCTAAGCTCTCCGAGCAAACCGATAGATNCNATGAAA
TGGTGGAGGCGATGAAGAAAGTTGCTCAGCTTGATGTGGAGCTAACTGTGGAAGAGAGGAATCTTGTATCT
GTANGGTACAAGAATGTGATTGGCGCAAGGAGAGCATCATNGANAATACTATNTNCCATTGAGCAAANNGA
AGAGNCCAAGGGANATGATNANAATGTCANGAGGCTTANAGAATTATCGTAAGAGANTTGAAGATGANCTT
GCTAAAGTTTGTAATGACATCTTGTCTGTCATTGATAACCATCTCATTCCATCTNCTAACCCTTGTGGAGT
CAACTGTCTTTTTCTACACAAATGAAAAGGAGATTACCTATNACTATCTTNCCNGANTTCANTTCTNGTGC
TGANCGCAAAGNAGCTCAAATCCAGTCTTCTTGANCCATATTNCGCTGCTNTGCCNGCCTCACAAAAANGA
NCTGCACCCCNCNCTTCCATTTAACTTGGCTTGGCCTTNANCNNTTCNATNTTCNCNTTGANAANCNTNNA
CNTCCNNCCNTAAGCCCCTGNCANTTTNCCTAACCTCGCATTTCNAACGAATGCCATTTGCNTAACTCTTG
TACNCTCCCCAANCNACGGAAANNCTTCCACNNNANCCCCNCCTCCTTTTTTATTNNCCCCTTACCTANNA
AAACCNATNNTCNACCTTTTNTNGNGACNTTCNNNNCTCTTTTATTANNGGAATCGCCTNTTTATAAATAC
NNCCACNCGGCTNCCTNTATTTGACCCCCCTNAAAANNTACCAACTCTTTTAACNNT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

TNGTCGTTTACGTGNGCCGNNCANCTGGACGAGNATGGGCACCACCNCGGCGAACAGCTCCTCGCCCTTGC
TNACCATTCTAGAGTTCTCATCNTGAGGCTCATCAGCACCTTTGGTTCTCTCATCTCCTTCCTCATTAAGG
TCTGAAGTCCACAAGGTGAGATTGTCTCTAAGTAGCTGCATAATAAGAGTGCTGTCTTTGTATGATTCCTC
GTTGAGGCTGTCAAGTTCAGCAATTGCATCATCGAATGCTTGCTTAGCCAATTGGCATGCGCTTTCGGGAG
AGTTCAAGATCTCATAGTAGAAAACTGAAAAGNTCAACGCCAAGCCAAGTCTAACTGGATGTGTGGGTGCC
AAACCATTCTCTGNAGCAGCAACAGCAGCCTTATATGCTTCAAGAGACTGATCTGCAGCTTCCTTGCGTTC
AGCACCAGAACTGAACTCCGCAAGATAGCGATAGTAATCTCCTTTCATTTTGTAGAAAAAGACAGTTGACT
CCACAGCGTTAGACGATGGAATGANATGCTTATCAATGACNGACAAGATGNCATTACAAACTTTAGCAAGC
TCATCTTCAACTCTCTTACGATAATTCTTAAGCCTCTTGACATTTTCATCATTTCCCTTGGACTCTTCCTT
CTNCTCAATGGAAGATAGNATTCTCCATGATGCTCTCCTTGCACCAATCACATTCTTTGTACCCTACAGAT
CAAGATTNCTCTCTTCCACAGTTAGCTCCNCTCAAGCNGAGCAAACTTTCTTCATTCGCCTCCACCATTTT
CATCGTATCTTTTCGGGNTTGNTCGGGAAAAGCTTTAGCCCAGTAAACCTCGCCTTTCCCCTCTCNATTCT
CCATT

Kappa Isoform

```
1      CTAGAACAAT GGCGACGACC TTAAGCAGAG ATCAATATGT CTACATGGCG
       GATCTTGTTA CCGCTGCTGG AATTCGTCTC TAGTTATACA GATGTACCGC

51     AAGCTCGCCG AGCAAGCCGA GCGTTACGAA GAGATGGTTC AATTCATGGA
       TTCGAGCGGC TCGTTCGGCT CGCAATGCTT CTCTACCAAG TTAAGTACCT

101    ACAGCTCGTA AGTGGAGCTA CACCGGCCGG TGAGCTGACC GTAGAAGAGA
       TGTCGAGCAT TCACCTCGAT GTGGCCGGCC ACTCGACTGG CATCTTCTCT

151    GGAATCTTCT CTCGGGCGCG TATAAGAACG TGATTGGATC TCTTCGCGCG
       CCTTAGAAGA GAGCCCGCGC ATATTCTTGC ACTAACCTAG AGAAGCGCGC

201    GCATGGAGAA TCGTGTCTTC GCTTGAGCAA AAGGAAGAGA GCAGGAAGAA
       CGTACCTCTT AGCACAGAAG CGAACTCGTT TTCCTTCTCT CGTCCTTCTT

251    CGAAGAACAC GTGTCGCTTG TTAAGGATTA CAGATCTAAA GTTGAGACTG
       GCTTCTTGTG CACAGCGAAC AATTCCTAAT GTCTAGATTT CAACTCTGAC

301    AGCTTTCTTC GATCTTTTCT GGGATTCTCA GGTTACTTGA TTCGCATCTA
       TCGAAAGAAG CTAGAAAAGA CCCTAAGAGT CCAATGAACT AAGCGTAGAT

351    ATTCCTTCAG CTACTGCCAG GGAGTCTAAG GTTTTTTACC TGAAGATGAA
       TAAGGAAGTC GATGACGGTC CCTCAGATTC CAAAAAATGG ACTTCTACTT

401    AGGAGATTAT CATCGTTATT TGGCTGAGTT TAAATCTGGT GATGAGAGGA
       TCCTCTAATA GTAGCAATAA ACCGACTCAA ATTTAGACCA CTACTCTCCT

451    AAACTGCTGC TGAAGATGCT ATGATCGCTT ACAAAGCTGC TCAGGACGTT
       TTTGACGACG ACTTCTACGA TACTAGCGAA TGTTTCGACG AGTCCTGCAA

501    GCAGTTGCTG ATCTAGCACC TACACATCCG ATCAGGCTTG GTTTGGCTCT
       CGTCAACGAC TAGATCGTGG ATGTGTAGGC TAGTCCGAAC CAAACCGAGA

551    TAACTTCTCA GTGTTTTACT ACGAGATTCT CAACTCTTCA GAGAAAGCTT
       ATTGAAGAGT CACAAAATGA TGCTCTAAGA GTTGAGAAGT CTCTTTCGAA

601    GTAGCATGGC GAAACAGGCT TTTGAAGAAG CCATTGCTGA GCTGGACACA
       CATCGTACCG CTTTGTCCGA AAACTTCTTC GGTAACGACT CGACCTGTGT

651    TTGGGAGAGG AGTCATACAA GGACAGTACT CTCATCATGC AGTTGCTAAG
       AACCCTCTCC TCAGTATGTT CCTGTCATGA GAGTAGTACG TCAACGATTC

701    GGACAATCTA ACCCTTTGGA CCTCCGATAT GCAGGAGCAG ATGGATGAGG
       CCTGTTAGAT TGGGAAACCT GGAGGCTATA CGTCCTCGTC TACCTACTCC

751    CCTCTAGAAT GGTGAGCAAG GGCGAGGAGC TGTTCACCGG GGTGGTGCCC
       GGAGATCTTA CCACTCGTTC CCGCTCCTCG ACAAGTGGCC CCACCACGGG

801    ATCCTGGTCG AGCTGGACNG CGACGTAAAC GGCCACAAGT TCAGCGTGTC
       TAGGACCAGC TCGACCTGNC GCTGCATTTG CCGGTGTTCA AGTCGCACAG
```

Kappa Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

GANCCTTCCTCTATATAAGGAAGTTCATTTNNTTTGGAGAGAACACGGGGGACTCTAGAACAATGGCGACG
ACCTTAAGCAGAGATCAATATGTCTACATGGCGAAGCTCGCCGAGCAAGCCGAGCGTTACGAAGAGATGGT
TCAATTCATGGAACAGCTCGTAAGTGGAGCTACACCGGCCGGTGAGCTGACCGTAGAAGAGAGGAATCTTC
TCTCGGTCGCGTATAAGAACGTGATTGGATCTCTTCGTGCGGCATGGAGAATCGTGTCTTCGATTGAGCAA
AAGGAAGAGAGCAGGAAGAACGAAGAACACGTGTCGCTTGTTAAGGATTACAGATCTAAAGTTGAGACTGA
GCTTTCTTCGATCTGTTCTGGGATTCTCAGGTTACTTGATTCGCATCTAATTCCTTCAGCTACTGCCAGTG
AGTCTAAGGTTTTTTACCTGAAGATGAAAGGAGATTATCATCGNTATTTGGCTGAGTTTAAATCTGGTGAT
GAGAGGAAAACTGCTGCTGAAGATACTATGATCGCTTACAAAGCTGCTCAGGACGTTGCAGTTGCTGATCT
AGCACCTACACATCCGATCAGGCTTGGTTTGGCTCTTAACTTCTCAGTGTTTTACTACGAGATTCTCAACT
CTTCAGAGAAAGCTTGTANCATGGCNAAACAGGCTTTTGAANAANCCATTGCTGANCTGGACACATTGGGA
AGAGGAGTCATACAAGGACAGTACTCTCATCATGCACTTGCCTAANGGACNATCTAACCCTTTTGGACCTC
CNATATTCCANGGAGCANATNGGATTAGGCCCNTANAATGGTNAACCNNGGGCCAAN

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

TTGNAGCCCCCTGCTAGANCANNATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTCTA
GAGGCCTCATCCATCTGCTCCTGCATATCGGAGGTCCAAAGGGTTAGATTGTCCCTTAGCAACTGCATGAT
GAGAGTACTGTCCTTGTATGACTCCTCTCCCAATGTGTCCAGCTCAGCAATGGCTTCTTCAAAAGCCTGTT
TCGCCATGCTACAAGCTTTCTCTGAAGAGTTGAGAATCTCGTAGTAAAACACTGAGAAGTTAAGAGCCAAA
CCAAGCCTGATCGGATGTGTAGGTGCTAGATCAGCAACTGCAACGTCCTGAGCAGCTTTGTAAGCGATCAT
AGTATCTTCAGCAGCAGTTTTCCTCTCATCACCAGATTTAAACTCAGCCAAATAACCGATGATAATCTCCT
TTCATCTTCAGGTAAAAAACCTTAGACTCACTGGCAGTAGCTGAAGGAATTANATGCGAATCAAGTAACCT
GAGAATCCCAGAACAGATCGAAGAAAGCTCAGTCTCAACTTTAGATCTGTAATCCTTAACAAGCGACACNT
GTTCTTNGTTCTTCCTGCTCTCTTCCTTTTGCTCAATCCGAAGACACGATTCTCCATGCCGCACGAAGAGA
TCCAATCACCGTTCNTATACNGCNACCGAGAGAAGATTCCTCTCTTCTACCGTCAAGTTCACCGGCCCGGT
GTAACTCCACTTACGAACCTGTTCCTTGNATTGAACCATCNTCNTCCCTAAACCGCTCNGCCTTGCTCGGC
GAAGCNTTCCCNTGGTNGACNTNATTGAACCTCCTGCTTAAAGGNCCCCCTCTTTGCTCCTANAACTCCCC
CCCCT

Lambda Isoform

```
 1    CTAGAACAAT GGCGGCGACA TTAGGCAGAG ACCAGTATGT GTACATGGCG
      GATCTTGTTA CCGCCGCTGT AATCCGTCTC TGGTCATACA CATGTACCGC

 51   AAGCTCGCCG AGCAGGCGGA GCGTTACGAA GAGATGGTTC AATTCATGGA
      TTCGAGCGGC TCGTCCGCCT CGCAATGCTT CTCTACCAAG TTAAGTACCT

101   ACAGCTCGTT ACAGGCGCTA CTCCAGCGGA AGAGCTCACC GTTGAAGAGA
      TGTCGAGCAA TGTCCGCGAT GAGGTCGCCT TCTCGAGTGG CAACTTCTCT

151   GGAATCTCCT CTCTGTTGCT TACAAAAACG TGATCGGATC TCTACGCGCC
      CCTTAGAGGA GAGACAACGA ATGTTTTTGC ACTAGCCTAG AGATGCGCGG

201   GCCTGGAGGA TCGTGTCTTC GATTGAGCAG AAGGAAGAGA GTAGGAAGAA
      CGGACCTCCT AGCACAGAAG CTAACTCGTC TTCCTTCTCT CATCCTTCTT

251   CGACGAGCAC GTGTCGCTTG TCAAGGATTA CAGATCTAAA GTTGAGTCTG
      GCTGCTCGTG CACAGCGAAC AGTTCCTAAT GTCTAGATTT CAACTCAGAC

301   AGCTTTCTTC TGTTTGCTCT GGAATCCTTA AGCTCCTTGA CTCGCATCTG
      TCGAAAGAAG ACAAACGAGA CCTTAGGAAT TCGAGGAACT GAGCGTAGAC

351   ATCCCATCTG CTGGAGCGAG TGAGTCTAAG GTCTTTTACT TGAAGATGAA
      TAGGGTAGAC GACCTCGCTC ACTCAGATTC CAGAAAATGA ACTTCTACTT

401   AGGTGATTAT CATCGGTACA TGGCTGAGTT TAAGTCTGGT GATGAGAGGA
      TCCACTAATA GTAGCCATGT ACCGACTCAA ATTCAGACCA CTACTCTCCT

451   AAACTGCTGC TGAAGATACC ATGCTCGCTT ACAAAGCAGC TCAGGATATC
      TTTGACGACG ACTTCTATGG TACGAGCGAA TGTTTCGTCG AGTCCTATAG

501   GCAGCTGCGG ATATGGCACC TACTCATCCG ATAAGGCTTG GTCTGGCCCT
      CGTCGACGCC TATACCGTGG ATGAGTAGGC TATTCCGAAC CAGACCGGGA

551   GAATTTCTCA GTGTTCTACT ATGAGATTCT CAATTCTTCA GACAAAGCTT
      CTTAAAGAGT CACAAGATGA TACTCTAAGA GTTAAGAAGT CTGTTTCGAA

601   GTAACATGGC CAAACAGGCT TTTGAGGAGG CCATAGCTGA GCTTGACACT
      CATTGTACCG GTTTGTCCGA AAACTCCTCC GGTATCGACT CGAACTGTGA

651   CTGGGAGAGG AATCCTACAA AGACAGCACT CTCATAATGC AGTTGCTGAG
      GACCCTCTCC TTAGGATGTT TCTGTCGTGA GAGTATTACG TCAACGACTC

701   GGACAATTTA ACCCTTTGGA CCTCCGATAT GCAGGAGCAG ATGGACGAGG
      CCTGTTAAAT TGGGAAACCT GGAGGCTATA CGTCCTCGTC TACCTGCTCC

751   CCTCTAGAAT GGTGAGCAAG GGCGAGGAGC TGTTCACCGG GGTGGTGCCC
      GGAGATCTTA CCACTCGTTC CCGCTCCTCG ACAAGTGGCC CCACCACGGG

801   ATCCTGGTCG AGCTGGACNG CGACGTAAAC GGCCACAAGT TCAGCGTGTC
      TAGGACCAGC TCGACCTGNC GCTGCATTTG CCGGTGTTCA AGTCGCACAG
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Lambda Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

ATAANGAAGTTCATTTNTTTGGAGAGAACACGGGGGACTCTAGAACAATGGCGGCGACATTAGGCAGAGAC
CAGTATGTGTACATGGCGAAGCTCGCCGANCANGCGGAGCGTTACGAAGAGATGGTTCAATTCATGGAACA
GCTCGTTACAGGCGCTACTCCAGCGGAAGAGCTCACCGTTGAAGAGAGGAATCTCCTCTCTGTTGCTTACA
AAAACGTGATCGGATCTCTACGCGCCGCCTGGAGGATCGTGTCTTCNATTGAGCANAAGGAAGAGAGTANG
AAGAACGACGAGCACGTGTCGCTTGTCAAGGATTACAGATCTAAAGTTGANTCTGAGCTTTCTTCTGTTTG
CTCTGGAATCCTTAANCTCCTTGACTCGCATTTGATCCCATCTGCTGGACCGAGTGAGNCTAANGTCTTTN
ACTTNGAAGATGAAAGGTGATTATCNTCGGCACATGGCTGAGNTTCAGNCTGGTGATGATAGGANAACTGC
TNCTGAANNATACCCTGCTCGCTTACAAAGNACATCAGGATATCNCAACCTGTNGATATGGCACNTACTCA
TTCCNNTAAGGCTTCGNTCTGGCCCTGGAATTTCTCNGNGTNTCTCTATGAGATNCNNAANNTNTTCAGAC
CAACNCTTGTNATNATNGCCNTACNNGCTTCTNANGAGGCCATACCCNNAACCTTTGACCNCTCTNGNNGC
AGNACGGANTCCTTCTANAGCCCAGCNCTNCTCCATAANTNTAAGTTTNCTNNAACGGTACCTNCTTTAAA
TCCTTTTCGGACTCCTCCTNNTATTGCCCANTATCTNTCTTGNNCNTNAGCCCTNNTNTCAATCNCTCNAC
CNATNTNGCCNANNT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

CCACCTTCTAANGNTNTCNNTNNANNTAACAANTCNTTATNCNNNNNNCNNCCNCCCCNNNNCCCCCCAGA
AAAAANNGNGGNGNNNNNNNNNTNNTGGGGTNANATNGGAATATTGGTCGATTCGTCGCCGATCACTCGTA
CGGAGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTCTAGAGGCCTCGTCCATCTGCTCC
TGCATATCGGAGGTCCAAAGGGTTAANNTGTCCCTCATCAACTGCATTATGAGAGTGCTGTCTTTGTAGGA
TTCCTCTCCCAGAGTGTCAAGCTCAGCTATGGCCTCCTCAAAAGCCTGTTTGGCCATGTTACAAGCTTTGT
CTGAAGAATTGAGAATCTCATAGTAGAACACTGAGAAATTCAGGGCCAGACCAAGCCTTATCGGATGAGTA
GGTGCCATATCCGCAGCTGCGATATCCTGAGCTGCTTTGTAAGCNANCATGGTATCTTCAACAGCANNTTT
CCTCTCATCACCAGACTTAAACTCANCCATGTACCGATGATAATCACCTTTCATCTTCNAGTAAAAGACCT
TAGACTCACTCGCTCCAGCAGATGGGATCACATGCCAGTCAAGGAGCTTAAGGATTCATAGCAAACNGNAN
CAAAGCTCATACTCANCTTTACATCTGTAATNCTTGACAATGCNACNCGAGCTCGTCGCCCTTCCTACTCT
CTTCCTTNTTGCTCAATCGAAGACNCGATCCTCCANACCGGNCCGTANACGAACCCAANCACTTTTTTTNN
AAATCAACANTAATAGGACATNTCTCTTCTTNACNGTGAGCTCTTTTCGNTTGGNNNTANCCGCCTCTNAN
CTAAGCTGCCTCCNTNANATTGNACCTNATTCTNTTCCTAACCNCCTCCCCCTGCCTNCNGTAANCCTTCT
NCCTGCACACNTANNATGNNTCTCTTGNNCNNANTNNTTGTCCCCCATTTGNTCCAANANANCCCCCCNNN
G

Omega Isoform

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1       CTAGTATGGC GTCTGGGCGT GAAGAGTTCG TATACATGGC TAAGCTCGCG
        GATCATACCG CAGACCCGCA CTTCTCAAGC ATATGTACCG ATTCGAGCGC

51      GAGCAAGCGG AGAGGTACGA AGAGATGGTA GAGTTTATGG AGAAAGTCTC
        CTCGTTCGCC TCTCCATGCT TCTCTACCAT CTCAAATACC TCTTTCAGAG

101     CGCCGCCGTT GATGGCGATG AACTCACCGT AGAAGAGCGA AATCTTCTCT
        GCGGCGGCAA CTACCGCTAC TTGAGTGGCA TCTTCTCGCT TTAGAAGAGA

151     CCGTCGCTTA TAAGAATGTG ATTGGTGCTC GCCGTGCCTC GTGGCGTATC
        GGCAGCGAAT ATTCTTACAC TAACCACGAG CGGCACGGAG CACCGCATAG

201     ATTTCATCGA TCGAGCAGAA GGAAGAGAGC CGTGGTAACG ATGACCACGT
        TAAAGTAGCT AGCTCGTCTT CCTTCTCTCG GCACCATTGC TACTGGTGCA

251     CACGGCGATC CGTGAATATA GGTCTAAGAT CGAGACGGAA CTCTCCGGAA
        GTGCCGCTAG GCACTTATAT CCAGATTCTA GCTCTGCCTT GAGAGGCCTT

301     TCTGCGACGG AATCCTTAAG TTGCTTGACT CTAGACTCAT CCCTGCCGCT
        AGACGCTGCC TTAGGAATTC AACGAACTGA GATCTGAGTA GGGACGGCGA

351     GCTTCTGGTG ATTCCAAGGT CTTTTACCTT AAGATGAAGG GAGATTATCA
        CGAAGACCAC TAAGGTTCCA GAAAATGGAA TTCTACTTCC CTCTAATAGT

401     CAGGTACTTG GCTGAGTTTA AGACTGGTCA AGAGAGGAAA GACGCCGCCG
        GTCCATGAAC CGACTCAAAT TCTGACCAGT TCTCTCCTTT CTGCGGCGGC

451     AACATACACT CGCCGCTTAC AAATCTGCTC AGGATATTGC TAATGCAGAG
        TTGTATGTGA GCGGCGAATG TTTAGACGAG TCCTATAACG ATTACGTCTC

501     CTTGCTCCAA CACACCCAAT TCGTCTTGGT CTTGCATTGA ACTTCTCTGT
        GAACGAGGTT GTGTGGGTTA AGCAGAACCA GAACGTAACT TGAAGAGACA

551     GTTCTATTAC GAGATCCTCA ATTCTCCTGA TCGTGCCTGT AACCTCGCCA
        CAAGATAATG CTCTAGGAGT TAAGAGGACT AGCACGGACA TTGGAGCGGT

601     AACAGGCCTT TGATGAGGCG ATTGCAGAGT TGGACACTCT TGGTGAAGAG
        TTGTCCGGAA ACTACTCCGC TAACGTCTCA ACCTGTGAGA ACCACTTCTC

651     TCATACAAAG ACAGTACCTT GATCATGCAG CTTCTTCGTG ACAATCTCAC
        AGTATGTTTC TGTCATGGAA CTAGTACGTC GAAGAAGCAC TGTTAGAGTG

701     TCTCTGGACA TCTGATATGC AGGATGATGC TGCGGATGAG ATCAAGGAAG
        AGAGACCTGT AGACTATACG TCCTACTACG ACGCCTACTC TAGTTCCTTC

751     CAGCAGCGCC AAAACCGACC GAGGAACAGC AGACTAGAAT GGTGAGCAAG
        GTCGTCGCGG TTTTGGCTGG CTCCTTGTCG TCTGATCTTA CCACTCGTTC

801     GGCGAGGAGC TGTTCACCGG GGTGGTGCCC ATCCTGGTCG AGCTGGACNG
        CCGCTCCTCG ACAAGTGGCC CCACCACGGG TAGGACCAGC TCGACCTGNC
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Omega Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

GACCTTCTCTTATAAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGTACAATGGCGTCTGGG
CGTGAAGAGTTCGTATACATGGCTAAGCTCGCGGAGCAAGCGGAGAGGTACGAAGAGATGGTAGAGTTTAT
GGAGAAAGTCTCCGCCGCCGTTGATGGCGATGAACTCACCGTAGAAGAGCGAAATCTTCTCTCCGTCGCTT
ATAAGAATGTGATTGGTGCTCGCCGTGCCTCGTGGCGTATCATTTCATCGATCGAGCAGAAGGAAGAGAGC
CGTGGTAACGATGACCACGTCGCGGCGATCCGTGAATATAGGTCTAAGATCGAGACGGAACTCTCCGGAAT
CTGCGACGGAATCCTTAAGTTGCTTGACTCTAGACTCATCCCTGCCGCTGCTTCTGGTGATTCCAAGGTCT
TTTACCTTAAGATGAAGGGAGATTATCACAGGTACTTGGCTGAGTTTAAGACTGGTCAAGAGAGGAAAGAC
GCCGCCGAACATACACTCGCCGCTTACAAATCTGCTCAGGATATTGCTAATGCAGACTTGCTCCAACACAC
CCAATTCGTCTTGGCTTGCATTGAACTTCTCTGTGTTCTATTACGAGATCCTCAATTCTCCTGATCGTGCC
TGTTACCTCGCCAAACANGCCTTTTATGAAGCGATTGCAGAATTTGGACACTCTTTGGTGAAGANNCNTAC
AAAGACAGTACCCTTGATCTTCNNCTTNTTCNTGGACNATTTTNACTTTNTTGGACCTTNTTGATATCCAG
GATTANTGCCTGNNGGATGAAAACNAAGGGAACCNNNNNCNCCCAAAACCCNCCCGNGGNANCNNCCNNAC
TTANAATTNGNNNANCNANGGGGCCAANGANCNTTTTCCCCGGGGNGGGNNGCCCNTCTGNTCAACNTGGA
CGGGNANCTAAAACNGNCNCNANTTTNANNCTTTTCCCNCNAGGGGCNANGGGNGANTCCCCCTTNNNGNA
ANCTNNCCTTNAATTNTTTTNANNCCCGNNANNTNCCGGCCTTGCCCCCTTTNACCCTTNNTTAGGTTNNN
TNTTNNCCNTNCCNCCCTTAAANA
NNNAN

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

CGTATACGTCGNCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACC
ATTCTAGTCTGCTGTTCCTCGGTCGGTTTTGGCGCTGCTGCTTCCTTGATCTCATCCGCAGCATCATCCTG
CATATCAGATGTCCAGAGAGTGAGATTGTCACGAAGAAGCTGCATGATCAAGGTACTGTCTTTGTATGACT
CTTCACCAAGAGTGTCCAACTCTGCAATCGCCTCATCAAAGGCCTGTTTGGCGAGGTTACAGGCACGATCA
GGAGAATTGAGGATCTCGTAATAGAACACAGAGAAGTTCAATGCAAGACCAAGACGAATTGGGTGTGTTGG
AGCAAGCTCTGCATTAGCAATATCCTGAGCAGATTTGTAAGCGGCGAGTGTATGTTCGGCGGCGTCTTTCC
TCTCTTGACCAGTCTTAAACTCAGCCAAGTACCTGTGATAATCTCCCTTCATCTTAAGGTAAAAGACCTTG
GAATCACCAGAAGCAGCGGCAGGGATGAGTCTAGAGTCAAGCAACTTAAGGATTCCCGTCGCANATTCCGG
AGAGTTCCGTCTCGATCTTAGACCTATATTCACGGATCGCCGCGACGTGGTCATCGTTACCACNGNTCTCT
TNCTTTNCTCGATCGATGAAATGATACGCCACGANGCACNGCGAGCCCCAATCACATTCTTATAAGCNACC
GGAGAGAANAATTCGCTCTTNTTCCGNGAGTTCATCCCCTTCACNGGGGGGGGAGACTTTNTCCATAAACT
CTTCCATCTCTTTNNACCTTTCCNCTTTGCTCCGCGAGCTTAACCNTTGTTTTCCAAACTTTTTCACNCCC
AAACCCCNTTGTACTAAAATCCCCCCGGGTTTNTCCANATGAAANTGAACTTTCCTTTTTNAAAAGGAAAG
GNCNTTCCCAAAAGGAAAANGGGGATTGNGNNGTNNTNCCCTTTCANTNNGNGGGGGNANATTNNCATTCA
ATCCCCCTTTCCTTTTAAAAANANNGNNTGGGNACCNNNTTTTTTTTTTTCCCNAAGCCCCCNCNGGGGGG
GGGGGGGGCCNTTTTTTGGGNNCNCNNNNNNGNNNNAAAAGNTCTTTTNANNNANNGCT

Phi Isoform

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1     CTAGAACAAT GGCGGCACCA CCAGCATCAT CCTCGGCGAG GGAAGAGTTC
      GATCTTGTTA CCGCCGTGGT GGTCGTAGTA GGAGCCGCTC CCTTCTCAAG

51    GTGTACCTCG CAAAGCTCGC AGAGCAAGCG GAACGTTACG AAGAAATGGT
      CACATGGAGC GTTTCGAGCG TCTCGTTCGC CTTGCAATGC TTCTTTACCA

101   TGAATTCATG GAAAAAGTCG CTGAAGCCGT TGACAAAGAC GAACTCACCG
      ACTTAAGTAC CTTTTTCAGC GACTTCGGCA ACTGTTTCTG CTTGAGTGGC

151   TCGAAGAACG TAATCTCCTC TCCGTCGCTT ACAAAAACGT AATCGGCGCT
      AGCTTCTTGC ATTAGAGGAG AGGCAGCGAA TGTTTTTGCA TTAGCCGCGA

201   CGTCGTGCTT CCTGGAGAAT CATCTCTTCC ATTGAACAAA AAGAAGAGAG
      GCAGCACGAA GGACCTCTTA GTAGAGAAGG TAACTTGTTT TTCTTCTCTC

251   TCGTGGTAAC GATGACCATG TGACCACGAT CCGTGATTAC AGAAGCAAGA
      AGCACCATTG CTACTGGTAC ACTGGTGCTA GGCACTAATG TCTTCGTTCT

301   TCGAATCTGA GTTATCGAAA ATCTGTGACG GTATTCTTAA GCTTCTTGAT
      AGCTTAGACT CAATAGCTTT TAGACACTGC CATAAGAATT CGAAGAACTA

351   ACTAGACTTG TTCCTGCTTC TGCTAATGGA GATTCTAAGG TTTTTTACCT
      TGATCTGAAC AAGGACGAAG ACGATTACCT CTAAGATTCC AAAAAATGGA

401   TAAGATGAAG GGAGATTATC ATAGGTATTT GGCTGAGTTT AAGACTGGAC
      ATTCTACTTC CCTCTAATAG TATCCATAAA CCGACTCAAA TTCTGACCTG

451   AAGAGAGGAA AGATGCTGCT GAACATACTC TCACCGCTTA CAAAGCTGCT
      TTCTCTCCTT TCTACGACGA CTTGTATGAG AGTGGCGAAT GTTTCGACGA

501   CAGGATATTG CTAATGCTGA ATTGGCTCCA ACGCATCCGA TTCGTCTTGG
      GTCCTATAAC GATTACGACT TAACCGAGGT TGCGTAGGCT AAGCAGAACC

551   TTTGGCTTTG AATTTCTCTG TGTTTTACTA TGAGATTCTT AATTCTCCAG
      AAACCGAAAC TTAAAGAGAC ACAAAATGAT ACTCTAAGAA TTAAGAGGTC

601   ATCGTGCTTG TAATCTCGCT AAGCAGGCGT TTGATGAAGC CATTGCTGAG
      TAGCACGAAC ATTAGAGCGA TTCGTCCGCA AACTACTTCG GTAACGACTC

651   TTAGATACTC TTGGAGAGGA GTCATACAAG GATAGTACCT TGATCATGCA
      AATCTATGAG AACCTCTCCT CAGTATGTTC CTATCATGGA ACTAGTACGT

701   GCTTCTTCGT GACAATCTTA CTCTCTGGAC TTCTGACATG CAGGACGAAA
      CGAAGAAGCA CTGTTAGAAT GAGAGACCTG AAGACTGTAC GTCCTGCTTT

751   GTCCGGAGGA GATTAAAGAA GCAGCAGCAC CAAAGCCTGC TGAAGAACAG
      CAGGCCTCCT CTAATTTCTT CGTCGTCGTG GTTTCGGACG ACTTCTTGTC

801   AAGGAGATCT CTAGAATGGT GAGCAAGGGC GAGGAGCTGT TCACCGGGGT
      TTCCTCTAGA GATCTTACCA CTCGTTCCCG CTCCTCGACA AGTGGCCCCA

851   GGTGCCCATC CTGGTCGAGC TGGACNGCGA CGTAAACGGC CACAAGTTCA
      CCACGGGTAG GACCAGCTCG ACCTGNCGCT GCATTTGCCG GTGTTCAAGT
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Phi Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

TCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGAACACGGGGGACTCTAGAACAATGGCGGCACCACCA
GCATCATCCTCGGCGAGGGAAGAGTTCGTGTACCTCGCAAAGCTCGCAGAGCAAGCGGAACGTTACGAAGA
AATGGTTGAATTCATGGAAAAAGTCGCTGAAGCCGTTGACATAGACGAACTCACCGTCGAAGAACGTAATC
TCCTCTCCGTCGCTTACAAAAACGTAATCGGCGCTCGTCGTGCTTCCTGGAGAATCATCTCTTCCATTGAA
CAAAAAGAAGAGAGTCGTGGTAACGATGACCATGTGACCACGATCCTTGATTACAGAAGCAAGATCGAATC
TGAGTTATCGAAAATCTGTGACGGTATTCTTAAGCTTCTTGATACTAGACTTGTTCCTGCTTCTGCTAATG
GAGATTCTAAGGTTTTTTACCTTAAGATGAAGGGAGATTATCATAGGTATTTGGCTGAGTTTAAGACTGGT
CAAGAGAGGAAAGATGCTGCTGAACATACTCTCACCGCTTACAAAGCTGCTCAGGATATTGCTAATGCTGA
ATTGGCTCCAACGCATCCGATTCGTCTTGGTTTGGCTTTGAATTTCTCTTGTGTTTTCTATGAAGATTCTT
AATTCTCCAGATCGTGCTTGNAATCTCCTTAACAGGCGTTTGATGAACCCTTGCTGAGTTAGATACTCTTG
GANAGGAGTCATCCANGGATAGTACCTTGGTTCATGCATCTTCTTTCGTGACATTNNTACTTCTTTTGGGA
CTTCTGANATGCAGGGACANAAAAGTCCGGGNNGGAGNATTAAAGAAANCANCTGCCCCANAACCCTGGTT
GNAAAANCTTCNNGNAAANNCTNTTGAATGGGGNAACNATGGGCNNANGANCTTGTTCNCCGGGGTGGGGG
CCCATTCCTGGNNAAACCTGGACGGNGGAAATTAAAACGGCCACCAAGTTTNNNCGTTNTCCCGGCTAAGG
GCANAGGGCNTATCCCCNCTTCTGGTNTGTTCACCCTTGAANGTNATTTGCNNCCCCGG

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

GTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTCTAG
AGATCTCCTGCTGTTCTTCAGCAGGCTTTGGTGCTGCTGCTTCTTTAATCTCCTCCGGACTTTCGTCCTGC
ATGTCAGAAGTCCAGAGAGTAAGATTGTCACGAAGAAGCTGCATGATCAAGGTACTATCCTTGTATGACTC
CTCTCCAAGAGTATCTAACTCAGCAATGGCTTCATCAAACGCCTGCTTAGCGAGATTACAAGCACGATCTG
GAGAATTAAGAATCTCATAGTAAAACACAGAGAAATTCAAAGCCAAACCAAGACGAATCGGATGCGTTGGA
GCCAATTCAGCATTAGCAATATCCTGAGCAGCTTTGTAAGCGGTGAGAGTATGTTCAGCAGCATCTTTCCT
CTCTTGACCAGTCTTAAACTCAGCCAAATACCTATGATAATCTCCCTTCATCTTANGGTAAAAAACCTTAG
AATCTCCATTAGCAGAAGCAGGAACAAGTCTAGTATCAAGAAGCTTAAGAATACCGTCACAGATTTTCGAT
AACTCANATTCGATCTTGCTTCTGTAATCAAGGATCGTGGTCACATGGTCATCGNTACCACGACTCTTTCT
TTTTGTTCAATGGAAGAAGATGATTCTCCANGAAGCACGACGAGCCGCCGAATTNCGTTTTTGGTAAGCCG
ACCNGAGAAGGANATTACGTTCTTTNGACGGNGANGTTCNNTNTATGNNAACCGGGTTTNGGCGGNCNTTT
NTCCNATGGAATTNCANCCATTTCTTCTTANCNGTTNNGCTTGGTTNTGGCNAAGCTNTTGCNAANGGANC
NCNGAANTTNTTCCTTTGNCCNGAGGANGAATGCTTNGTGGGGGGCCNCCACTTGTTCNCNNAAGANCTCN
CCGGNGGGCTTTNCNTCAANGNNAANGNAATNTCCTTTTATTCNAANAAAGGGCCTGCTANAGGANAGTGG
GGAGNNGNNCGNAANCCNTAANGNNCGGGGNGAAATNCATTAANNCTCANGGCTTANANACCN

Upsilon Isoform

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 1    CTAGAACAAT GTCTTCTGAT TCGTCCCGGG AAGAGAATGT GTACTTGGCC
      GATCTTGTTA CAGAAGACTA AGCAGGGCCC TTCTCTTACA CATGAACCGG

51    AAGTTAGCCG AGCAAGCTGA GCGTTACGAG GAAATGGTTG AGTTCATGGA
      TTCAATCGGC TCGTTCGACT CGCAATGCTC CTTTACCAAC TCAAGTACCT

101   GAAAGTTGCA AAGACCGTGG AGACCGAGGA ACTTACTGTT GAAGAGAGGA
      CTTTCAACGT TTCTGGCACC TCTGGCTCCT TGAATGACAA CTTCTCTCCT

151   ATCTCTTGTC TGTTGCTTAC AAGAACGTGA TTGGTGCTAG GAGAGCTTCT
      TAGAGAACAG ACAACGAATG TTCTTGCACT AACCACGATC CTCTCGAAGA

201   TGGAGGATTA TCTCTTCCAT TGAGCAGAAG GAAGATAGCA GGGGCAACAG
      ACCTCCTAAT AGAGAAGGTA ACTCGTCTTC CTTCTATCGT CCCCGTTGTC

251   TGATCATGTT TCGATTATCA AGGATTACAG AGGCAAGATT GAAACTGAGC
      ACTAGTACAA AGCTAATAGT TCCTAATGTC TCCGTTCTAA CTTTGACTCG

301   TCAGCAAGAT TTGTGATGGC ATTTTGAACC TTCTTGAGGC TCATCTCATT
      AGTCGTTCTA AACACTACCG TAAAACTTGG AAGAACTCCG AGTAGAGTAA

351   CCTGCTGCTT CTTTGGCTGA GTCCAAAGTT TTTTACCTGA AGATGAAGGG
      GGACGACGAA GAAACCGACT CAGGTTTCAA AAAATGGACT TCTACTTCCC

401   AGATTATCAT CGGTACCTTG CTGAATTCAA GACTGGTGCT GAGAGGAAAG
      TCTAATAGTA GCCATGGAAC GACTTAAGTT CTGACCACGA CTCTCCTTTC

451   AAGCTGCTGA GAGCACTCTT GTTGCCTACA AGTCTGCTCA GGATATTGCT
      TTCGACGACT CTCGTGAGAA CAACGGATGT TCAGACGAGT CCTATAACGA

501   CTTGCTGATC TGGCTCCCAC TCACCCAATC AGACTGGGGC TTGCTCTTAA
      GAACGACTAG ACCGAGGGTG AGTGGGTTAG TCTGACCCCG AACGAGAATT

551   CTTCTCTGTT TTCTACTATG AGATTCTCAA CTCATCTGAT CGTGCGTGTA
      GAAGAGACAA AAGATGATAC TCTAAGAGTT GAGTAGACTA GCACGCACAT

601   GTCTCGCAAA GCAGGCTTTT GATGAGGCAA TCTCGGAGCT AGACACATTG
      CAGAGCGTTT CGTCCGAAAA CTACTCCGTT AGAGCCTCGA TCTGTGTAAC

651   GGAGAGGAAT CATACAAGGA CAGTACATTG ATCATGCAGC TTCTCCGTGA
      CCTCTCCTTA GTATGTTCCT GTCATGTAAC TAGTACGTCG AAGAGGCACT

701   CAATCTCACC CTCTGGACTT CTGACCTCAA TGACGAAGCT GGTGATGATA
      GTTAGAGTGG GAGACCTGAA GACTGGAGTT ACTGCTTCGA CCACTACTAT

751   TCAAGGAAGC CCCGAAAGAG GTGCAGAAAG TTGATGAACA AGCCCAACCA
      AGTTCCTTCG GGGCTTTCTC CACGTCTTTC AACTACTTGT TCGGGTTGGT

801   CCACCTTCGC AGTCTAGAAT GGTGAGCAAG GGCGAGGAGC TGTTCACCGG
      GGTGGAAGCG TCAGATCTTA CCACTCGTTC CCGCTCCTCG ACAAGTGGCC

851   GGTGGTGCCC ATCCTGGTCG AGCTGGACNG CGACGTAAAC GGCCACAAGT
      CCACCACGGG TAGGACCAGC TCGACCTGNC GCTGCATTTG CCGGTGTTCA
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Upsilon Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

TGNNNACGCCCGCAGNACCTTCTCTATATAAGGAAGTTNTTTCTTTGGAGAGAACACGGGGGACTCTAGAA
CAATGTCTTCTGATTCGTCCCGGGAAGAGAATGTGTACTTGGCCAAGTTAGCCGAGCAAGCTGAGCGTTAC
GAGGAAATGGTTGAGTTCATGGAGAAAGTTGCAAAGACCGTGGAGACCGAGGAACTTACTGTTGAAGAGAG
GAATCTCTTGTCTGTTGCTTACAAGAACGTGATTGGTGCTAGGAGAGCTTCTTGGAGGATTATCTCTTCCA
TTGAGCAGAAGGAAGATAGCAGGGGCAACAGTGATCATGTTTCGATTATCAAGGATTACAGAGGCAAGATT
GAAACTGAGCTCANCAAGATTTGTGATGGCATTTTGAACCTTCTTGAGGCTCATCTCATTCCTGCTGCTTC
TTTGGCTGAGTCCAAAGTTTTTTACCTGAAGATGAAGGGAGATTATCATCGGTACCTTGCTGAATTCAAGA
CTGGTGCTGAGAGGAAAGAAGCTGCTGAGAGCACTCTTGTTGCCTACAAGTCTGCTCAGGATATTGCTCTT
GCTGATCTGGCTCCCACTCACCCAATCAGACTGGGGCTTGCTCTTAACTTCTCTGTTTTCTACTATGAGAT
TCTCAACTCATCTGATCGTGCGTGTAGTCTCNCAAAGCANGCTTTTGATGAGGCAATCTCGGAGCTAGACA
CATTGGGAGANGAATCATTCAAGGACAGNACATTGATCATGCAGCTTCTCCGTGACAATCTCACCCTCTGG
ACTTCTGCCTCAANTGACCAAACTGGTGATGATATCAACGGAAGCCCCGANNT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

GNNTNANGANNCCCCNNNGGTTTGCCGTCNCGTAAACTCCGCTCGACTNNAATGGGCACCACCCNGGTGAA
CAGCTCCTCGCCCTTGCTCACCATTCTAGACTGCGAAGGTGGTGGTTGGGCTTGTTCATCAACTTTCTGCA
CCTCTTTCGGGGCTTCCTTGATATCATCACCAGCTTCGTCATTGAGGTCAGAAGTCCAGAGGGTGAGATTG
TCACGGAGAAGCTGCATGATCAATGTACTGTCCTTGTATGATTCCTCTCCCAATGTGTCTAGCTCCGAGAT
TGCCTCATCAAAAGCCTGCTTTGCGAGACTACACGCACGATCAGATGAGTTGAGAATCTCATAGTAGAAAA
CAGAGAAGTTAAGAGCAAGCCCCAGTCTGATTGGGTGAGTGGGAGCCAGATCAGCAAGAGCAATATCCTGA
GCAGACTTGTAGGCAACAAGAGTGCTCTCAGCAGCTTCTTTCCTCTCAGCACCAGTCTTGAATTCAGCAAG
GTACCGATGATAATCTCCCTTCATCTTCAGGTAAAAAACTTTGGACTCAGCCAAAGAAGCAGCAGGAATGA
GATGAGCCTCAAGAAGGTTCAAAATGCCATCACAAATCTTGCTGAGCTCAGTTTCAATCTTGCCTCTGTAA
TCCTTGATAATCGAACATGATCACTGNTGCCCCTGCTATCTTCCTTCTGCTCAATGGAAGAGATAATCCTC
AAGAAGCTCTCCTAGCACCAATCACGTTCTTGGAAGCAACAGACAAGANAATCCTCTCTTCAACAGTAAGT
TCCTCGGTCTCCACGGTCTTTTGCACTTTNTTCATGAACTAACCATTTCCTCGTAACNCTTAACTTGGCTC
GNTAACTGGGCCAAGNNCACATTNTNTTCCCGGGACGAATCANAAAAAATTGTTTTNNAATCCCCCGGGTT
NTTTCAAATGAAANGAANTTCNTTTTTANANGAAGGGNCTTNCNAAGGAAAANGGGAATNGGNCNNNTTCC
TTNCNCNNGGGNAAATCCNTNANCCNCTTGTTTNAAAAANNGGNNGGACNNTTTTTTTNCCNANNNNCC

Nu Isoform

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1      CTAGAACAAT GTCGTCTTCT CGGGAAGAGA ATGTGTACTT AGCCAAGTTA
       GATCTTGTTA CAGCAGAAGA GCCCTTCTCT TACACATGAA TCGGTTCAAT

51     GCTGAGCAAG CTGAACGTTA TGAGGAAATG GTTGAGTTCA TGGAGAAAGT
       CGACTCGTTC GACTTGCAAT ACTCCTTTAC CAACTCAAGT ACCTCTTTCA

101    TGCAAAGACT GTTGACACCG ATGAGCTTAC TGTCGAAGAG AGAAACCTCT
       ACGTTTCTGA CAACTGTGGC TACTCGAATG ACAGCTTCTC TCTTTGGAGA

151    TGTCTGTTGC TTACAAGAAC GTCATTGGTG CTAGGAGAGC TTCCTGGAGG
       ACAGACAACG AATGTTCTTG CAGTAACCAC GATCCTCTCG AAGGACCTCC

201    ATCATATCTT CCATTGAACA GAAGGAAGAA AGCAGAGGAA ACGATGATCA
       TAGTATAGAA GGTAACTTGT CTTCCTTCTT TCGTCTCCTT TGCTACTAGT

251    TGTTTCCATT ATCAAGGACT ACAGAGGAAA GATCGAAACT GAACTCAGCA
       ACAAAGGTAA TAGTTCCTGA TGTCTCCTTT CTAGCTTTGA CTTGAGTCGT

301    AAATCTGTGA TGGAATACTC AATCTTCTGG ATTCTCACCT TGTTCCCACT
       TTTAGACACT ACCTTATGAG TTAGAAGACC TAAGAGTGGA ACAAGGGTGA

351    GCATCTTTGG CCGAGTCCAA AGTCTTTTAC CTCAAAATGA AAGGAGATTA
       CGTAGAAACC GGCTCAGGTT TCAGAAAATG GAGTTTTACT TTCCTCTAAT

401    CCACAGGTAC CTTGCTGAGT TTAAGACTGG AGCTGAGAGG AAAGAAGCTG
       GGTGTCCATG GAACGACTCA AATTCTGACC TCGACTCTCC TTTCTTCGAC

451    CTGAGAGCAC TCTGGTTGCT TACAAGTCAG CTCAGGATAT TGCACTTGCT
       GACTCTCGTG AGACCAACGA ATGTTCAGTC GAGTCCTATA ACGTGAACGA

501    GATTTAGCTC CTACTCATCC GATTAGACTG GGACTTGCTC TTAACTTCTC
       CTAAATCGAG GATGAGTAGG CTAATCTGAC CCTGAACGAG AATTGAAGAG

551    TGTCTTCTAC TACGAGATTC TCAACTCACC TGATCGTGCC TGCAGTCTCG
       ACAGAAGATG ATGCTCTAAG AGTTGAGTGG ACTAGCACGG ACGTCAGAGC

601    CAAAACAGGC TTTTGATGAG GCCATTTCTG AGCTGGATAC ATTAGGAGAA
       GTTTTGTCCG AAAACTACTC CGGTAAAGAC TCGACCTATG TAATCCTCTT

651    GAATCATACA AAGACAGTAC GTTGATAATG CAACTTCTCC GTGACAATCT
       CTTAGTATGT TTCTGTCATG CAACTATTAC GTTGAAGAGG CACTGTTAGA

701    GACCCTTTGG AACTCTGACA TCAATGATGA GGCGGGCGGT GATGAGATCA
       CTGGGAAACC TTGAGACTGT AGTTACTACT CCGCCCGCCA CTACTCTAGT

751    AGGAGGCGTC AAAACATGAG CCGGAAGAGG GGAAACCAGC TGAGACAGGG
       TCCTCCGCAG TTTTGTACTC GGCCTTCTCC CCTTTGGTCG ACTCTGTCCC

801    CAGTCTAGAA TGGTGAGCAA GGGCGAGGAG CTGTTCACCG GGGTGGTGCC
       GTCAGATCTT ACCACTCGTT CCCGCTCCTC GACAAGTGGC CCCACCACGG

851    CATCCTGGTC GAGCTGGACN GCGACGTAAA CGGCCACAAG TTCAGCGTGT
       GTAGGACCAG CTCGACCTGN CGCTGCATTT GCCGGTGTTC AAGTCGCACA
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Nu Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

CCAGACCCTTCCTCTATATAAGGAAGTTNATTTNATTTGGAGAGAACACGGGGGACTCTAGAACAATGTCG
TCTTCTCGGGAAGAGAATGTGTACTTAGCCAAGTTAGCTGAGCAAGCTGAACGTTATGAGGAAATGGTTGA
GTTCATGGAGAAAGTTGCAAAGACTGTTGACACCGATGAGCTTACTGTCGAAGAGAGAAACCTCTTGTCTG
TTGCTTACAAGAACGTCATTGGTGCTAGGAGAGCTTCCTGGAGGATCATATCTTCCATTGAACAGAAGGAA
GAAAGCAGAGGAAACGATGATCATGTTTCCATTATCAAGGACTACAGAGGAAAGATCGAAACTGAACTCAG
CAAAATCTGTGATGGAATACTCAATCTTCTGGATTCTCACCTTGTTCCCACTGCATCTTTGGCCGAGTCCA
AAGTCTTTTACCTCAAAATGAAAGGAGATTACCACAGGTACCTTGCTGAGTTTAAGACTGGAGCTGAGAGG
AAAGAAGCTGCTGAGAGCACTCTGGTTGCTTACAAGTCAGCTCAGGATATTGCACTTGCTGATTTAGCTCC
TACTCATCCGATTAGACTGGGACTTGCTCTTAACTTCTCTGTCTTCTACTACGAGATTCTCAACTCACCTG
ATCGTGCCTGCAGTCTCNCAAAACAGGCTTTTGATGAGGCCATTTCTGAGCTGGATACATTAGGAGAAGAA
TCATACAAAGACAGTACGTTGATAATGCAACTTCTTCCGTGACAATCTGACCCTTTGGAACTCTGACATCA
ATGATNANGCGGGCCGGNGATGANATCAAAGGAGGCGTCAAAANCATTAACCCGNAAANNGGAAACCACCT
TAAACCAGGCACTTTANAATCT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

GTTCGTTTCGTTTCGTCCGCTCGACCGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCA
TTCTAGACTGCCCTGTCTCAGCTGGTTTCCCCTCTTCCGGCTCATGTTTTGACGCCTCCTTGATCTCATCA
CCGCCCGCCTCATCATTGATGTCAGAGTTCCAAAGGGTCAGATTGTCACGGAGAAGTTGCATTATCAACGT
ACTGTCTTTGTATGATTCTTCTCCTAATGTATCCAGCTCAGAAATGGCCTCATCAAAAGCCTGTTTTGCGA
GACTGCAGGCACGATCAGGTGAGTTGAGAATCTCGTAGTAGAAGACAGAGAAGTTAAGAGCAAGTCCCAGT
CTAATCGGATGAGTAGGAGCTAAATCAGCAAGTGCAATATCCTGAGCTGACTTGTAAGCAACCAGAGTGCT
CTCAGCAGCTTCTTTCCTCTCAGCTCCAGTCTTAAACTCANCAAGGTACCTGTGGTAATCTCCTTTCATTT
TGAGGTAAAAGACTTTGGACTCGGCCAAAGATGCAGTGGGAACAANGTGAGAATCCAGAAGATTGAGTATT
CCATCACAGATTTTGCTGAGTTCCAGTTTCGATCTTTCCTCTTGTNGTCCTTGATAATGGAAACATGATCA
TCGTTTCCTCTGCTTTCCTTCCTTCTGTTCAATGGAAGATATGATCCTTNCAGGGANGCTCTCCTACACCA
ATGACCTTTCTTGTTAAGCNACACGACAAGANGGTTTCTCCTCTTCCNNCAGNAAGCNTCATCCGGTGTTN
ACAAGTTTTTTGCAACCTTNCTTCCNATGAACCTCAACNCATTACCNTCATAAACGTTCAANCTTGGCT

Chi Isoform

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  1 CTAGAACAAT GGCGACACCA GGAGCTTCCT CAGCAAGAGA TGAATTCGTA
    GATCTTGTTA CCGCTGTGGT CCTCGAAGGA GTCGTTCTCT ACTTAAGCAT

 51 TACATGGCGA AACTCGCCGA ACAAGCAGAG CGTTACGAAG AAATGGTTGA
    ATGTACCGCT TTGAGCGGCT TGTTCGTCTC GCAATGCTTC TTTACCAACT

101 ATTCATGGAG AAAGTCGCGA AAGCTGTTGA CAAAGACGAA CTCACCGTCG
    TAAGTACCTC TTTCAGCGCT TTCGACAACT GTTTCTGCTT GAGTGGCAGC

151 AAGAACGTAA TCTCCTCTCC GTCGCTTACA AAAACGTCAT CGGAGCTCGT
    TTCTTGCATT AGAGGAGAGG CAGCGAATGT TTTTGCAGTA GCCTCGAGCA

201 CGTGCTTCGT GGAGAATCAT TTCATCGATC GAACAAAAGG AGGAGTCTCG
    GCACGAAGCA CCTCTTAGTA AAGTAGCTAG CTTGTTTTCC TCCTCAGAGC

251 CGGTAACGAT GACCACGTTT CGTTGATCCG TGACTACAGA AGCAAAATCG
    GCCATTGCTA CTGGTGCAAA GCAACTAGGC ACTGATGTCT TCGTTTTAGC

301 AAACGGAACT TTCTGATATC TGTGACGGAA TCCTTAAGCT TCTCGATACG
    TTTGCCTTGA AAGACTATAG ACACTGCCTT AGGAATTCGA AGAGCTATGC

351 ATTCTCGTTC CCGCTGCTGC TTCTGGAGAT TCGAAGGTGT TTTATCTGAA
    TAAGAGCAAG GGCGACGACG AAGACCTCTA AGCTTCCACA AAATAGACTT

401 GATGAAAGGT GATTATCATA GGTACTTGGC TGAGTTCAAA TCTGGTCAAG
    CTACTTTCCA CTAATAGTAT CCATGAACCG ACTCAAGTTT AGACCAGTTC

451 AGAGGAAAGA TGCTGCTGAA CATACTCTTA CTGCTTACAA AGCTGCTCAG
    TCTCCTTTCT ACGACGACTT GTATGAGAAT GACGAATGTT TCGACGAGTC

501 GATATTGCTA ATTCTGAATT GGCTCCAACG CATCCGATTC GTCTTGGTCT
    CTATAACGAT TAAGACTTAA CCGAGGTTGC GTAGGCTAAG CAGAACCAGA

551 TGCGTTGAAC TTCTCTGTGT TTTACTATGA GATTCTCAAT TCTCCAGATC
    ACGCAACTTG AAGAGACACA AAATGATACT CTAAGAGTTA AGAGGTCTAG

601 GTGCTTGTAA TCTCGCTAAG CAGGCGTTTG ATGAAGCAAT TGCTGAATTG
    CACGAACATT AGAGCGATTC GTCCGCAAAC TACTTCGTTA ACGACTTAAC

651 GATACTCTTG GTGAAGAGTC ATACAAGGAC AGTACTTTGA TTATGCAGCT
    CTATGAGAAC CACTTCTCAG TATGTTCCTG TCATGAAACT AATACGTCGA

701 TCTTCGTGAT AACCTCACTC TCTGGGCTTC TGATATGCAG GACGATGTTG
    AGAAGCACTA TTGGAGTGAG AGACCCGAAG ACTATACGTC CTGCTACAAC

751 CTGATGACAT CAAAGAAGCA GCACCAGCAG CAGCAAAACC CGCTGACGAG
    GACTACTGTA GTTTCTTCGT CGTGGTCGTC GTCGTTTTGG GCGACTGCTC

801 CAACAATCCT CTAGAATGGT GAGCAAGGGC GAGGAGCTGT TCACCGGGGT
    GTTGTTAGGA GATCTTACCA CTCGTTCCCG CTCCTCGACA AGTGGCCCCA

851 GGTGCCCATC CTGGTCGAGC TGGACNGCGA CGTAAACGGC CACAAGTTCA
    CCACGGGTAG GACCAGCTCG ACCTGNCGCT GCATTTGCCG GTGTTCAAGT
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Chi Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

AGGNNNAGGGGGNTTTTNTTTTTANACTTTTCTTATAAGGAAGTTATTTCTTTGGAGAGAACACGGGGGAC
TCTAGAACAATGGCGACACCAGGAGCTTCCTCAGCAAGACATGAATTCGTATACATGGCGAAACTCGCCGA
ACAAGCAGAGCGTTACGAAGACATGGTTGAATTCATGGAGAGAGTCGCGAAAGCTGTTGACAAAGACGAAC
TCACCGTCGAAGAACGTAATCTCCTCTCCGTCGCATCCAAAAACGTCATCGGAGCTCGTCGTGCTTCGTGG
AGAATCATTTCATCGATCGAACAAAAGGAGGAGTCTCGCGGTAACGATGACCACGTTTCGTTGATCCGTGA
CCACAGAAGCAAAATCGAAACGGAACTTTCTGATATCTGTGACGGAATCCTTAAGCTTCTCGATACGATTC
TCGTTCCCGCTGCTGCTTCTGGAGATTCGAAGGTGTTTTATCTGAAGATGAAAGGTGATTATCATAGGTAC
TTCGCTGAGTTCAAATCTGGTCAAGAGAGGAAAGATGTTGCTGAACATACTCTTACTGCTTACAAAGCTGC
TCAGGATATTGCTAATTCTGAATTGGCTCCAACGCATCCGATTCGTCTTGGTCTTGCGTTGAACTTCTCTG
TGTTTTACTATGAGATTCTCAATTCTCCAGATCGTGCTTGTAATCTCGCTAANCAGGCGTTTGATGAAGCA
ATTGCTGAATTGGATACTCTTGGNGAAGAGTCATACAAGGACAGCACTTTTATTNTGCANCTTCTTCGTGA
TAANCCTNACTCTNTTGGACTTTNTGATATTGCANGACNATGTTGCTGATGACATCNAANAANCCNCCCCA
ACANCNGNCAAACCCNCTTGCCNAACNACAATTCTCTTNAAT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

GCNATTTGCCTCCNTTCGACCGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATTCTA
GAGGATTGTTGCTCGTCAGCGGGTTTTGCTGCTGTTGGTGCTGCTTCTTTGATGTCATCAGCAACATCGTC
CTGCATATCAGAAGTCCAGAGAGTGAGGTTATCACGAAGAAGCTGCATAATAAAGTACTGTCCTTGTATGA
CTCTTCACCAAGAGTATCCAATTCAGCAATTGCTTCATCAAACGCCTGCTTAGCGAGATTACAAGCACGAT
CTGGAGAATTGAGAATCTCATAGTAAAACACAGAGAAGTTCAACGCAAGACCAAGACGAATCGGATGCGTT
GGAGCCAATTCAGAATTAGCAATATCCTGAGCAGCTTTGTAAGCAGTAAGAGTATGTTCAGCAACATCTTT
CCTCTCTTGACCAGATTTGAACTCACGCGAANTACCTATGATAATCACCTTTCATCTTCAAGATAAAACAC
CTTCGAATCTCCAGAAGCAGCAACNGGGAACGAGAATCCGTNTCCGAGAANCTTAANGATTCCGTCACAGA
ATATCAAGAANAGTTNCGGTTNCGAATNTTGCTTCTGTGGGTCNACCNGATTCAACCGAAAACGTGGTCAT
ACNNTNCCGCNNAGACTCCCCCNTTTTTGTTCGAATCCNNCTGAAANTGGATTCCTCCTCCGAAANCTCCG
ACTAAGCNTCCACATTNANCNTTCCTTTNTGNTCGCCCACTTCGAANNNNGCAATNTCTCTCGCTCTTCAN
NNTCTCTACTGTACNCNCTNTACACGCTACNTTCGNGCNCACCNCCNNCTTCTATCNTAACGTGCCCTCAC
CTANCTCNCNTNGNGACTTCNNNCTACTNTCACTTATACCTNTANNNCATNANNACCACCCNCTTCNCGGN
CNNNTNCNNTTACCCGNCGNNNGGCTTNCTNNNNCTNTNCCTCCTCT

Psi Isoform

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4951  AGACCCTTCC TCTATATAAG GAAGTTCATT TCATTTGGAG AGAACACGGG
      TCTGGGAAGG AGATATATTC CTTCAAGTAA AGTAAACCTC TCTTGTGCCC

5001  GGACTCTAGA ACAATGTCGA CAAGGGAAGA GAATGTTTAC ATGGCGAAAT
      CCTGAGATCT TGTTACAGCT GTTCCCTTCT CTTACAAATG TACCGCTTTA

5051  TAGCCGAACA AGCTGAACGT TACGAAGAAA TGGTTGAATT CATGGAGAAA
      ATCGGCTTGT TCGACTTGCA ATGCTTCTTT ACCAACTTAA GTACCTCTTT

5101  GTTGCGAAAA CTGTTGATGT TGAGGAACTT TCAGTTGAAG AGAGGAATCT
      CAACGCTTTT GACAACTACA ACTCCTTGAA AGTCAACTTC TCTCCTTAGA

5151  TCTCTCTGTT GCTTACAAGA ACGTGATTGG AGCGAGAAGA GCTTCGTGGA
      AGAGAGACAA CGAATGTTCT TGCACTAACC TCGCTCTTCT CGAAGCACCT

5201  GAATCATTTC TTCGATTGAG CAGAAAGAAG AGAGCAAAGG GAACGAAGAT
      CTTAGTAAAG AAGCTAACTC GTCTTTCTTC TCTCGTTTCC CTTGCTTCTA

5251  CATGTTGCTA TTATCAAGGA TTACAGAGGA GAGATTGAAT CCGAGCTTAG
      GTACAACGAT AATAGTTCCT AATGTCTCCT CTCTAACTTA GGCTCGAATC

5301  CAAAATCTGT GATGGGATTT TGAATGTTCT TGAAGCTCAT CTTATTCCTT
      GTTTTAGACA CTACCCTAAA ACTTACAAGA ACTTCGAGTA GAATAAGGAA

5351  CTGCTTCACC AGCTGAATCT AAAGTGTTTT ATCTTAAGAT GAAGGGTGAT
      GACGAAGTGG TCGACTTAGA TTTCACAAAA TAGAATTCTA CTTCCCACTA

5401  TATCATAGGT ATCTTGCTGA GTTTAAGGCT GGTGCTGAAA GGAAAGAAGC
      ATAGTATCCA TAGAACGACT CAAATTCCGA CCACGACTTT CCTTTCTTCG

5451  TGCTGAAAGC ACTTTGGTTG CTTACAAGTC TGCTTCCGAC ATTGCCACTG
      ACGACTTTCG TGAAACCAAC GAATGTTCAG ACGAAGGCTG TAACGGTGAC

5501  CTGAGTTAGC TCCTACTCAC CCGATAAGGC TTGGTCTTGC ACTCAACTTC
      GACTCAATCG AGGATGAGTG GGCTATTCCG AACCAGAACG TGAGTTGAAG

5551  TCTGTGTTTT ACTATGAAAT CCTCAACTCG CCTGATCGTG CTTGCAGCCT
      AGACACAAAA TGATACTTTA GGAGTTGAGC GGACTAGCAC GAACGTCGGA

5601  CGCAAAGCAG GCGTTTGATG ATGCAATCGC TGAGTTAGAT ACATTGGGTG
      GCGTTTCGTC CGCAAACTAC TACGTTAGCG ACTCAATCTA TGTAACCCAC

5651  AGGAATCATA CAAGGACAGT ACACTGATTA TGCAGCTTCT TAGAGACAAT
      TCCTTAGTAT GTTCCTGTCA TGTGACTAAT ACGTCGAAGA ATCTCTGTTA

5701  CTCACTCTCT GGACTTCAGA TATGACTGAC GAAGCAGGAG ATGAGATTAA
      GAGTGAGAGA CCTGAAGTCT ATACTGACTG CTTCGTCCTC TACTCTAATT

5751  GGAGGCATCA AAGCCCGATG GTGCCGAGTC TAGAATGGTG AGCAAGGGCG
      CCTCCGTAGT TTCGGGCTAC CACGGCTCAG ATCTTACCAC TCGTTCCCGC
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Psi Isoform

Sequence using a forward primer to the tail end of the 35S promoter (5’CGCACAATCCCACTATCCTT3’):

CAGGACCTTTCCTCTTATAAGGAAGTTCATTTNATTTGGAGAGAACACGGGGGACTCTAGAACAATGTCGA
CAAGGGAAGAGAATGTTTACATGGCGAAATTAGCCGAACAAGCTGAACGTTACGAAGAAATGGTTGAATTC
ATGGAGAAAGTTGCGAAAACTGTTGATGTTGAGGAACTTTCAGTTGAAGAGAGGAATCTTCTCTCTGTTGC
TTACAAGAACGTGATTGGAGCGAGAAGAGCTTCGTGGAGAATCATTTCTTCGATTGAGCAGAAAGAAGAGA
GCAAAGGGAACGAAGATCATGTTGCTATTATCAAGGATTACAGAGGAAAGATTGAATCCGAGCTTAGCAAA
ATCTGTGATGGGATTTTGAATGTTCTTGAAGCTCATCTTATTCCTTCTGCTTCACCAGCTGAATCTAAAGT
GTTTTATCTTAAGATGAAGGGTGATTATCATAGGTATCTTGCTGAGTTTAAGGCTGGTGCTGAAATGAAAA
AGCTGCTGAAAGCACTTTGGTTGCTTACAAGTCTGCTTCCGACATTGCCACTGCTGAGTTAGCTCCTACTC
ACCCGATAAGGCTTGGTCTTGCACTCAACTTCTCTGTGTTTTACTATGAAATCCTCAACTCGCCTGATCGT
GCTTGCAGCCTCNCAAAGCAGGCGTTTGATGATGCAATCGCNTGAGTTAGATACATTTGGGTGAGGGAATC
ATTACAAGGGCCAGTNCACTGATTTATTCNACNTTCTTAGAGACAATCCTCNCTCTCTTGGACTTTCNGAA
TATGACTGACCAAANCNNGGAGATTGAGATAAAGGAGGCATCANAGCCCCGATGGTGCCNANTCTATAATG
GGTGAGCNCGGGCCAANGAGCTGTNCNCCNGGGGTNGTCT

Sequence using a reverse primer to the beginning of GFP (5’CGGACACGCTGAACTTG3’):

NGTCGTTNACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTC
ACCATTCTAGACTCGGCACCATCGGGCTTTGATGCCTCCTTTATCTCATCTCCTGCTTCGTCAGTCATATC
TGAAGTCCAGAGAGTGAGATTGTCTCTAAGAAGCTGCATAATCAGTGTACTGTCCTTGTATGATTCCTCAC
CCAATGTATCTAACTCAGCGATTGCATCATCAAACGCCTGCTTTGCGAGGCTGCAAGCACGATCAGGCGAG
TTGAGGATTTCATAGTAAAACACAGAGAAGTTGAGTGCAAGACCAAGCCTTATCGGGTGAGTAGGAGCTAA
CTCAGCAGTGGCAATGTCGGAAGCAGACTTGTAAGCAACCAAAGTGCTTTCAGCAGCTTTTTCATTTCAGC
ACCAGCCTTAAACTCAGCAAGATACCTATGATAATCACCCTTCATCTTAAGATAAAACACTTTAGATTCAG
CTGGTGAAGCAGAAGGAATAAGATGAGCTTCAAGAACATTCAAAATCCCATCACAGATTTTGCTAAGCTCG
GATTCAATCTTTCCTCTGTAATCCTTGATAATAGCAACATGATCTTCGTTCCCTTTGCTCTCTTCTTTCTG
CTCAATCGAAGAAATGATTCTCCACGAAGCTCTTCTCGCTCCAATCACGTTCTTGTAAGCAACAGAGAGAA
GATTCCTCTCTTCAACTGAAAGTTCCTCACATCAACAGTTTTCGCAACTTTCTCCTGAATTCAACCATTTC
TTCGTAACGNTCAGCCTTGCTCGGCTAATTTCGCCCTGTCAAACATTCN

LIST OF REFERENCES

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408: 796-815.

Aitken A (1995) 14-3-3 proteins on the MAP. Trends Biochem Sci 20: 95-97.

Bachmann M, Huber JL, Athwal GS, Wu K, Ferl RJ, Huber SC (1996) 14-3-3 proteins associate with the regulatory phosphorylation site of spinach leaf nitrate reductase in an isoform-specific manner and reduce dephosphorylation of Ser-543 by endogenous protein phosphates. FEBS Lett 398:26-30.

Bihn EA, Paul AL, Wang SW, Erdos GW, Ferl RJ (1997) Localization of 14-3-3 proteins in the nuclei of arabidopsis and maize. Plant J 12: 1439-1445.

Brandt J, Thordal-Christensen H, Vad K, Gregersen PL, Collinge DB (1992) A pathogen-induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. Plant J 2: 815-820.

Chen Z, Fu H, Liu D, Chang PF, Narasimhan M, Ferl R, Hasegawa PM, Bressan RA (1994) A NaCl-regulated plant gene encoding a brain protein homology that activates ADP ribosyltransferase and inhibits protein kinase C. Plant J 6: 729-740.

Chung HJ, Sehnke PC, Ferl RJ (1999) The 14-3-3 proteins: cellular regulators of plant metabolism. Trends Plant Sci 4: 367-371.

DeLille JM, Sehnke PC, Ferl RJ (2001) The arabidopsis 14-3-3 family of signaling regulators. Plant Physiol 126: 35-38.

de Vetten NC, Ferl RJ (1994) Two genes encoding GF14 (14-3-3) proteins in Zea mays. Structure, expression, and potential regulation by the G-box binding complex. Plant Physiol 106: 1593-1604.

de Vetten NC, Lu GH, Ferl RJ (1992) A maize protein associated with the G-box binding complex has homology with brain regulatory proteins. Plant Cell : 1295-1307.

Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Gross RW, Williams LT (1994) Activation of Raf-1 by 14-3-3 proteins. Nature 371: 612-614.

Ferl RJ (1996) 14-3-3 proteins and signal transduction. Annual Review of Plant Physiol. 47: 49-73.

Finnie C, Borch J, Collinge DB (1999) 14-3-3 proteins: eukaryotic regulatory proteins with many functions. Plant Mol Biol 40: 545-554.

Hirsch S, Aitken A, Bertsch U, Soll, J (1992) A plant homologue to mammalian brain 14-3-3 protein and protein kinase C inhibitor. FEBS Lett 296: 222-24.

Ichimura T, Isobe T, Okuyama T, Takahashi N, Araki K, Kuwano R, Takahashi Y (1988) Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. Proc Natl Acad Sci U S A 85: 7084-7088.

Ichimura T, Isobe T, Okuyama T, Yamauchi T, Fujisawa H (1987) Brain 14-3-3 protein is an activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca2+,calmodulin-dependent protein kinase II. FEBS Lett 219: 79-82.

Ichimura T, Sugano H, Kuwano R, Sunaya T, Okuyama T, Isobe T (1991) Widespread distribution of the 14-3-3 protein in vertebrate brains and bovine tissues: correlation with the distributions of calcium-dependent protein kinases. J Neurochem 56: 1449-1451.

Jarillo JA, Capel J, Leyva A, Martinez-Zapater JM, Salinas J (1994) Two related low-temperature-inducible genes of Arabidopsis encode proteins showing high homology to 14-3-3 proteins, a family of putative kinase regulators. Plant Mol Biol 25: 693-704.

Kidou S, Umeda M, Kato A, Uchimiya H (1993) Isolation and characterization of a rice cDNA similar to the bovine brain-specific 14-3-3 protein gene. Plant Mol Biol 21: 191-194.

Korthout HA, de Boer AH (1994) A fusicoccin binding protein belongs to the family of 14-3-3 brain protein homologs. Plant Cell 6: 1681-1692.

Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann N, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M et al., (2001) Initial sequencing and analysis of the human genome. Nature 409: 860-921.

Laughner B, Lawrence SD, Ferl RJ (1994) Two tomato fruit homologs of 14-3-3 mammalian brain proteins. Plant Physiol 105: 1457-1458.

Leffers H, Madsen P, Rasmussen HH, Honore B, Andersen AH, Walbum E, Vandekerckhove J, Celis JE (1993) Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signalling pathway. J Mol Biol 231: 982-998.

Lu G, DeLisle AJ, de Vetten NC, Ferl RJ (1992) Brain proteins in plants: an Arabidopsis homolog to neurotransmitter pathway activators is part of a DNA binding complex. Proc Natl Acad Sci U S A 89: 11490-11494.

Lu G, de Vetten NC, Sehnke PC, Isobe T, Ichimura T, Fu H, van Heusden GP, Ferl RJ (1994a) A single Arabidopsis GF14 isoform possesses biochemical characteristics of diverse 14-3-3 homologues. Plant Mol Biol 25: 659-667.

Lu G, Sehnke PC, Ferl RJ (1994b) Phosphorylation and calcium binding properties of an Arabidopsis GF14 brain protein homolog. Plant Cell 6: 501-510.

Marra M, Fullone MR, Fogliano V, Pen J, Mattei M, Masi S, Aducci P (1994) The 30-kilodalton protein present in purified fusicoccin receptor preparations is a 14-3-3-like protein. Plant Physiol 106: 1497-1501.

Martin H, Rostas J, Patel Y, Aitken A (1994) Subcellular localisation of 14-3-3 isoforms in rat brain using specific antibodies. J Neurochem 63: 2259-2265.

Moore BW, Perez VJ. (1967) Specific acidic proteins of the nervous system. In Carlson, F.D. (ed.) *Physiological and Biochemical Aspects of Nervous Integration*. Prentice-Hall, Inc, The Marine Biological Laboratory, Woods Hole, Massachusetts, pp. 343-359.

Moorhead G, Douglas P, Cotelle V, Harthill J, Morrice N, Meek S, Deiting U, Stitt M, Scarabel M, Aitken A, MacKintosh C (1999) Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. Plant J 18:1-12

Morgan A, Burgoyne RD (1992) Exo1 and Exo2 proteins stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. Nature 355: 833-836.

Nielsen PJ (1991) Primary structure of a human protein kinase regulator protein. Biochim Biophys Acta 1088: 425-428.

Oecking C, Eckershorn C, Weiler EW (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. FEBS Lett 352:163-166.

Pan S, Sehnke PC, Ferl RJ, Gurley WB (1999) Specific interactions with TBP and TFIIB in vitro suggest that 14-3-3 proteins may participate in the regulation of transcription when part of a DNA binding complex. Plant Cell 11: 1591-1602.

Patel Y, Martin H, Howell S, Jones D, Robinson K, Aitken A (1994) Purification of 14-3-3 protein and analysis of isoforms in chicken brain. Biophys Acta 1222:405-409.

Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM (1994) Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. Science 266: 129-133.

Robinson K, Jones D, Patel Y, Martin H, Madrazo J, Martin S, Howell S, Elmore M, Finnen MJ, Aitken A (1994) Mechanism of inhibition of protein kinase C by 14-3-3 isoforms. 14-3-3 isoforms do not have phospholipase A2 activity. Biochem J 299: 853-861.

Rosenquist M, Sehnke PC, Ferl RJ, Sommarin M, Larsson C (2000) Evolution of the 14-3-3 protein family: does the large number of isoforms in multicellular organisms reflect functional specificity? J Mol Evol 51: 446-458.

Sehnke PC, Chung HJ, Wu K, Ferl RJ (2001) Regulation of starch accumulation by granule-associated plant 14-3-3 proteins. Proc Natl Acad Sci U S A 98: 765-770.

Sehnke PC, Ferl RJ. (2000) Plant 14-3-3s: Omnipotent Metabolic Phosphopartners? Science's STKE.

Sehnke PC, Henry R, Cline K, Ferl RJ (2000) Interaction of a plant 14-3-3 protein with the signal peptide of a thylakoid-targeted chloroplast precursor protein and the presence of 14-3-3 isoforms in the chloroplast stroma. Plant Physiol 122: 235-242.

Swanson KD, Ganguly R (1992) Characterization of a Drosophila melanogaster gene similar to the mammalian genes encoding the tyrosine/tryptophan hydroxylase activator and protein kinase C inhibitor proteins. Gene 113: 183-190.

Token A, Ellis CA, Sellers LA, Aitken A (1990) Protein kinase C inhibitor proteins. FEBS Lett 191: 421-429.

Toroser D, Athwal GS, Huber SC (1998) Site-specific regulatory interaction between spinach leaf sucrose-phosphate synthase and 14-3-3 proteins. FEBS Lett 435: 110-114.

van Heusden GP, Wenzel TJ, Lagendijk EL, de Steensma HY, van den Berg JA (1992) Characterization of the yeast BMH1 gene encoding a putative protein homologous to mammalian protein kinase II activators and protein kinase C inhibitors. FEBS Lett 302: 145-150.

Wu K, Rooney MF, Ferl RJ (1997) The Arabidopsis 14-3-3 multigene family. Plant Physiol 114: 1421-1431.

BIOGRAPHICAL SKETCH

Justin Marcus DeLille was born in Little Rock, Arkansas in February 1977. He graduated Valedictorian from Fayetteville High School in 1995. He attended the University of Arkansas, Fayetteville, Arkansas on a full academic scholarship. Justin graduated Cum Laude in May 1999 with a bachelors degree in microbiology. He entered the graduate program in plant molecular and cellular biology in August 1999 and began work on his Master of Science degree. He has accepted a position in the Master of Science in Business Management program at the University of Florida business school.