SUBNUCLEAR LOCALIZATION AND INTERACTION OF

SELECTED AUX/IAA AND ARF PROTEINS *IN VIVO*

by

JOHN C. MAJSZTRIK

Under the Direction of Campbell Joseph Nairn

ABSTRACT

The plant hormone auxin has been studied for many years and is instrumental for plant growth and development. Auxin regulates gene transcription of at least five families including the Aux/IAA family. Aux/IAA and ARF proteins are known to interact through conserved domains in both the Aux/IAA and ARF families. This research is the first to report individual nuclear and sub-nuclear localization patterns of the *Arabidopsis* ARF and Aux/IAA protein *in vivo*, focusing on ARF1 and IAA17. Several co-localizations of proteins from the same and different families exhibited protein recruitment to structures and locations individual proteins did not localize to when expressed alone. This work strongly supports the putative interactions between ARF and Aux/IAA proteins *in vivo.* Northern Blot and semi-quantitative RT PCR showed that ARF1 and IAA17 were co-expressed in tissues sampled. The role of putative nuclear localization signals was investigated by localization of deletion constructs of IAA17 and ARF1.

INDEX WORDS: Auxin Response Factor (ARF), Aux/IAA, Fluorescent protein, Nuclear localization

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B.S., The College of New Jersey, 2001

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2004

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August 2004

DEDICATION

This thesis would not have been possible without the help of many people. I wanted to thank my parents for always providing loving guidance and caring support throughout my life. Thank you for helping me realize and pursue my goals, and do everything that you could for me throughout my life. I would also like to thank Andrew, Paul, and Vanessa, you have always been there for me in whatever capacity necessary. I also wanted to thank my Grandfather and Great Aunt Martha for their support and Thank you also to Shannon, the Love of my life, for your support, encouragement, understanding and love throughout this process. May we always continue to grow closer to one another. Thanks must also be given to God; through Him all things are possible.

ACKNOWLEDGEMENTS

I would like to thank Joe for all of his guidance and help that he has provided along the way. You helped to increase my knowledge of Molecular Biology and science in general in so many ways. Dr. Jeff Dean also provided helpful critical reading of this thesis in its beginning stages during his proposal writing class and through to the final copy. I appreciate everything that you have done for me. I would also like to thank the rest of my committee, Dr. Mark Farmer, for his help on the confocal microscope, Dr. Sarah Covert and Dr. Scott Merkle for their help and critical review of this work.

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Chapter 1 Literature Review

Introduction

Plants have evolved the ability to live autonomously using relatively simple molecules and compounds from their environment, and thus making them capable of synthesizing everything they need to live. Although plants use simple molecules to synthesize new compounds, the biosynthetic pathways involved are far from simple. Researchers have been studying plants for many years and have made many discoveries, but still are far from a complete understanding of how plants function. One area that still has unanswered questions is the production and function of plant hormones.

Plant hormones, such as auxin, cytokinin, gibberellins, abscisic acid and ethylene, control many aspects of growth and development. Plant hormones are similar to animal hormones in that they are synthesized in small amounts, but are able to produce dramatic effects. Plant hormones often affect areas far from where they are produced and act as signaling molecules that are able to control diverse processes in a cell. Auxin is a plant hormone that has been studied for many years, and has been found in green algae, mosses and vascular plants.

Auxin

Auxins are a class of plant hormones or plant growth regulators that play a key role in many aspects of plant growth and development. Indole-3-acetic acid or IAA is the predominant auxin found in plants. IAA is important in controlling cell division, elongation and differentiation (Worley et al., 2000) tropic responses, apical dominance, cambial cell division, root initiation, and differentiation of vascular tissue (Hagen and Guilfoyle, 2002). Auxin signals

are rapidly transferred into a variety of responses including changes in the direction of growth, shoot and root branching, and vascular differentiation (Leyser, 2001). Auxin is produced in young organs, mainly in the plant apex and leaves, and is asymmetrically transported through the plant by membrane proteins called auxin efflux and influx carriers (Estelle, 2001). Auxin is transported basipetally from the shoots to the base of the plant and down through the roots. The concentration of auxin in a cell determines to a large degree the effects it has on the cell (Leyser and Berleth, 1999).

The effect of auxin on a plant begins early in development, starting with hypocotyl elongation. Hypocotyl elongation allows a dicotyledonous plant to emerge from the soil, in order to initiate the photosynthetic machinery, and is arguably one of the most important processes at the beginning of germination. There are two distinct developmental pathways that a plant can follow, which can overlap under normal conditions. Skotomorphogenesis occurs in the dark, and photomorphogenesis occurs in the light. In order to start on the correct pathway, there are several different photoreceptors that are able to detect the different quantities and qualities of light including light in the red and far-red area of the spectrum. These pathways, along with plant hormones such as auxin and cytokinin, are also able to influence hypocotyl elongation (Nakazawa et al., 2001).

Auxin plays a major role in early development and plays a key role throughout the life of the plant. In addition to being involved in cell division, elongation and differentiation, auxin is also involved in wood formation, and effects cambial activity and xylem development (Sundberg et al., 2000). Auxin is involved in maintaining cambium as meristematic tissue, which is not terminally differentiated. By removing auxin, the cambial cells differentiate into axial parenchyma (Savidge, 1983). A class of auxin induced transcription factors, Aux/IAAs, are

differentially expressed along a developmental gradient in wood forming tissue in hybrid aspen (*Populus tremula L.*x *Populus tremuloides Michx.*) (Moyle et al., 2002). Aux/IAA genes were down-regulated during the transition from active cambium into dormancy (Moyle et al., 2002). Developmental processes such as dormancy, which are regulated by auxin, are thought to vary with changes in cellular content, sensitivity of cells to auxin and polar auxin transport (Leyser and Berleth, 1999).

IAA has been studied for years, but the pathways behind IAA biosynthesis and how it is sensed by cells is poorly understood (Bartel, 1997). Once IAA is produced, it is transported through the plant by polar auxin transport from the apical meristem and leaves to the roots. The mechanisms of auxin sensing by cells are still under investigation. Polar auxin transport is believed to regulate a number of developmental processes, including tropism and apical dominance. Plants can regulate IAA concentrations by conjugating IAA to amino acids or sugars, or convert tryptophan (an IAA precursor) to IAA to keep free IAA at constant levels (Hobbie and Estelle, 1994). Auxin can act as an herbicide, growth retardant or growth stimulator, depending on the concentration at which it is applied (Guilfoyle et al., 1998b).

Many pathways have been proposed for auxin synthesis. In *Arabidopsis thaliana*, the highest levels of IAA synthesis occur in young leaves (less than 0.5mm in length) where cells are rapidly dividing, and decreases 100 fold as leaves expand to their full size (Ljung et al., 2001). All parts of young *Arabidopsis* plants including cotyledons, expanding leaves and root tissues, have the ability to produce IAA *de novo* (Ljung et al., 2001). There also appears to be feedback inhibition of IAA biosynthesis. When the level of auxin reaches a certain concentration, auxin synthesis is stopped until the concentration is lowered (Ljung et al., 2001).

Understanding gene regulation in plants is a difficult and complex task. There are many factors that are involved in controlling such different and large scale processes as flowering, branching, rooting, and wood formation. Two gene families that have been found to play a role in controlling gene regulation in early auxin induced genes are the Aux/IAA and auxin response (ARF) gene families.

Aux/IAA genes

Auxin affects the transcription of at least five gene families, which are called primary response genes because of their rapid induction in response to auxin. These are the Aux/IAA, SAUR (Small Auxin Up-Regulated), GH3 like, Aminocyclopropane-1-Carboxylic acid Synthase (ACS) and glutatione-S-transferase (GH2/4-like) gene families (Abel and Theologis, 1996). Research is currently underway to determine how auxin affects these gene families.

The Aux/IAA family of genes has been widely studied, including their regulation and transcription. Aux/IAA genes are not found outside of the plant kingdom, but are conserved among monocots, dicots and gymnosperms (Hagen and Guilfoyle, 2002). Like most genes that code for regulatory proteins, Aux/IAA genes are strictly regulated at many levels (Colon-Carmona et al., 2000). The encoded proteins interact with the auxin response factor (ARF) family through protein-protein interactions, and are thought to regulate early gene transcription (Kim et al., 1997). The focus of this thesis is on the localization and interaction of members of the Aux/IAA and ARF gene families.

Aux/IAA genes were first isolated as auxin-responsive mRNAs from soybean (Walker and Key, 1982). The Aux/IAA family has 29 putative genes in *A. thaliana*, which range in size from 20-35 kD (Hagen and Guilfoyle, 2002). Aux/IAA genes were identified in *Arabidopsis*

based on sequence similarity and two-hybrid screens (Abel et al., 1995; Kim et al., 1997), with additional genes being discovered by the *Arabidopsis* genome project (Reed, 2001).

Aux/IAA genes are induced at different times after the addition of auxin. IAA1, IAA2, IAA3, IAA5, and IAA6 are induced within 5 minutes of auxin addition, while IAA7, IAA8, IAA9, and IAA10 are induced within 30 minutes of auxin addition (Park et al., 2002). There are likely to be differences in cell- and/or tissue specific-expression patterns for different ARF and Aux/IAA genes (Ulmasov et al., 1999a). This reduces the number of ARFs and Aux/IAAs that are expressed at one time, thereby reducing the number of interactions that can occur *in planta*.

Aux/IAAs are specifically induced by auxin in the cell. When elongating regions of hypocotyls or epicotyls are excised and incubated in auxin-free medium, Aux/IAA mRNAs are rapidly depleted, and are rapidly induced again by addition of auxin (Hagen and Guilfoyle, 2002). Addition of cycloheximide, a protein synthesis inhibitor, shows that Aux/IAA genes are regulated by pre-existing components. No new proteins are being made in response to auxin that influence IAA gene induction, so all of the necessary components were present in the cell before cycloheximide treatment. Specificity of IAA induction on Aux/IAA genes was demonstrated by adding other plant hormones and analogues, which did not cause induction. (Hagen and Guilfoyle, 2002).

The expression of some Aux/IAA genes have distinct spatial and temporal patterns, pointing to a diversity of auxin responses in different tissues and organs (Liscum and Reed, 2002). This differential expression may play a large role in determining which ARF and Aux/IAA genes can interact in the cell, which may in part determine whether transcription is activated or repressed. Aux/IAAs are thought to auto regulate themselves and cross regulate other Aux/IAAs via negative feedback (Park et al., 2002). Aux/IAA and ARF proteins both

function as transcriptional regulators with all Aux/IAA proteins acting as putative repressors, and some ARF proteins either being activators or repressors of transcription (Liscum and Reed, 2002).

ARF proteins putatively bind with their DNA binding domain (DBD) (Figure 1-2 to the auxin response element (AuxRE) in the promoter region of early auxin-induced genes (Figure 1-1). ARF proteins also interact with IAA proteins through shared domains III and IV. These domains are conserved in both gene families and have been shown to mediate protein-protein interactions (Park et al., 1997).

Aux/IAA genes have four conserved domains one through four. Domains I-IV have 8, 14, 17, and 40 residues respectively (Guilfoyle et al., 1998b). The function of Domain I remains unknown, but in combination with domain II, may interact with phytochrome A and be involved in regulating photosynthesis (see Liscum and Reed, 2002 for review). Domain I might also play a role in homodimerization of Aux/IAA proteins (Ouellet et al., 2001).

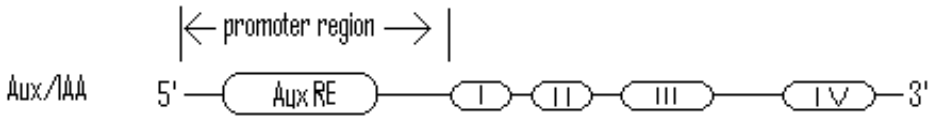


Figure 1-1. Schematic diagram of Aux/IAA gene with Auxin Response Element (AuxRE) of the promoter and domains I-IV.

Domain II is responsible for destabilizing the protein, most likely through interactions with the ubiquitin pathway (Worley et al., 2000; Colon-Carmona et al., 2000). The ubiquitin proteasome degradation pathway is believed to be the main regulator of Aux/IAA protein abundance in the cell. If Aux/IAA proteins are regulated by this pathway, it may partially explain why Aux/IAA proteins are short-lived inside the cell. Domain II is conserved in 24 of 29

Aux/IAA genes in *Arabidopsis*, so it is reasonable to hypothesize that the Aux/IAA proteins are degraded by this pathway (Gray et al., 2001). Rapid degradation of Aux/IAA proteins is necessary for normal auxin response (Worley et al., 2000). Nine different known mutations in Aux/IAA genes involve a single amino acid substitution in domain II, and function to stabilize the protein (Liscum and Reed, 2002). These mutations produce severe developmental phenotypes, highlighting the important role that rapid Aux/IAA turnover has on plant development (Liscum and Reed, 2002). Aux/IAA genes from hybrid aspen (*Populus tremula L. x Populus tremuloides michx.),* PttIAA3 and PttIAA4, have a substitution of a glycine for an aspartic acid in domain II that is known to regulate the stability of IAA proteins (Moyle et al., 2002). Domain II putatively has an additional function as a nuclear localization signal. Part of domain II and the conserved KR (lysine and arginine) residues between domains I and II in nearly all Aux/IAA genes are believed to function as nuclear localization signals (Moyle et al., 2002).

Domain III and IV are putative protein-protein interaction domains. These domains are reported to allow interaction between other Aux/IAA proteins and with ARF proteins. Domain III is reported to have other functions beyond forming protein-protein dimers. It is predicted to have an amphipathic βαα fold similar to the βαα fold of prokaryotic transcriptional repressors, such as Arc and Metj (Abel et al., 1994). In prokaryotes, this βαα fold mediates dimerization of Arc monomers and DNA recognition of operator half-sites by Arc dimers. It is possible that Aux/IAA proteins might also regulate transcription of secondary gene expression in response to auxin (Morgan et al., 1999).

Research has suggested that Aux/IAA proteins have two nuclear localization signals (Abel and Theologis, 1995). In addition to a role in protein-protein interaction, domain IV is

also reported to contain a third nuclear localization signal (NLS)(Abel and Theologis, 1995). One NLS is hypothesized to import the Aux/IAA protein into the nucleus, while others may be responsible for sub-nuclear localization of the protein, but none have been tested *in vivo* (Abel and Theologis, 1995).

Aux/IAA mutants

Much of what is known about the Aux/IAA gene family, and the effect of auxin on plants was discovered through the isolation and characterization of mutants. Plants with mutations in Aux/IAA genes show altered response to auxin and/or changes in growth patterns because of their altered sensitivity to auxin. Mutations are often a useful way to assist in determining the function of a gene. When a gene no longer functions because of a mutation, there may be a change in phenotype of the resulting plants. This phenotypic change, along with other information, can be used to determine the function of the gene. There are mutants for several genes in both the Aux/IAA and ARF gene families that have been isolated and used to study the role of functional proteins.

The first auxin-resistant mutants were discovered by growing plants on an inhibitory concentration of auxin. In general, auxin mutants are agravitrophic and have defects in apical dominance and cell elongation. All auxin mutants have been shown to have defects in root gravitropism. They also show changes in auxin levels or response to auxin, as well as other plant hormones (Hobbie and Estelle, 1994)

The *axr3*/IAA17 mutant has two semidominant alleles, *axr3-3* and *axr3-1* (Hobbie and Estelle, 1994). They are 1000x less sensitive to exogenously applied IAA than wild-type plants and are significantly less sensitive than any other auxin-resistant mutant that has been identified. The phenotype of *axr3* is opposite that of another mutant, *axr1*. Both *axr3-1* and *axr3-3* have

severe phenotypes that are consistent with an over-response to auxin, including short agravitrophic roots with increased number of adventitious roots and reduced number of root hairs (Knox et al., 2003). The a*xr3* mutant produces a single unbranched inflorescence, while wild-type lines have 2-5 branches and *axr1* is highly branched (Hobbie and Estelle, 1994). *Axr3* plants have about 8 roots stemming from the hypocotyls, and the roots are agravitrophic, twisted and lack root hairs. Wild-type plants have one or two main roots growing from the base of the hypocotyls, but none from the hypocotyl itself (Hobbie and Estelle, 1994).

*Shy2/*IAA3 (short hypocotyl) is semidominant. The phenotype occurs because of a stabilizing mutation in domain II and affects root hair development and hypocotyls length. *axr3*/IAA17 blocks root hair initiation and elongation, while *shy2*/IAA3 promotes initiation of root hair development and prolongs hair elongation (Knox et al., 2003). *Shy2* causes leaf formation in the dark, so it is thought to regulate normal plant development (Tian and Reed, 1999). Gain and loss of function *shy2* mutations affect auxin dependent root growth, lateral root formation and timing of gravitropism (Tian and Reed, 1999). Thus *shy2*/IAA3 appears to regulate multiple auxin responses in roots.

The phenotype of *shy2-2* in both dark and light includes curled up leaves, and early flowering in the light. Plants with this allele can also make leaves in the dark. The *shy2-3* allele is less severe than *shy2-2* and has expanded cotyledons without extensive leaf formation in the dark (Tian and Reed, 1999). The *shy2-1* allele produces curled leaves in the light. In the dark it is able to make true leaves, inflorescence stems and flowers (Tian and Reed, 1999). This information suggests IAA3 as being both a positive and negative regulator of the auxin response in plants. It was also found that the levels of expression of early auxin-induced genes were

decreased in *shy2-1*/IAA3, compared to wild-type (Oono et al., 2002). The protein is expressed ectopically in hypocotyl, cotyledon, petioles, and root vascular tissue (Oono et al., 2002).

The mutation *axr2-1*/IAA7 has an extreme dwarf phenotype and is caused by an amino acid substitution in domain II. Mutant plants are deficient in both root and shoot gravitropism, the hypocotyl and stems are shorter than wild-type, and the roots are resistant to auxin (Nagpal et al., 2000). It is thought that the phenotype is due to a decrease in cell elongation and not a reduction in cell number. This mutation disrupts auxin action early on, and is most likely involved in perception or transduction of the auxin signal (Hobbie and Estelle, 1994). From mutation analysis, it is hypothesized that IAA7 controls development in light-grown seedlings. Dark-grown seedlings have a short hypocotyl and make leaves, therefore, *axr2* is sufficient to induce morphological responses that are normally induced by light (Nagpal et al., 2000).

The solitary root mutation *slr1*/IAA14 also has an amino acid substitution in domain II. This mutant lacks lateral roots, and the phenotype cannot be rescued by adding exogenous auxin. Adding exogenous auxin to a wild-type plant will greatly increase the number of lateral roots. It is thought that *slr1* blocks cell division of pericycle cells in lateral root initiation (Fukaki et al, 2002). Mutants are also defective in root hair formation, and have agravitrophic roots and hypocotyls. The aboveground phenotype of *slr1* includes small leaves, short, thin inflorescences, and a reduced number of inflorescence stems (Fukaki et al, 2002). It is thought that *slr1* increases apical dominance, because of the phenotypic features. The varied effects of mutations in the Aux/IAA gene family on growth and development might reflect the functional differences between IAA proteins and differences in expression regarding organ and tissue specificity and response to auxin (Fukaki et al, 2002). IAA proteins may have both overlapping and distinct functions in plant growth and development.

Auxin mutants

In addition to mutations in ARF and Aux/IAA genes, there are other mutants that exhibit altered responses to auxin. These mutants have putative functions in Aux/IAA turnover in the cell. *Aux1* has several different alleles, all of which are recessive (Hobbie and Estelle, 1994). Homozygous individuals have a complete loss of gravitropism and delayed hypocotyl elongation, but have wild-type aerial structures. Mutant plants are resistant to auxin, ethylene and cytokinin, and roots are deficient in touch-induced root tip rotation (Hobbie and Estelle, 1994). This is possibly due to starch-containing amyloplasts sedimenting more slowly in *aux1* mutants than in wild type plants. Amyloplasts are thought to be important for the detection of gravity (Hobbie and Estelle, 1994).

Another auxin mutant is *axr1*. All eight *axr1* mutations, are recessive. The *axr1* plants are resistant to IAA, 2,4-D, NAA, ethylene, and cytokinin. Morphological defects include reduced stem length, stamen length, apical dominance, and hypocotyl elongation, leaf wrinkling, increased root elongation, decreased root gravitropism and decreased root branching (Liscum and Reed, 2002). Cell size is similar to wild type so reduced size is due to a reduced cell number. All differences from the wild type can be explained by decreased auxin sensitivity. The action of the AXR1 gene is believed to be related to ubiquitin-activating enzyme E1 (Leyser et al., 1993), but AXR1 is not believed to function as an E1 enzyme (Hobbie and Estelle, 1994).

Auxin response factor (ARF) genes

The movement of auxin into and out of the cells by polar auxin transport is the primary control of auxin concentration in cells. ARF proteins have been shown to interact with other proteins to regulate transcription of the Aux/IAA gene family and other gene families (for reviews see Guilfoyle et al., 1998a, Hagen and Guilfoyle, 2002). Aux/IAA genes are auxin

induced (Abel et al., 1995), while the ARF family has not been found to undergo auxin-dependent transcriptional changes (Ulmasov et al., 1999b)

ARF proteins contain a DNA binding domain (DBD), a middle region (MR) that separates the DBD from domain III, domain III, and domain IV (Figure 1-2). The DBD has been shown to interact with the TGTCTC site in AuxREs, a conserved promoter motif in many of the early auxin induced genes (Guilfoyle and Hagen, 2001). The middle region is thought to function as either an activator or repressor of transcription in different members of the gene family (Ulmasov et al., 1999a). Domains III and IV are involved in interactions with other ARF and Aux/IAA proteins.

There are 23 ARF-related genes in the *Arabidopsis* genome ranging in size from 64-129 kD (Guilfoyle et al., 1998b; Hagen and Guilfoyle, 2002). ARF23 is most likely a pseudogene, due to the presence of a stop codon in the DBD and a lack of sequence downstream of the DBD. In addition, ARFs 12, 13, 14,15, 20, 21, and 22 may be pseudogenes because no ESTs or mRNA have been reported for these genes (Guilfoyle et al., 1998b). These genes all map to a small region on chromosome 1 and have closely related DNA sequences (Hagen and Guilfoyle, 2002). ARF3 lacks the carboxyl-terminal domains III and IV that are used for homo- and heterodimerization with other ARF or Aux/IAA proteins (Liscum and Reed, 2002). It is thought that ARF3 is not able to form dimers with other ARF or Aux/IAA proteins. If ARF3 is able to interact with other proteins it is most likely through an unknown coupling protein or other factor (Liscum and Reed, 2002). The remaining ARFs are all thought to be biologically active.

Auxin response elements (AuxRE) are upstream of the start site of many early auxin-induced genes, including the Aux/IAA family. The AuxRE site can be either simple, having

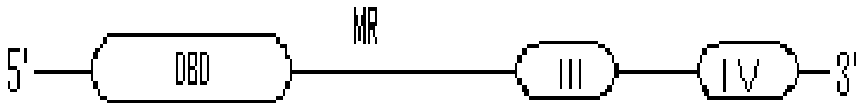


Figure 1-2. Schematic diagram of an Auxin Response Factor (ARF) gene. ARFs contain a DNA Binding Domain (DBD), a middle region (MR), and domains III and IV.

only a TGTCTC element, or it can have constitutive or coupling elements, in the vicinity that can further affect ARF binding (Ulmasov et al., 1999b). The constitutive/coupling elements present may influence which particular transcription factor binds to the TGTCTC element (Ulmasov et al., 1999b).

There are two main conserved sequences TGTCTC and (G/T)GTCCCAT known to be in the promoters of early auxin-responsive genes, including Aux/IAA genes (Guilfoyle et al., 1998b). These two elements have been found in many auxin-responsive genes, but their functional significance still needs to be assessed in many cases (Guilfoyle et al., 1998b). In the TGTCTC motif, the first 4 bases are critical for ARF1 and ARF5 binding *in vitro*, while nucleotide substitutions in position 5 and 6 are tolerated, especially at position 5, but may reduce binding (Guilfoyle et al., 1998b).

The DNA binding domains of ARF proteins has been shown to recognize and bind specifically to AuxREs (Ulmasov et al., 1999a). The DBD is about 350 amino acids long and is highly conserved throughout the ARF gene family and is necessary and sufficient for binding to the AuxRE (Ulmasov et al., 1999b). Using the PSORT program, five putative nuclear localization signals (NLS) were identified in the DBD and middle region of ARFs 1, 2, 3, 4, 9, 10, 13, and 16 (Guilfoyle and Hagen, 2001). The DBD in *monopterous*/ARF5 was tested by

GUS fusion and was found to contain more than one NLS with varying selectivity for localizing to the nucleus (Hartdke and Berleth,1998).

The middle region, between the DBD and domain III, is variable among members of the ARF family. Ulmasov et al. (1999a) noted that although the MR is variable there is an amino acid preference among the ARF proteins. ARF5, 6, 7, and 8 all have a preference for the amino acids glutamine, leucine and serine (Q/L/S-rich) and are putative activators of transcription, while ARF1 has a preference for proline, serine and threonine (P/S/T-rich) and is a putative repressor of transcription (Ulmasov et al., 1999a). Other ARFs do not seem to have an amino acid preference and were not found to have increased activation or repression compared to controls.

Domains III and IV are involved in forming dimers with other ARF proteins, and Aux/IAA proteins, which also have conserved domains III and IV. These domains have been shown to interact in two hybrid assays forming both homo- and heterodimers (Liscum and Reed, 2002). ARF-ARF homodimers, as well as ARF-Aux/IAA heterodimers, are able to stably bind DNA (Ulmasov et al., 1999b). It is thought that Aux/IAA-ARF and ARF-ARF dimers are able to influence ARF binding, depending on whether the specific ARF protein is a repressor or activator of transcription (Liscum and Reed, 2002). Certain combinations of proteins would either activate or repress transcription, either increasing or decreasing the expression of the gene they are regulating. Aux/IAA-Aux/IAA homodimers have not been found to stably bind DNA.

There are 3 known ARF mutants, *ettin*/ARF3, *nph4/*ARF7 and *monopteros*/ARF5 (*mp*). Because of its phenotype, the *mp* gene is thought to play a key role in organizing the basal body region of the *Arabidopsis* embryo. In the *monopteros* mutant, apical structures (i.e. shoot meristem and cotyledons) are present but basal structures such as hypocotyl, radicle and root

meristem are missing and the plant is without any root-specific morphological features. It is thought that this gene contributes to apical-basal pattern formation in the embryo (Berleth and Jurgens, 1993). Przemeck et al. (1996) found that these mutants form normal rosettes and root systems, but inflorescences fail to form lateral flowers, or lateral flowers are greatly reduced. It was also found that the auxin transport capacity of the inflorescence axis is impaired and the vascular strands are distorted. ARF5 likely promotes cell axialization and cell file formation at many stages of plant development. Thus, the functional gene ARF5, may be required for initiating the files of elongated cells that form the hypocotyl and root axis.

The DBD of ARF1 and ARF5/MP/IAA24 are both able to bind to the same functionally defined promoter element of auxin-induced genes; therefore, these proteins are able to regulate the same gene (Hardtke and Berleth, 1998). In a study by Hardtke and Berleth (1998), IAA24 was reported to be a partially represented cDNA and is thought to be part of ARF5, because 865 out of 866 amino acids are identical. They also reported that MP/IAA24 has 3 NLSs including a bi-partite localization signal. It is now believed that *monopteros* is an ARF5 mutant not an IAA24 mutant.

E*ttin/*ARF3 (*ett)* is characterized by an increase in perianth organ number, a decrease in stamen number and anther formation, and defects in apical-basal patterning in the gynoecium (Sessions et al., 1997). It is thought that *ett* conveys regional identity to floral meristems and affects perianth organ number and spacing, stamen formation, and regional differentiation in stamens and the gynoecium.

N*ph4/*ARF7 plants have disrupted hypocotyls, root phototropism and hypocotyl gravitropism (Liscum and Briggs, 1996). *Nph4* plants also have defects in apical hook and phytochrome-dependent hypocotyl curvature of etiolated seedlings (Stowe-Evans et al., 1998).

Mutant plants are resistant to exogenous auxin, and the early-response genes including Aux/IAA show reduced expression levels with and without exogenous auxin (Guilfoyle and Hagen, 2001). These data argue for ARF7 functioning as a transcription factor regulating auxin-response genes required for growth (Harper et al., 2000).

Model of early auxin gene regulation

A model has been proposed for how auxin regulates Aux/IAA genes (based on Hagen and Guilfoyle, 2002; Gray and Estelle, 2000; Gray et al., 1999, Gray et al., 2001;Liscum and Reed, 2002). ARFs are always believed to be bound to the auxin response element by their DBD. Aux/IAAs genes are either repressed or activated, depending on which ARF is bound to the AuxRE, and what protein (ARF or Aux/IAA) is bound through domains III and IV of that ARF protein. Binding is controlled in part by the auxin concentration. ARF and Aux/IAA protein that is free in the cell are thought to be bound as homo- and heterodimers.

At low concentrations of auxin, ARF activator protein is thought to be deactivated by association with Aux/IAA repressor proteins through domain III and IV. These dimers most likely form whether or not an ARF is bound to the AuxRE in the promoter. Tissue-specific expression patterns of both ARF and Aux/IAA proteins will limit the number of possible interactions between ARF and IAA proteins, and which ARF can bind the TGTCTC element.

Increasing auxin concentration activates transcription, leading to a dissociation of repressor proteins, and association of activator proteins. An early step in activation is thought to be removal and rapid degradation of Aux/IAA proteins, perhaps by the ubiquitin-proteasome degradation pathway (Worley et al., 2000). Phosphorylation of Aux/IAA proteins in response to phytochrome A might initiate this process. Once the Aux/IAA proteins are removed and degraded, activation occurs and the early-response genes are transcribed. Transcription might be

further enhanced by an activating ARF binding to another ARF already bound to the promoter. Different cells may exhibit different responses to auxin because they express different sub-sets of ARFs, Aux/IAAs, or other factors that interact with these regulators. Aux/IAA genes that contain AuxREs would respond to auxin via activated transcription, resulting in increased levels of mRNA and protein. Aux/IAA proteins may function as repressors of their own transcription by negative feedback, when auxin levels are low. At high auxin concentrations, early auxin-response genes remain activated for at least several hours.

Aux/IAA protein signaling and degradation

Proteins are not only modified by the addition of small groups such as phosphates, but also by covalent binding of other proteins such as ubiquitin (Marx, 2001). Ubiquitin is a putative signaling molecule that targets other proteins for degradation by the 26s proteasome. Ubiquitin, one of the most conserved proteins known in eukaryotes, may be attached to other proteins to mark them for degradation by the 26s proteasome (del Pozo et al., 1998). The ubiquitin-proteasome degradation pathway is a mechanism that the cell uses to quickly remove specific proteins. This pathway plays a key role in a number of cellular events, such as cell cycle transitions, metabolic regulation, stress response and differentiation (Gray et al., 1999). The ubiquitin pathway is believed to occur in the following stepwise fashion (from Gray and Estelle, 2000).

Ubiquitin is first activated by the formation of a thiol-ester bond between its C-terminus and a cysteine residue within the ubiquitin-activating enzyme (E1). Ubiquitin is then transesterified to a member of a family of ubiquitin-conjugating enzymes (E2). With the assistance of a ubiquitin ligase (E3), ubiquitin is covalently attached to an NH_2 group of a lysine residue within the substrate protein. Reiteration of these reactions attaches additional ubiquitin

proteins to a specific lysine residue within the conjugated ubiquitin, and results in the generation of a polyubiquitin chain. The 26s proteasome recognizes this chain and degrades the tagged protein, releasing free ubiquitin in the process.

E3 ligases are a diverse and complex group, with four types characterized (Dharmasiri and Estelle, 2002). The E3 enzyme recognizes specific proteins and/or residues in two ways and therefore is able to target specific proteins for degradation (Gray et al., 1999). First, the E3 enzyme forms a catalytic intermediate with ubiquitin, and secondly brings the E2 and substrate into close proximity to facilitate ubiquitination (Gray and Estelle, 2000). This rapid breakdown of Aux/IAA protein by the ubiquitin pathway serves a necessary role in the cell. Half-lives of Aux/IAA genes have been reported to be 5-80 minutes, but stabilizing mutations in domain II have been shown to increase that time up to 550 minutes or more (Park et al., 2002). The short half-lives of Aux/IAAs ensure that they will not accumulate to high levels or persist after synthesis has terminated (Worley et al., 2000).

Photomorphogenesis

Seedlings have two possible developmental pathways, skotomorphogenesis or photomorphogenesis. Skotomorphogenesis is characterized by closed cotyledons on an apical hook and elongated hypocotyls, while photomorphogenesis is characterized by short hypocotyls and expanded green cotyledons (Osterlund et al., 1999). The change from skotomorphic growth to photomorphic growth is thought to involve sensing light quality and light quantity (Nakazawa et al., 2001). Light is perceived by several complementing photoreceptors including phyA, phyB and CRY1, which are involved in far-red-, red- and blue-light-dependent hypocotyl elongation respectively, during seedling development (Osterlund et al., 1999).

It is thought that COP1 acts downstream of these photoreceptors in plants, and is regulated by them (Osterlund et al., 1999). In the dark, COP1 is activated and transported into the nucleus (Osterlund et al., 1999). When the plants are transferred to the light the nuclear abundance of COP1 is reduced (Osterlund et al., 1999). Exclusion of COP1 from the nucleus may determine which pathway the plant takes.

Recent studies have shown that COP1 is able to interact with multiple transcription factors and regulate gene expression, thereby suppressing photomorphogenic development (Osterlund et al., 1999). COP1 has been shown to closely interact with the transcription factor HY5, a positive regulator of photomorphogenesis, which is degraded in the dark (Bahrami et al., 2002). HY5 binds to the G-box, a light-responsive promoter element, and is essential for activating light-inducible genes that have the G-box motif in their promoters (Osterlund et al., 1999).

The COP1 pathway is thought to tie into other proteins and transcription factors, including the ARF and Aux/IAA gene families. Mutants of Aux/IAA and ARF that show altered light response suggest that light may play a role in altering auxin responses. It is believed that phytochromes alter Aux/IAA protein activity directly (Liscum and Reed, 2002). Phy A has been shown to phosphorylate Aux/IAA proteins *in vitro* (Colon-Carmona et al., 2000). *In vivo,* phytochrome A is activated in the cytoplasm and translocates into the nucleus, where it can phosphorylate substrates like the Aux/IAA proteins (Liscum and Reed, 2002). This in turn, is thought to play a role in regulating the stability of Aux/IAA proteins (Liscum and Reed, 2002) because most proteins that are broken down by the ubiquitin proteasomes pathway are first phosphorylated (Maniatis, 1999).

The purpose of this thesis was to localize individual proteins and investigate the interactions between two proteins in the same cell using fluorescent protein markers *in vivo*. This is the first research to determine the sub-nuclear localization of any ARF or Aux/IAA protein *in vivo*. This is also the first research to determine the sub-cellular co-localization patterns of proteins in these families. The main focus of this research was on the localization and co-localization of ARF1 and IAA17 proteins in carrot protoplasts. ARF6, ARF7, IAA2 and IAA3 were also localized individually, with ARF6 and IAA3 also being co-localized with ARF1. This research supports putative protein-protein interactions of these two families, which has not been documented *in vivo.*

The ARF and Aux/IAA gene families are involved in gene regulation and this research is important for our understanding of how these genes interact *in vivo* and understanding gene regulation in plants. These genes have also been shown to be differentially expressed in wood forming tissue and have broader implications of being useful in forestry to begin understanding the steps involved in wood formation in trees.

Chapter 2 Materials and Methods

Fluorescent protein vector construction

Primers for all constructs were designed using *Arabidopsis thaliana* genome sequences incorporating enzyme restriction sites present in the multiple cloning site of the fluorescent protein fusion expression vectors (Figure 2-1). RT PCR was performed using total RNA extracted from 4 week-old *A. thaliana* plants to generate cDNA clones for genes of interest. RNA extraction was carried out as described elsewhere (Sambrook and Russell 2001). Superscript Reverse Transcriptase III (Invitrogen) was used for reverse transcription of plant RNA and PCR reactions were run following the manufacturer's protocol (Invitrogen). A mixture of Pfu (20%) and Taq (80%) was used to increase fidelity during PCR amplification. All cDNA clones were subjected to DNA sequence analysis.

The 5' forward primers (Appendix 1) used for amplification and cloning of genes into expression vectors that fuse a fluorescent protein to the C-terminus (Figure 2-1) included a ribosomal binding site (RBS), TCGCCACC. All 5' primers contain a restriction enzyme recognition sequence, a RBS upstream of the start codon of the gene. The 3' primers for inserts being cloned into vector with fluorescent protein fused to the N-terminus contain a stop codon. The 3' primers for inserts with fluorescent protein fused to the C-terminus have the stop codon deleted for translation through the fluorescent protein coding region.

Following amplification, PCR fragments were digested for 3-4 hours at 37° C using appropriate enzymes and buffers (New England Biolabs). Digested PCR fragments were gel purified after electrophoresis in a 1% agarose gel following the manufacturer's protocol

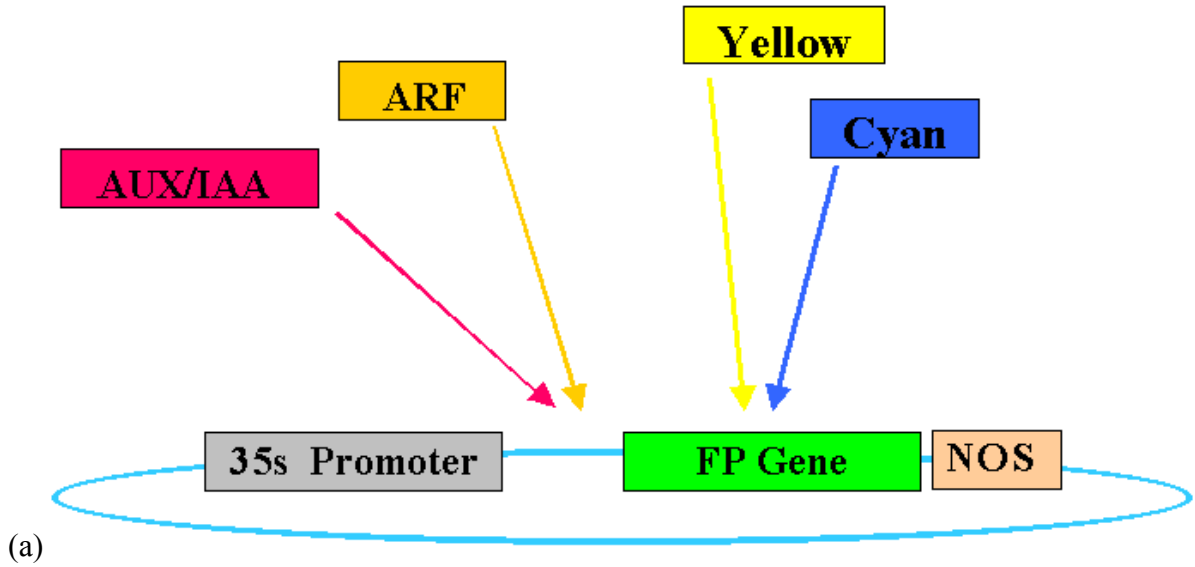
(Qiagen). Fragments were quantitated and ligated into fluorescent protein vector (Figure 2-1), that had been digested with appropriate restriction enzymes. Control vectors contain only fluorescent protein coding regions (Figure 2-1). Approximately 20-80 ng of insert was ligated into 100-200 ng of vector using T4 DNA ligase and following manufacturer's protocol (New England Biolabs). After ligation, constructs were transformed into XL-1 blue competent cells (Sambrook and Russell, 2001), and plated onto agar with 50 mg/ml ampicillin. Colonies were screened for positive clones using PCR primers designed to amplify inserts between the 35S promoter and the fluorescent protein gene. Positive clones were grown overnight in 5 ml LB broth, glycerol stocks were made and plasmid was purified using Qiagen miniprep, according to the manufacturer's instructions. Plasmid constructs were digested using cloning enzymes to check junctions. Constructs were also sequenced in the 5' and 3' directions for verification that no errors were introduced into the sequences during PCR amplification.

Constructs for protoplast transfections were amplified in 500-1000 mL LB broth with 50 mg/ml ampicillin. Plasmid DNA was isolated either by running on a cesium chloride gradient (Sambrook and Russell, 2001), or using maxiprep kits following manufacturer's protocol (Sigma). Plasmid was resuspended in 10 mM Tris Cl pH 8.0.

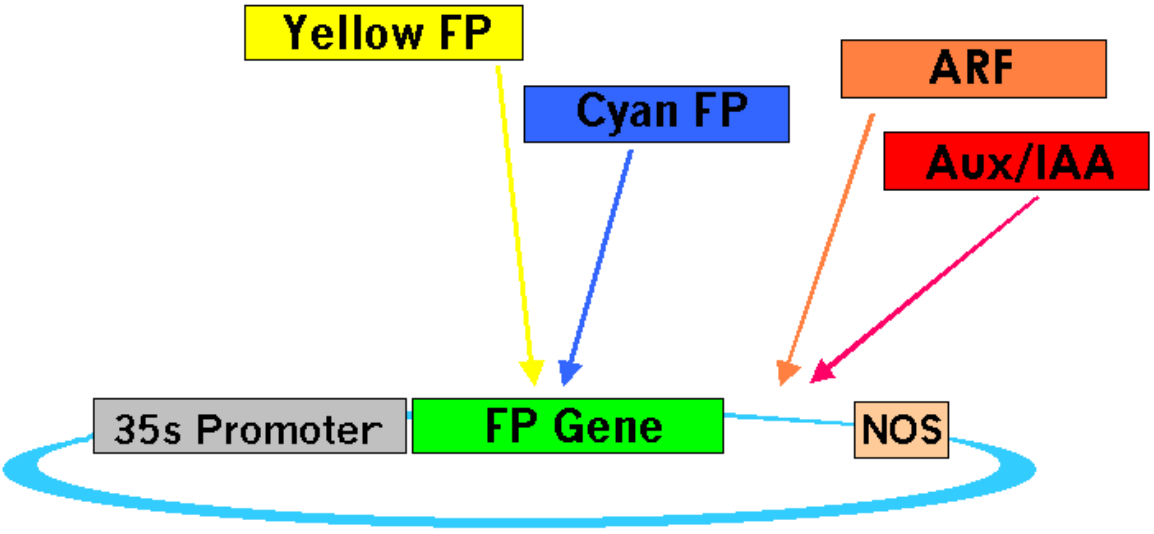
Carrot cell digestion protocol

Solutions for carrot digestion were modified from Liu et al. (1994) (Appendix 2). All steps were carried out at room temperature. Cells from a 50 ml culture of 3-4 day old carrot suspension culture were pelleted by centrifuging at 150x g for 5 minutes without deceleration. Supernatant was aspirated and cells were resuspended in 25-50 ml driselase. Solution was then poured into a sterile deep well petri plate. Cells were incubated in the dark for 6 hours with 40 RPM shaking. Digestion time varied and was determined empirically. After digestion, cells

were decanted into a 50 ml conical tube and pelleted at 100x g for 5 minutes without deceleration.



(a)



(b)

Figure 2-1. Fluorescent protein vector. a) Fluorescent protein expression vector with fluorescent protein gene fused to the C-terminus. b) Fluorescent protein expression vector with fluorescent protein gene fused to the N-terminus.

Supernatant was decanted and cells were resuspended in 30 ml 1x W5 solution. An aliquot of cells was removed and counted using a haemocytometer. Cells were centrifuged at 100x g for 5 minutes. Supernatant was aspirated and 20 ml 1x W5 solution was added and cells were resuspended. Cells were centrifuged at 100x g for 5 minutes, the supernatant was aspirated and cells were resuspended in MC solution to a cell density of 2 x 10^6 cells/ml.

Transformations were carried out in 2 ml tubes (Phenix) using 5-20 ug of DNA for each construct (20 ul total volume). Using wide-bore tips (Rainin), 300 ul of resuspended protoplasts were added to DNA, followed immediately by 300 ul PEG CM. Cells were incubated for 5 minutes, and then transferred to sterile 15 ml tubes containing 4 ml of room temperature carrot media with or without NAA. Cells were then mixed by inverting and placed in the dark horizontally for 24 hours.

Cells were imaged using a Leica confocal SP2 microscope at the University of Georgia (Athens, Georgia, USA). Transformed and co-transformed constructs were also viewed using filters specific for each color fluorescent protein's emission spectra. Digital images were captured using Leica confocal software. All images were representative of the localization pattern of each construct. Each construct was localized at least 3 different times. Localization patterns seen consistently in each transformation were considered typical.

Northern Blot and semi-quantitative RT PCR

Arabidopsis thaliana ecotype Columbia seeds were planted in 4" pots in moistened potting soil, with two pots for each treatment. Treatments were uninduced and auxin-induced plants. Plants were harvested at 1, 2, 3, and 4 weeks, for a total of 8 treatments. 1 and 2 week pots were planted heavily while 3 and 4-week pots were thinned. After planting, pots were placed in trays; trays were filled with 2 cm water, covered in plastic wrap, and imbibed by

placing them in the dark at 4°C for 48 hours. After 4°C incubation, plants were placed in a growth chamber under long day conditions (18 hours light, 6 hours dark 22°C). Day 1 was determined by germination of the first seedlings. Plants for 3 and 4-week samples were thinned about 8 days after germination.

On days 6, 13, 20, and 27, seedlings were misted with either 10 um 1-napthalene acetic acid (NAA) or dH_2O. Plants were sprayed outside of the growth chamber and allowed to dry before returning to growth chamber. The following day, seedlings were treated again and harvested 2 hours after treatment. For harvesting at 1, 2, and 3 weeks whole seedlings were removed from the soil and washed in dH_2O to remove any loose soil. For 4-week samples, inflorescence stalks, including flowers and seedpods were dissected and collected. Rosette leaves were also dissected, collected, and washed briefly. Roots were removed from the soil and thoroughly washed with dH_2O. Excess water was removed from all samples. Samples were then placed in 50 ml conical tubes, flash frozen in liquid nitrogen, and stored at –80°C.

RNA isolation was carried out using Trizol® reagent (Invitrogen) and Phase lock gel (Eppendorf). 2.5-3 grams of tissue was ground using mortar and pestle under liquid nitrogen. Samples were then added to 30-35 mL Trizol® reagent and homogenized for 2 minutes using a tissue homogenizer. Samples were then added to a pre-spun 50 mL phase lock gel tube. RNA was extracted following Trizol® protocol. After extraction, RNA was precipitated using isopropanol and washed (Sambrook and Russell, 2001). Samples were resuspended in 2 ml RNase free 1 mM Tris pH 8.0. Spectrophotometric quantitation at 260 and 280 nm was used to determine quality and yield. 500 ug of total RNA was stored at –80°C for mRNA isolation, and remaining RNA was stored under ethanol at –20°C. 400 ul of Oligo dT Dynabeads® (Dynal Corp.) was used for each 500 ug sample of total RNA. Isolation of mRNA was carried out

following manufacturers protocol. Samples were eluted in 40 ul RNase free 1mM Tris pH 8.0 quantitated by spectrophotometry at 260 and 280 nm and stored at –80°C.

Duplicate denaturing agarose gels were run loading 1 ug of mRNA per lane and using the Northern Max kit (Ambion), following the manufacturer's protocol. Gel was run at 100v for 30 minutes, photographed, then transferred to a Biodyne A nylon membrane (Pall) by capillary blotting for 4 hours. Blots were then dried at 65°C for 15 minutes and UV crosslinked.

ARF1 and IAA17 inserts were digested out of plasmid vectors and gel purified (Qiagen). Purified ARF1 and IAA17 were labeled for 20 hours using Phototope probe labeling kit (New England Biolabs). Probes were purified using Probequant Sephadex spin columns according to the manufacturer's protocol (Amersham).

Hybridizations were carried out at 65°C overnight. Membranes were then washed and detected following detection kit protocol (New England Biolabs). Labeled membranes were exposed to Hyperfilm ECL (Amersham Biosciences) for 5 and 7 minutes.

To further purify mRNA, samples were subjected to a second round of mRNA selection using Oligo dT Dynabeads® following manufacturer's protocol (Dynal Corp). Samples were eluted in 40 ul RNase-free 1 mM Tris pH 8.0 and quantitated by spectrophotometry at 260 and 280 nm. Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen) following manufacturer's protocol using 100 ng of mRNA per reaction for all 12 samples. Samples were then used in 50 ul PCR reaction as follows:

5.0 ul 10x buffer
5.0 ul 2 mM dNTP's
5.0 ul 5' primer (5 uM)
5.0 ul 3' primer (5 uM)
0.5 ul taq polymerase
4.0 ul Magnesium
2.0 ul cDNA from RT reaction
23.5 ul ddH_2O

Sequence specific primers were developed for ARF1 and IAA17, and ACT2 and RPT3 (Appendix 1). Primers amplified fragments of approximately 400 bp. Reactions were set up in duplicate with one undergoing 25 and the other 35 amplification cycles following the program:

Step	Temp	Time
1	94°C	30 seconds
2	94°C	30 seconds
3	57°C	30 seconds
4	72°C	30 seconds
5	Go to step 2	(25 or 35 cycles)
6	72°C	10 minutes
7	4°C	Hold

Samples were run on a 1% agarose gel for 30 minutes at 100 volts and digitally imaged (Alpha Innotek).

Chapter 3 Results

ARF1 localization

ARF, Aux/IAA, mutant, and derived deletion fusion constructs were cloned into a fluorescent protein expression vector containing the CAMV 35s promoter and NOS terminator (Figure2-1). These constructs were transformed into carrot cell protoplasts by PEG mediated chemical uptake and visualized on a Leica confocal SP2 microscope. ARF1, IAA17 and *axr3-1* were ligated into vectors with fluorescent protein fused to both the C-terminus and N-terminus, with no discernable difference in their pattern of localization. No difference in localization was seen in any construct, when transformed cells were incubated with or without NAA overnight. Transformations were repeated at least three times per construct. All images are representative of typical localization patterns, which were specific and repeatable.

ARF1 protein had a specific, repeatable subcellular and sub-nuclear localization. This construct was localized individually at least 40 times during the course of the experiments. ARF1 shows peri-nucleolar localization as well as punctuate localization concentrated in the nucleus, but was also found in a punctate distribution in the cytoplasm (Figure 3-1). When multiple nuclei are present in the cell, ARF1 protein was localized to both nuclei and around both nucleoli (Figure 3-1), and was typical of the observed pattern of ARF1 localization. Approximately 50% of transformed cells exhibited peri-nucleolar localization with approximately 30% exhibiting protein localization throughout the nucleus and the remaining 30% exhibiting punctate localization inside and outside of the nucleus not associated with the nucleolus.

Transformed cells from each construct localized shared similar characteristics. About 30-40% of all transformed cells for all constructs had a localization pattern with protein diffused throughout the nucleus. The remaining 60-70% had a distinct repeatable punctate localization pattern that varied depending on the construct transformed. Constructs that had punctate localization also exhibited varying amounts of fluorescent protein diffused throughout the nucleus, not associated with structures.

When a control vector containing the promoter, fluorescent protein coding region, and NOS terminator was expressed in carrot protoplasts, fluorescent protein was distributed throughout the cytoplasm and nucleus (Figure 3-2). No punctate accumulations of fluorescent protein were observed in cells expressing the control construct. Structures observed in transformed cells did not appear to be different from those present in non-transformed cells (Figure 3-3). Non-transformed carrot cells were also found to contain multiple nucleoli. Fluorescent protein was never observed inside the nucleolus with any of the constructs tested. Fluorescent protein alone is small enough to passively diffuse through the nuclear membrane into the nucleus.

ARF1 deletion constructs

Deletion constructs containing fragments of the ARF1 coding region fused to a fluorescent protein were created to examine putative localization motifs within the ARF1 protein (Figure 3-4). Deletion constructs were transiently expressed in carrot protoplasts and the cells were visualized. ARF1 deletion 1 (ARF1-D1) and ARF1 deletion 2 (ARF1-D2) exhibited no nuclear localization or targeting to foci (Figure 3-6 and Figure 3-7). There was no observable difference between these deletion constructs and controls containing fluorescent protein alone.

ARF1-D1 contained the DNA Binding Domain (DBD), and ARF1-D2 contained the DBD and putative bi-partite NLS (Figure 3-5a).

ARF1 deletion construct 3 (ARF1-D3) and ARF1 deletion construct 4 (ARF1-D4) exhibited restored nuclear and sub-nuclear localization (Figure 3-8 and Figure 3-9). ARF1-D3 contained the DBD, the putative bi-partite NLS and putative NLS. ARF1-D4 included the DBD the two putative NLSs and the non-conserved MR (Figure 3-5a).

IAA17 localization

Cells expressing IAA17 fusion protein exhibited localization to foci or structures within the nucleus. Some cells contained a few (1-6) large structures, whereas other cells contained many (10-100) smaller structures. Both localization patterns were seen about equally. IAA17 localized to structures within the nucleus, but was not found surrounding the nucleolus (Figure 3-10). The localization of IAA17 was limited to structures inside the nucleus, which can be seen in the transmitted images of the cell.

***axr3-1/*IAA17 localization**

Mutant plants with *axr3-1/*IAA17 have a phenotype different from wild-type plants. These plants are auxin resistant and have a single, unbranched inflorescence. The roots are agravitrophic without root hairs that stem from the hypocotyl instead of the base of the hypocotyl. In order to determine if the *axr3-1* mutation affected localization, the coding region of the mutant allele was cloned into the fluorescent protein expression vector and transformed into carrot protoplasts. The mutant *axr 3-1*/IAA17 protein had the same localization pattern as wild-type IAA17 protein (Figure 3-11). The a*xr3-1* fusion exhibited nuclear and sub-nuclear localization to discrete foci. This construct localized to either a few large sub-nuclear structures,

depending on the cell, or many small sub-nuclear structures and was not found outside of the nucleus. Less intense, diffuse protein was also found throughout the nucleus (Figure 3-10b).

Co-localization of ARF1 with IAA17 and ARF1 with *axr3-1*

Co-transformation of ARF1 and IAA17 constructs in carrot protoplasts were carried out to examine putative protein-protein interactions *in vivo*. ARF1 was fused to cyan fluorescent protein (CFP) and IAA17 to yellow fluorescent protein (YFP). The CFP and YFP have different emission spectra, allowing discrete visualization of the two color variants. Cells expressing both constructs exhibited localization of both fusion proteins to identical regions and structures within the nucleus. Co-transfected ARF1, in addition to being peri-nucleolar, is distributed to the punctate structures where IAA17 is normally distributed when expressed alone (Figure 3-12 and 3-13). IAA17 fusion protein localizes to the peri-nucleolus and other structures previously seen only when ARF1 was expressed alone. ARF1 protein found outside of the nucleus did not have IAA17 associated with it, IAA17 was still found exclusively in the nucleus (Figure 3-12).

Co-transformation of ARF1 and *axr3-1* in carrot protoplasts was carried out to determine if *axr3-1* protein localized differently from IAA17 protein. Cells expressing both ARF1 and *axr3-1* exhibited protein localization similar to that of IAA17. Co-transformation exhibited overlapping localization of ARF1 and *axr3-1* in the peri-nucleolus and sub-nuclear foci (Figure 3-14, 3-15, and 3-16). This localization pattern was not seen when *axr3-1* was localized individually, but was observed when co-transformed with ARF1. *Axr3-1* was not found outside the nucleus when expressed with ARF1 (Figure 3-16).

IAA17 deletion constructs

IAA17 deletion constructs were made containing portions of the coding region fused to a fluorescent protein (Figure 3-17). IAA17 deletion construct 1 (IAA17-D1) (Figure 3-18), IAA17

deletion construct 2 (IAA17-D2) (Figure 3-19), and IAA17 deletion construct 3 (IAA17-D3) (Figure 3-20) did not localize to the sub-nuclear regions observed for the full-length IAA17 fusions. These constructs exhibited a pattern of localization like that of control GFP, and were observed throughout the cytoplasm and nucleus. IAA17-D3 contained both of the putative nuclear localization signals identified in the Aux/IAA family of transcription factors.
The IAA17 deletion construct 4 (IAA17-D4) (Figure 3-21) and IAA17 deletion construct 5 (IAA17-D5) (Figure 3-22) exhibited localization patterns indistinguishable from those observed for the full-length IAA17 fusion. IAA17-D4 contained domain III of the Aux/IAA family in addition to the two putative NLSs predicted for this family of transcription factors (Figure 3-5b).

Gene expression analysis of ARF1 and IAA17

One, two, and three week-old *Arabidopsis* plants were either sprayed with NAA or water 16 and 2 hours before harvesting. Treated and control four week old plants were harvested and dissected into inflorescence stems, rosette leaves and roots. Total RNA was isolated and poly A+ mRNA was purified using oligo dT Dynabeads®.

Arabidopsis mRNA was separated by denaturing agarose gel electrophoresis and transferred to nylon membranes by capillary blotting. Duplicate Northern blots were probed with either the ARF1 or IAA17 coding region. Northern blot analysis indicated ARF1 and IAA17 are co-expressed in all tissues examined (Figure 3-23 a).

Semi-quantitative RT PCR was also used to examine expression of the ARF1 and IAA17 genes in *Arabidopsis*. Gene specific primers were developed to amplify an approximately 400 bp fragment of ARF1 and IAA17 (Appendix 1). The actin gene ACT2 (An et al. 1996) and 26s proteasome AAA-ATPase subunit RPT-3 (AF123392) were used as internal controls. Following amplification, PCR reactions were subjected to agarose gel electrophoresis, stained with

Ethidium Bromide, and photographed. Results were consistent with Northern blot analysis and indicated that the ARF1 and IAA17 genes were co-expressed in all tissues examined (Figure 3-23 b).

ARF6, ARF7, IAA2 and IAA3 localization

Localization of other ARF and Aux/IAA genes was also examined using fluorescent protein fusions and carrot cell protoplast system. ARF6 showed a pattern of localization that was similar to ARF1. The ARF6 fusion protein exhibited a punctate distribution within the nucleus that appeared to be associated with sub-nuclear structures (Figure 3-24). The ARF6 fusion was also localized to the peri-nucleolar region observed for the ARF1 fusion in about 50% of transformed protoplasts. ARF6 was not seen in structures outside of the nucleus.

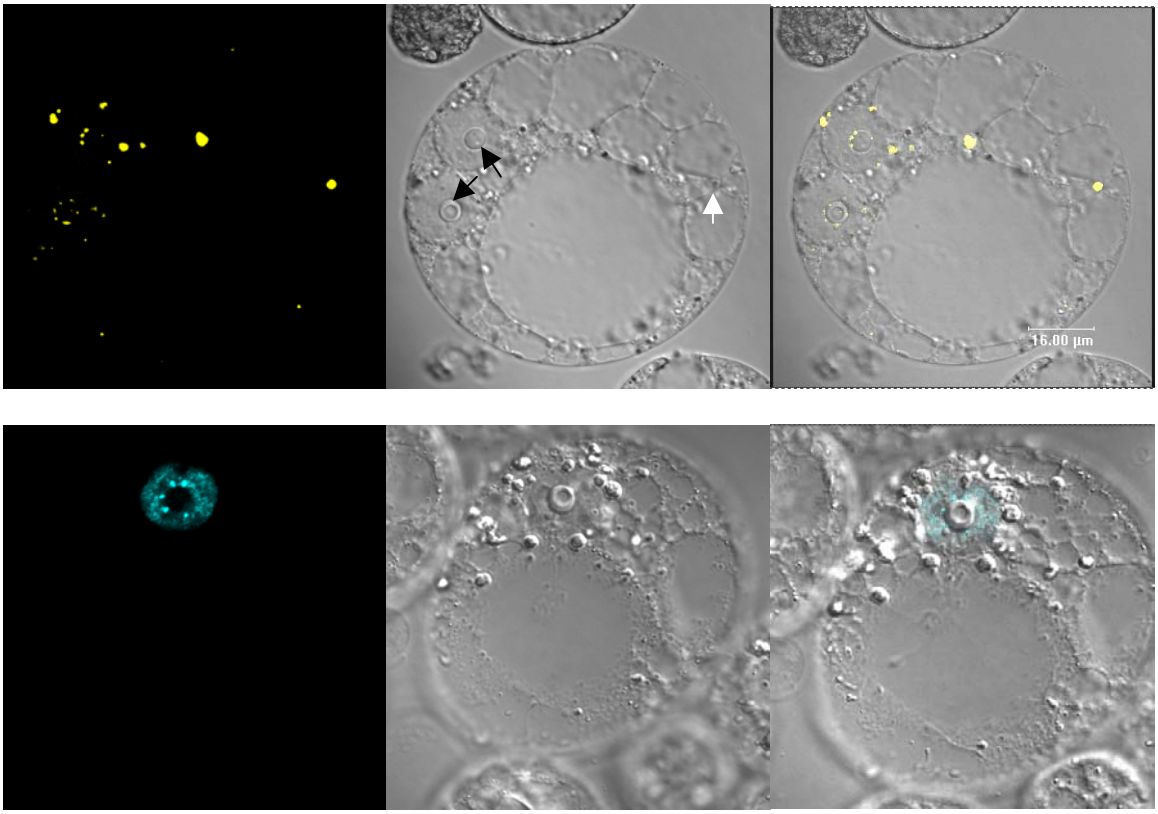
ARF1 (cyan) and ARF6 (yellow) were also co-localized. In cells where ARF1 and ARF6 were co-localized, a different localization pattern than that of individual localizations was observed. Some of the cells had ARF6 exclusively inside the nucleus, while ARF1 and ARF6 were co-localized in structures outside of the nucleus (Figure 3-25). Other cells had both ARF1 and ARF6 both exclusively inside the nucleus and associated with the peri-nucleolar region (Figure 3-26).

ARF 7 protein showed two distinct localization patterns. Some cells had a punctate pattern with protein distributed in structures inside and outside of the nucleus (Figure 3-27 a-b). In other cells, ARF 7 was seen not associated with any visible structures, but in a definite pattern (Figure 33-27 c-d).

The IAA2 fusion protein localized to the nucleus. It did not exhibit a punctate distribution, but was distributed throughout the nucleus except for the nucleolus (Figure 3-28).

IAA3 had a sub-cellular localization pattern with protein in either a few large structures or many small structures exclusively inside the nucleus (Figure 3-29). This pattern was similar to that of IAA 17.

Co-expression of ARF1 with IAA3 exhibited ARF1 distributed exclusively to structures outside of the nucleus, and IAA3 diffused exclusively in the nucleus, not associated with any structures (Figure 3-30).



A B C

Figure 3-1. Localization of ARF1 protein in transiently transfected carrot protoplasts.
A. Fluorescent image of ARF1 protein localization pattern.
B. Transmitted image of ARF1. Black arrows indicate the nucleoli. White arrow indicates a structure outside of the nucleus that accumulates ARF1 fluorescent protein.
C. Overlay image of A and B. ARF1 is inside the nucleus, around the nucleolus, and associated with structures outside of the nucleus.

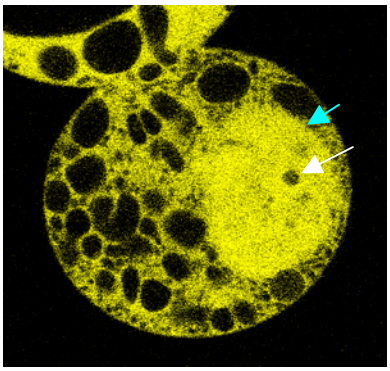
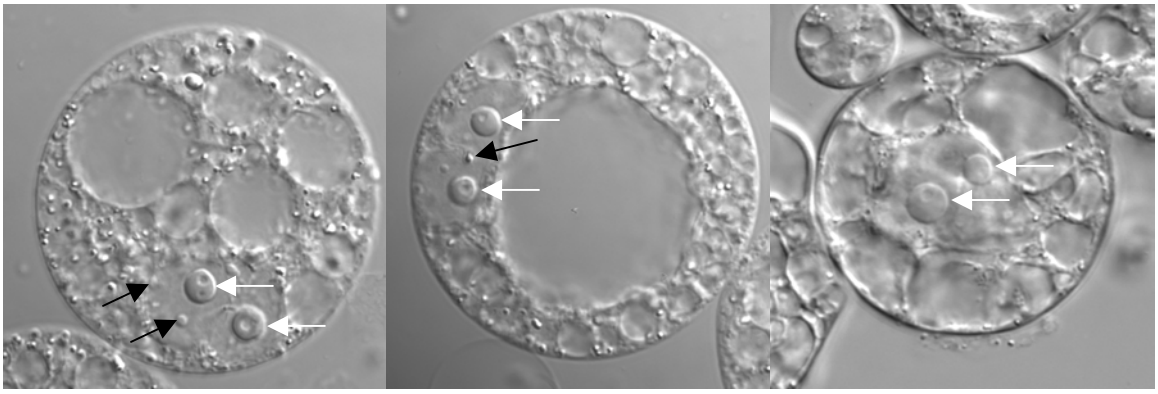


Figure 3-2. Control GFP vector expressed in carrot protoplast. Protein is found throughout the cell including the cytoplasm and nucleus. White arrow indicates the nucleolus. Blue arrow indicates the nucleus.



A B C

Figure 3-3. Transmitted images of non-transformed cells showing similar morphology and structures compared to transformed cells. White arrows indicate nucleoli, black arrows identify structures seen in transformed cells

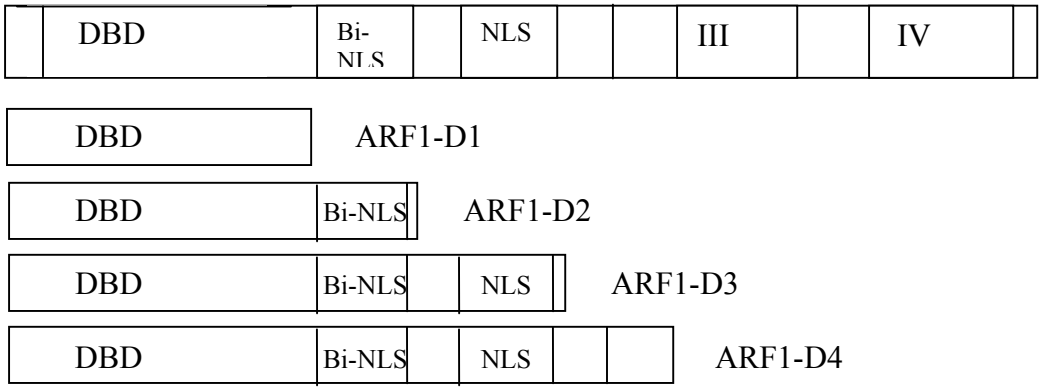


Figure 3-4. Schematic diagram of ARF1 deletion constructs. Diagram includes putative domains and localization signals reported for ARF1.

(a) ARF 1

|→ 5' primer | |→

1 ATGGCAGCTT CCAATCATTC ATCTGGTAAA CCTGGAGGAG TTTTAAGTGA

DNA binding domain

51 TGCTTTATGT AGGGAGCTCT GGCATGCCTG TGCTGGACCT CTTGTAACCC

101 TACCTCGTGA AGGGGAACGA GTTTATTATT TCCCTGAAGG CCACATGGAG

151 CAGCTCGAGG CATCAATGCA CCAAGGTTTG GAGCAACAGA TGCCTTCCTT

201 CAACCTCCCA TCTAAGATCC TCTGTAAAGT TATCAACATC CAGCGCAGGG

251 CAGAGCCCGA GACTGACGAA GTATATGCGC AAATAACCTT ATTGCCAGAA

301 CTGGATCAAA GCGAACCCAC TAGCCCAGAT GCCCCTGTTC AAGAACCTGA

351 AAAGTGCACC GTACATTCAT TTTGCAAGAC ACTAACTGCT TCAGACACAA

401 GCACACATGG TGGCTTCTCG GTGCTACGGA GACATGCAGA TGATTGTCTC

451 CCACCCTTGG ATATGTCCCA ACAACCACCG TGGCAAGAAT TGGTTGCAAC

| deletion primer 1 ←|

501 TGATTTGCAC AATAGTGAAT GGCATTTTAG GCACATTTTC CGAGGCCAAC

|→ ←| putative bipartite NLS |→

551 CAAGGCGTCA TTTGCTAACA ACTGGATGGA GTGTTTTTGT TAGCTCGAAG

←| | 3' deletion primer 2

601 AAACTAGTGG CTGGTGATGC TTTCATATTC TTAAGGGGTG AGAATGAAGA

←|

651 GCTCCGAGTA GGTGTTAGGC GGCACATGAG ACAACAGACT AATATCCCGT

701 CATCTGTCAT TTCAAGTCAT AGCATGCATA TTGGGGTCCT TGCAACAGCA

751 GCTCATGCCA TTACAACAGG AACAATCTTT TCTGTCTTCT ACAAGCCAAG

801 GACAAGTAGG TCAGAGTTTA TTGTGAGCGT CAATAGGTAT CTCGAAGCTA

851 AGACCCAGAA GCTGTCTGTA GGCATGCGTT TCAAGATGAG ATTCGAGGGG

901 GAAGAAGCTC CCGAGAAAAG GTTCAGTGGC ACAATAGTTG GTGTTCAGGA

951 AAATAAGTCT TCGGTCTGGC ATGATTCTGA ATGGAGATCG CTAAAGGTTC

1001 AATGGGACGA ACCCTCATCT GTATTTCGTC CTGAAAGAGT TTCACCTTGG

1051 GAACTTGAGC CCCTAGTTGC AAATAGTACT CCGTCTTCAC AACCTCAGCC

|→ putative NLS ←|

1101 TCCGCAAAGG AACAAACGAC CAAGACCTCC TGGTTTACCT TCACCAGCCA

| 3'deletion primer 3←| ←|

1151 CTGGTCCATC TGGTCCTGTT ACTCCAGATG GTGTGTGGAA ATCCCCGGCA

1201 GACACTCCTT CCTCAGTGCC ATTATTCTCT CCTCCTGCCA AAGCTGCTAC

1251 GTTTGGTCAT GGTGGGAACA AATCATTTGG AGTATCTATT GGATCAGCCT

1301 TTTGGCCCAC CAATGCAGAT AGTGCAGCTG AATCCTTTGC TTCAGCGTTT

1351 AACAATGAAT CTACTGAAAA GAAACAAACT AATGGAAATG TCTGTAGGCT

1401 TTTTGGGTTT GAGCTAGTTG AAAATGTTAA TGTGGATGAA TGTTTCTCTG

1451 CTGCCTCTGT GTCTGGTGCT GTCGCTGTAG ATCAACCTGT CCCATCCAAC

1501 GAGTTTGACT CTGGCCAGCA ATCTGAGCCA TTAAACATCA ACCAATCTGA

| 3’

1551 TATTCCTTCG GGGAGTGGTG ACCCTGAGAA ATCCTCTTTG AGGTCTCCTC

deletion primer 4 ←| |→ Domain III

1601 AAGAATCACA AAGTAGACAGATACGTAGCT GCACAAAGGT GCACATGCAA

1651 GGCAGTGCAG TAGGCAGAGC TATTGATTTG ACAAGGTCAG AGTGTTATGA

←|

1701 AGATCTGTTC AAGAAGCTGG AAGAGATGTT TGATATCAAG GGTGAACTCT

|→ Domain IV

1751 TAGAATCTAC CAAAAAATGG CAAGTCGTTT ACACCGATGA TGAAGATGAC

1801 ATGATGATGG TTGGTGATGA TCCATGGAAT GAGTTCTGTG GAATGGTGAG

←|

1851 GAAGATATTC ATCTACACAC CTGAGGAAGT GAAGAAACTTT CACCGAAGA

1901 ACAAACTCGC AGTCAATGCA AGGATGCAGC TCAAAGCTGA TGCAGAGGAA

| 3’ primer ←|

1951 AATGGGAATA CAGAGGGCAG AT CATCATCT ATGGCGGGAT CAAGATGA

(b) Aux/IAA17

|→ 5’ primer | |→ Domain I

1 ATGATGGGCA GTGTCGAGCT GAATCTGAGG GAGACTGAGC TGTGTCTTGG

←| |3’ del. Primer 1←| |→ ←| putative

51 TCTTCCCGGT GGAGATACAG TGGCTCCGGT AACCGGAAAC **AAGAGA**GGGT

bi-partite NLS |→ ←|

101 TCTCAGAGAC GGTTGATCTG **AAGCTAA**ATC TGAATAATGA GCCTGCAAAC

| 3’ deletion primer 2 ←|

151 AAGGAAGGAT CTACGACTCA TGACGTCGTG ACTTTTGATT CCAAGGAGAA

|→

201 GAGTGCTTGT CCTAAAGATC CAGCCAAACC TCCGGCCAAG GCACAAGTTG

Domain II |→ putative NLS ←| ←| |

251 TGGGATGGCC ACCGGTGAGA TCATACCGGA AGAACGTGAT GGTTTCCTGC

3’ del. Primer 3←| |→ Domain III

301 CAAAAATCAA GCGGTGGCCC GGAGGCGGCG GCGTTCGTGA AGGTATCAAT

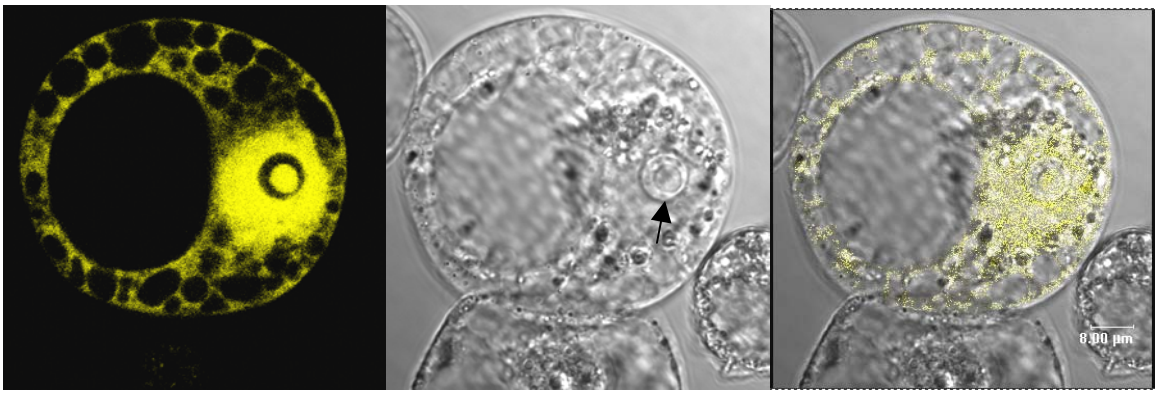
351 GGACGGAGCA CCGTACTTGA GGAAAATCGA TTTGAGGATG TATAAAAGCT

| 3' deletion primer 4 ←|
←|
401 ACGATGAGCT TTCTAATGCT TTGTCCAACA TGTTCAGCTC TTTTACCATG

451 GGCAAACATG GAGGAGAAGA AGGAATGATA GACTTCATGA ATGAGAGGAA
|→ Domain IV
501 ATTGATGGAT TTGGTGAATA GCTGGGACTA TGTTCCCTCT TATGAAGACA
| 3' del. primer 5 ←|
←|
551 AAGACGGTGA TTGGATGCTC GTCGGCGACG TTCCTTGGCC AATGTTCGTC

601 GATACATGCA AGCGTTTACG TCTCATGAAA GGATCGGATG CCATTGGTCT
| 3' primer ←|
651 CGCTCCGAGG GCGATGGAGA AGTGCAAGAG CAGAGCTTGA

Figure 3-5 a-b. Coding sequence of ARF1 (a) and IAA17 (b). The putative bi-partite localization signal is a conserved signal that contains two parts, both of which are required for localization. They are separated by a short non-conserved sequence of DNA. For Aux/IAA17, the putative NLS is located inside domain II, and 3' deletion primers 4 and 5 were made to amplify to the end of domains III and IV respectively. Text in red are conserved domains, in purple are putative nuclear localization signals and blue are sequence specific primers.



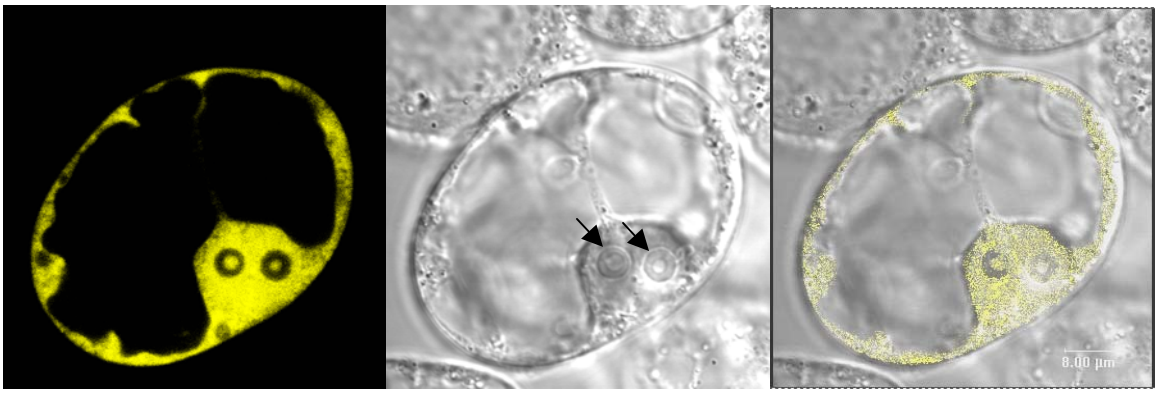
A B C

Figure 3-6. Localization of ARF1-D1 protein in transiently transfected carrot protoplasts.

A. Fluorescent image of ARF1-D1 protein.

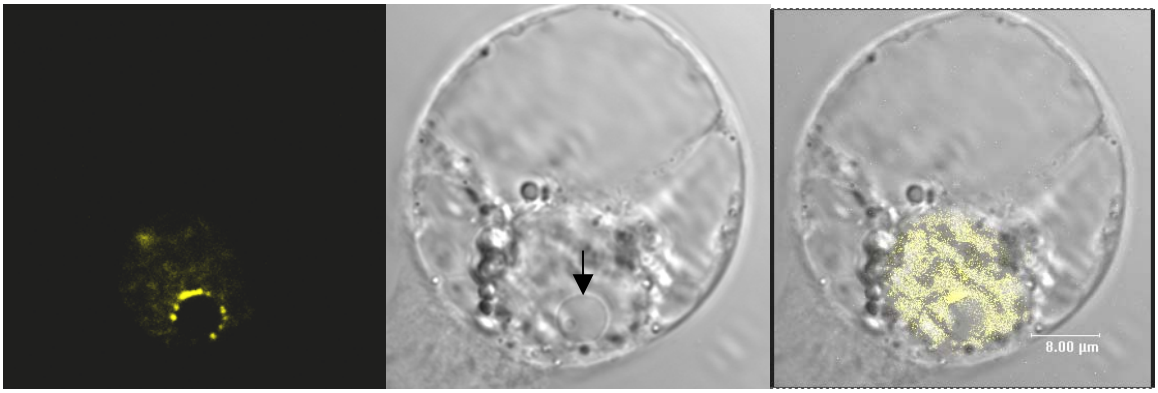
B. Transmitted image of construct including nucleus and nucleolus. Arrow indicates nucleolus.

C. Overlay of image A and B with protein throughout the cytoplasm and nucleus.



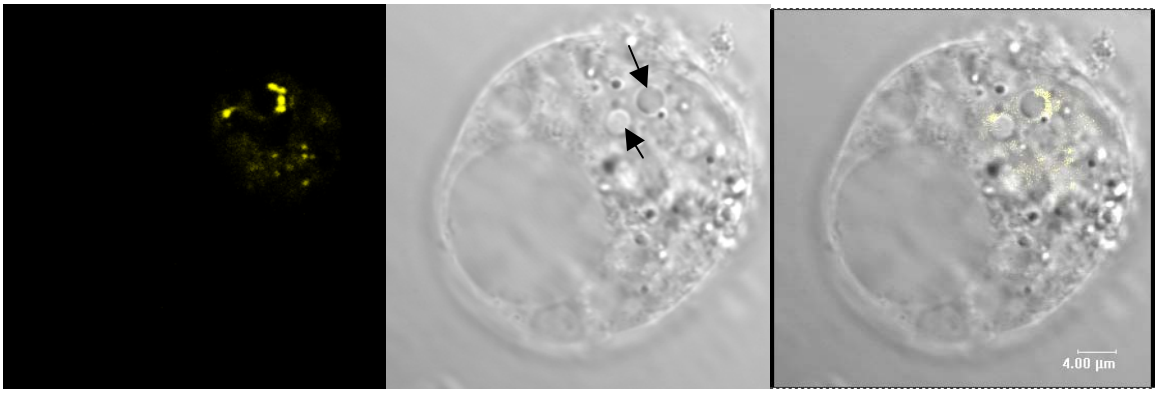
A B C

Figure 3-7. Localization of ARF1-D2 protein in transiently transfected carrot protoplasts.
A. Fluorescent image of ARF1-D2 protein.
B. Transmitted image of cell. Arrows indicate nucleoli
C. Overlay image of A and B.



A B C

Figure 3-8. Localization of ARF1-D3 protein in transiently transfected carrot protoplasts.
A. Fluorescent image of ARF1-D3 protein.
B. Transmitted image of cell. Arrow indicates the nucleolus.
C. Overlay image of A and B.



A B C

Figure 3-9. Localization of ARF1-D4 protein in transiently transfected carrot protoplasts.
A. Fluorescent protein localization pattern of ARF1-D4.

B. Transmitted image of cell. Arrows indicate nucleoli
C. Overlay image of A and B.

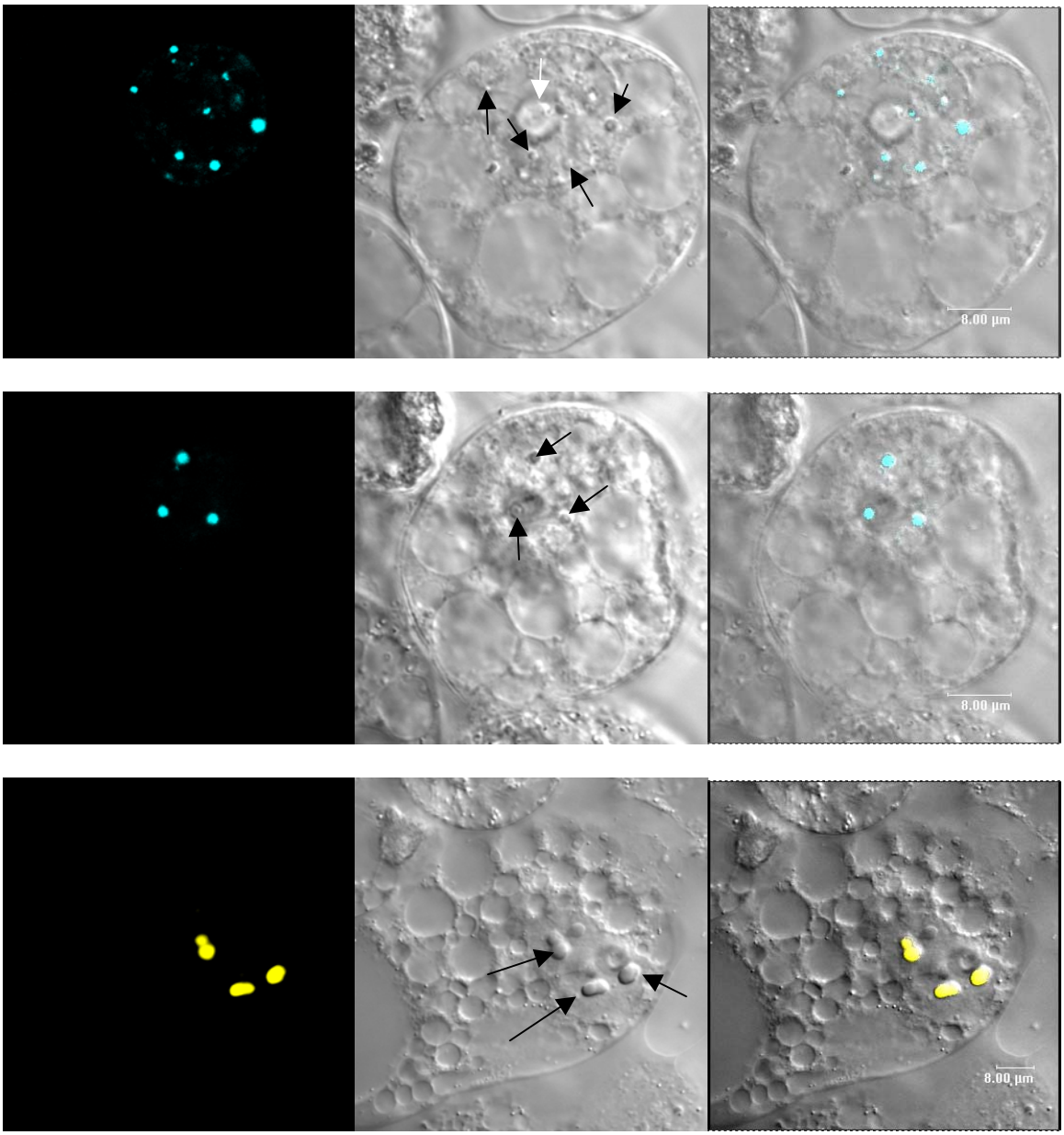


Figure 3-10. Localization of IAA17 protein in transiently transfected carrot protoplasts.
A. IAA17 fluorescent protein localization.
B. Transmitted image of construct. Black arrows indicate several structures in the nucleus that IAA17 protein localizes to. White arrow indicates the nucleus.
C. Overlay of A and B.

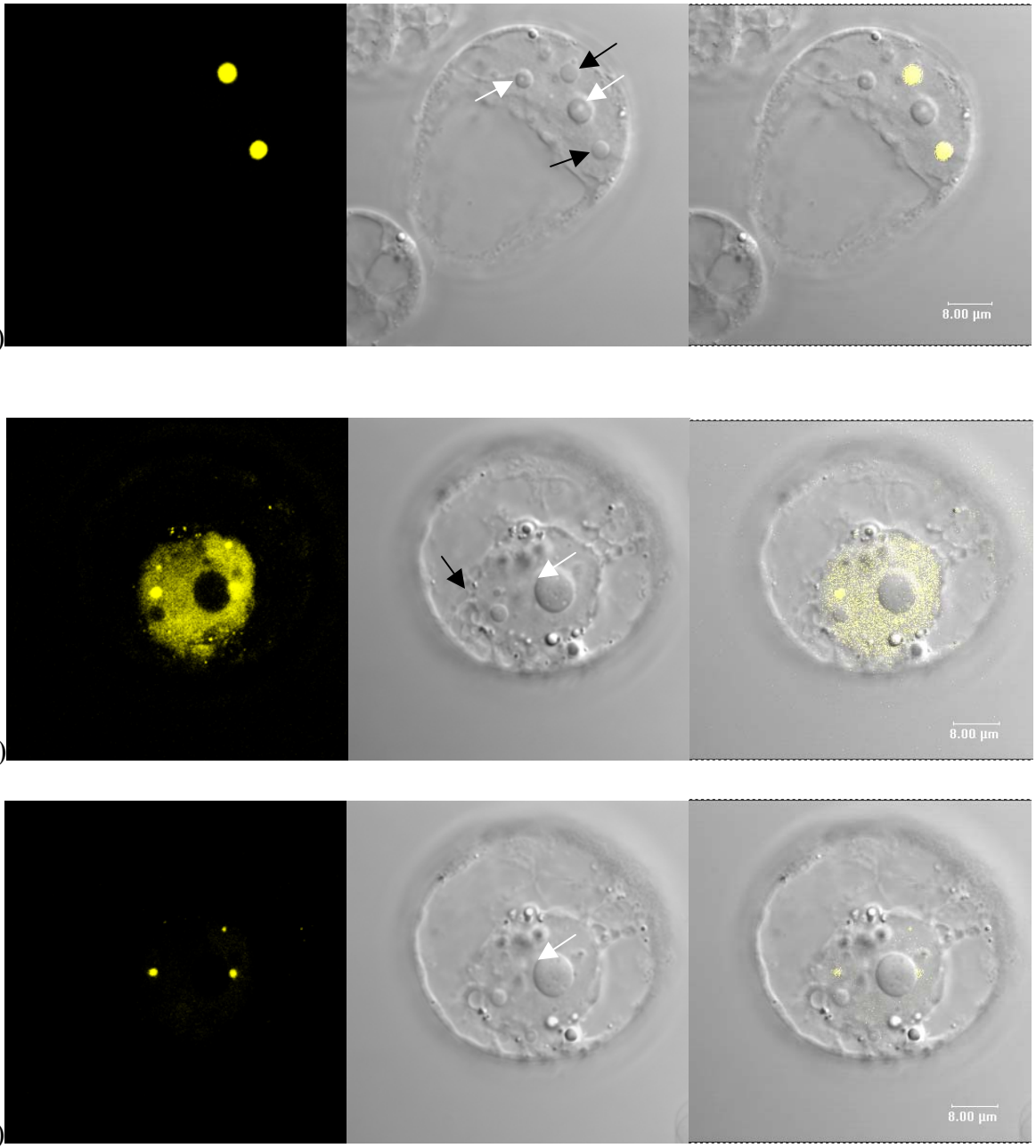


Figure 3-11 a-c. Localization of *axr3-1* protein in transiently transfected carrot protoplasts. Images on left are fluorescent expression, middle are transmitted images and on the right are overlay images of both. Black arrows indicate structures that *axr3-1* localizes to. White arrow indicates nucleoli.

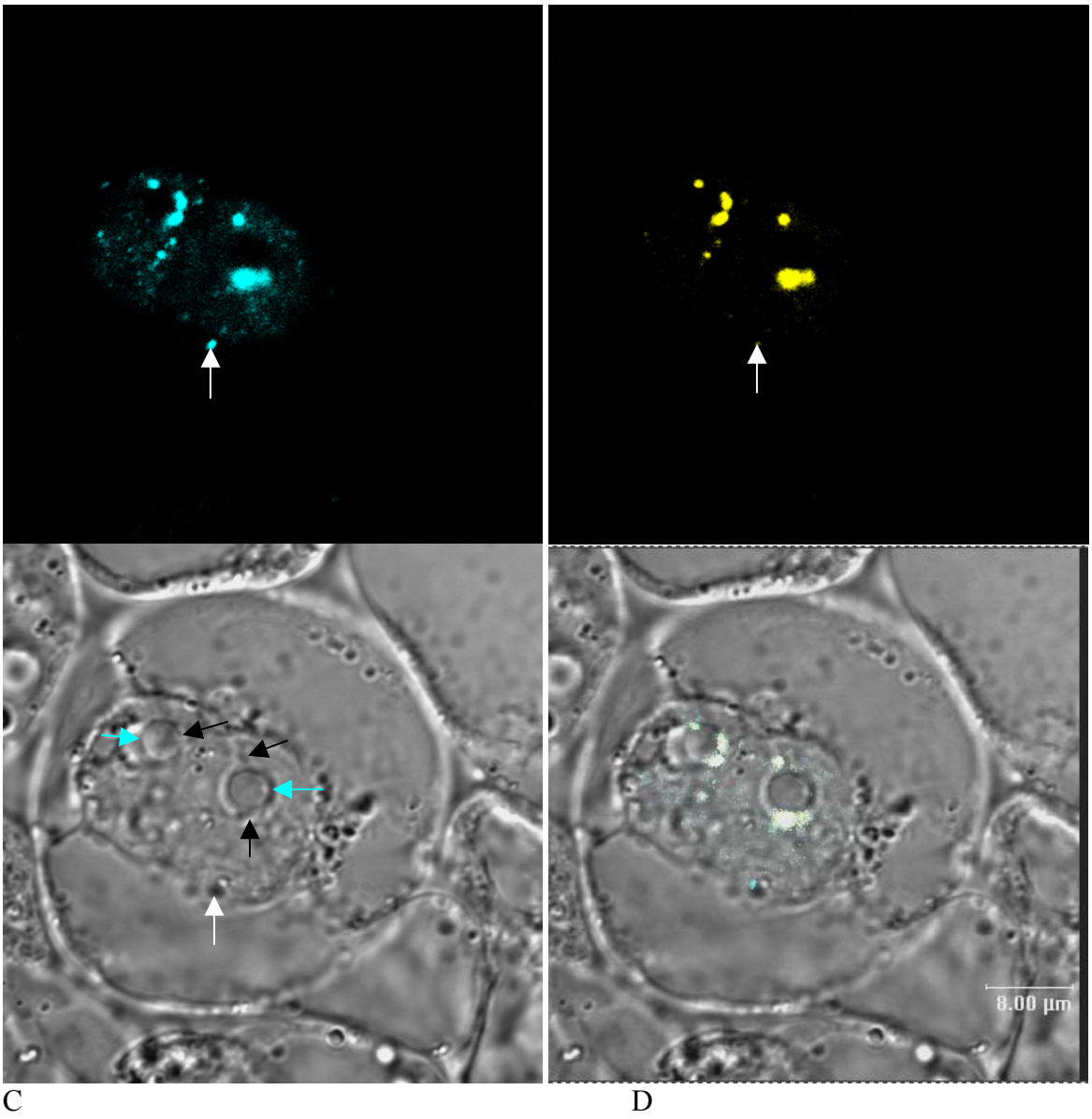


Figure 3-12. Co-localization of ARF1 and IAA17 protein in transiently transfected carrot protoplasts.

A. ARF1 protein localization. White arrow indicates localization of ARF1 that does not have IAA17 co-localized with it.
B. IAA17 protein localization. White arrow indicates location where ARF1 localized, but IAA17 did not.
C. Transmitted image of cell. Black arrows indicate overlap of localization of ARF1 and IAA 17. White arrow indicates structure that ARF1 localized to, but IAA17 did not. Blue arrows indicate nucleoli.
D. Overlay image of A, B, and C.

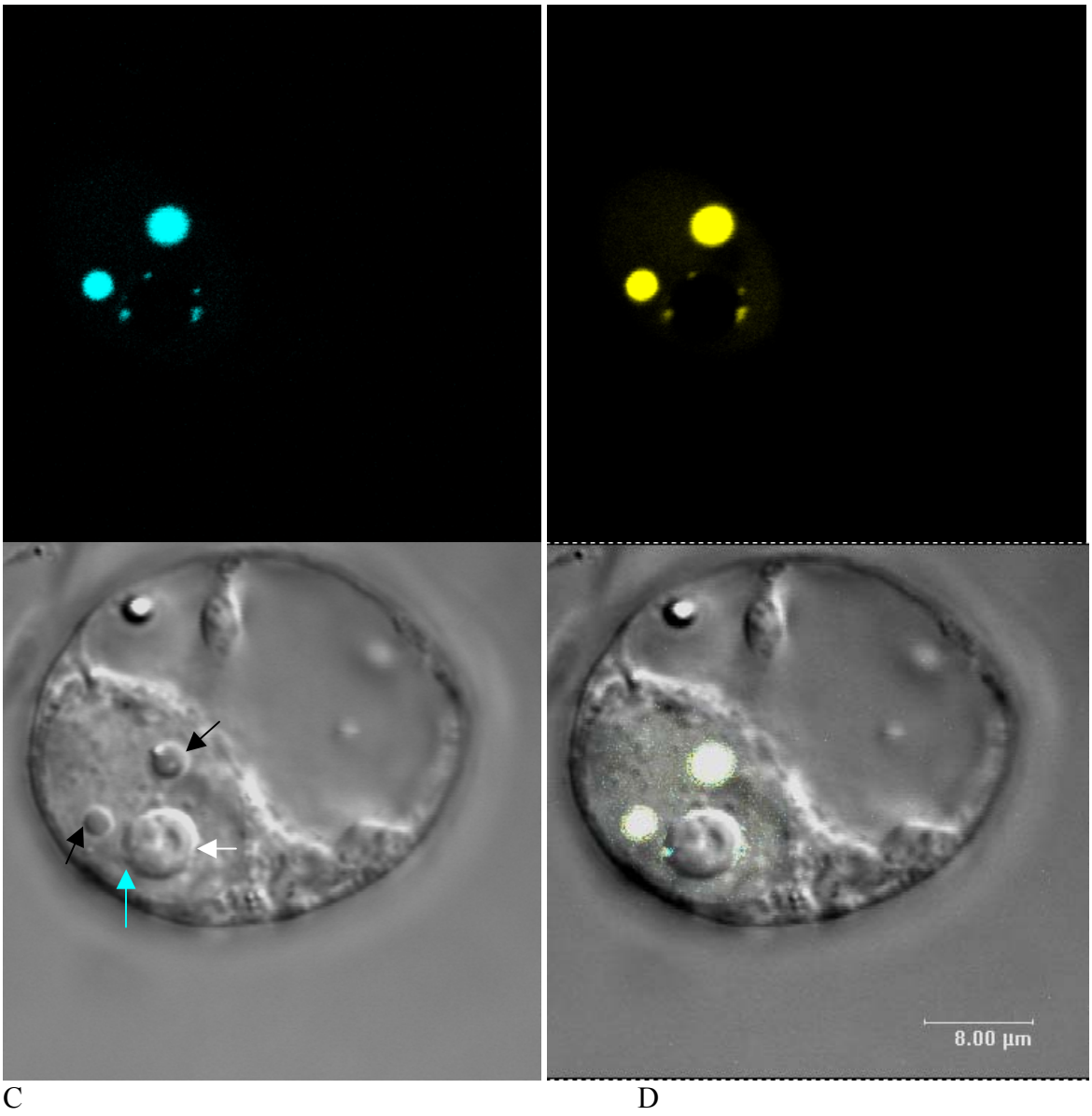


Figure 3-13. Co-localization of ARF1 and IAA17 protein in transiently transfected carrot protoplasts.

A. ARF1 localization.
B. IAA17 localization.
C. Transmitted image of cell. Black arrows indicate large structures inside the nucleus that IAA17 protein localized to individually. White arrow indicates the nucleolus. Blue arrow indicates a visible structure on nucleolus.
D. Overlay image of A, B, and C. Note: Bright areas where both colors appear are rendered as white.

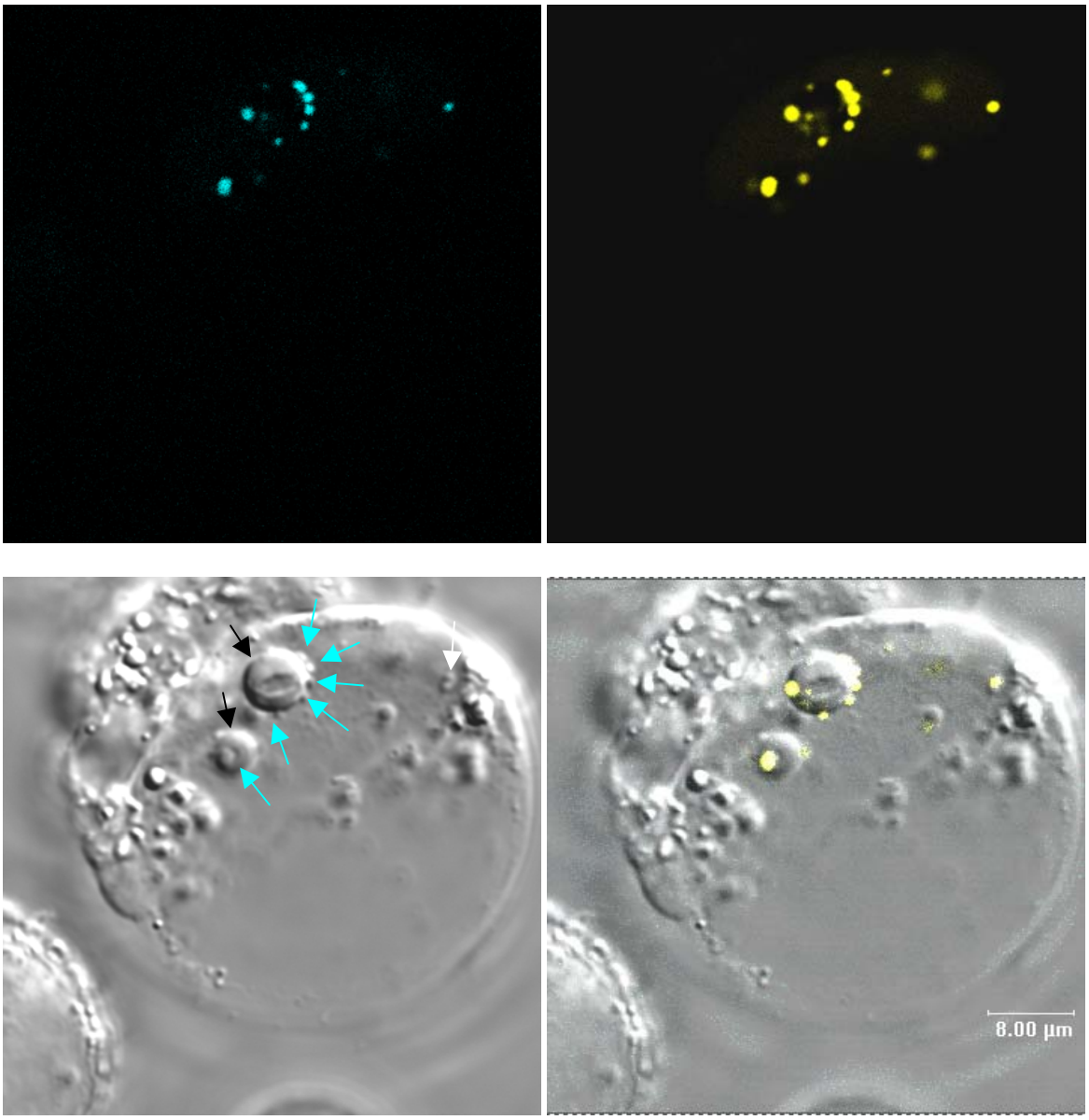


Figure 3-14. Co-localization of ARF1 and *axr3-1* protein in transiently transfected carrot protoplasts.

A. ARF1 protein localization.
B. *axr3-1* protein localization
C. Transmitted image of cell. Black arrows indicate nucleoli. Note visible structures around nucleolus that ARF1 and *axr3-1* localize to (blue arrows). White arrow indicates a nuclear structure with both proteins present.
D. Overlay image of A, B, and C.

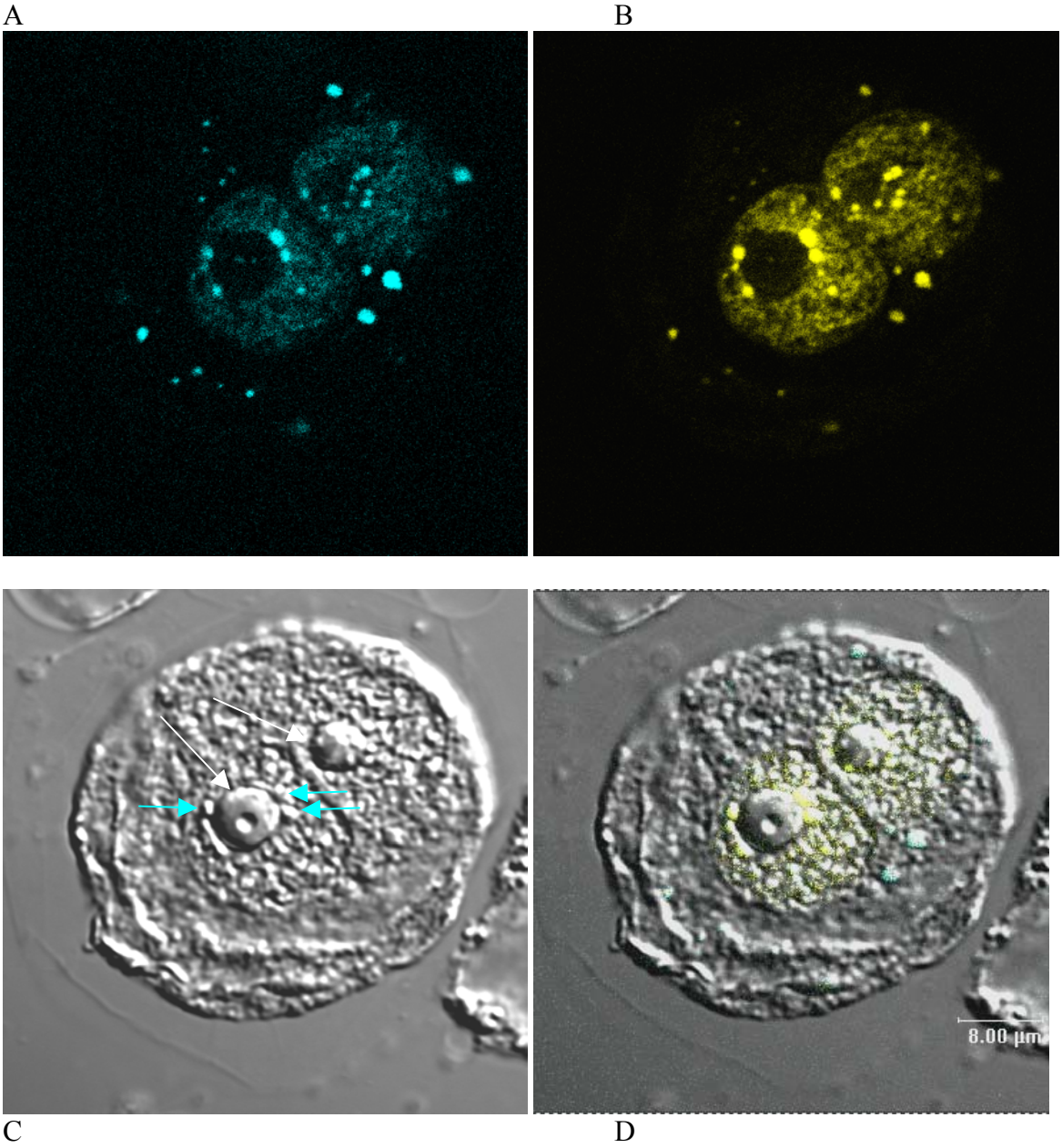


Figure 3-15. Co-localization of ARF1 and *axr3-1* protein in transiently transfected carrot protoplasts.

A. ARF1 protein localization pattern.
B. *axr3-1* protein localization pattern.
C. Transmitted image of cell. White arrows indicate nucleoli. Blue arrows indicate visible structures around nucleolus.
D. Overlay image of A, B, and C.

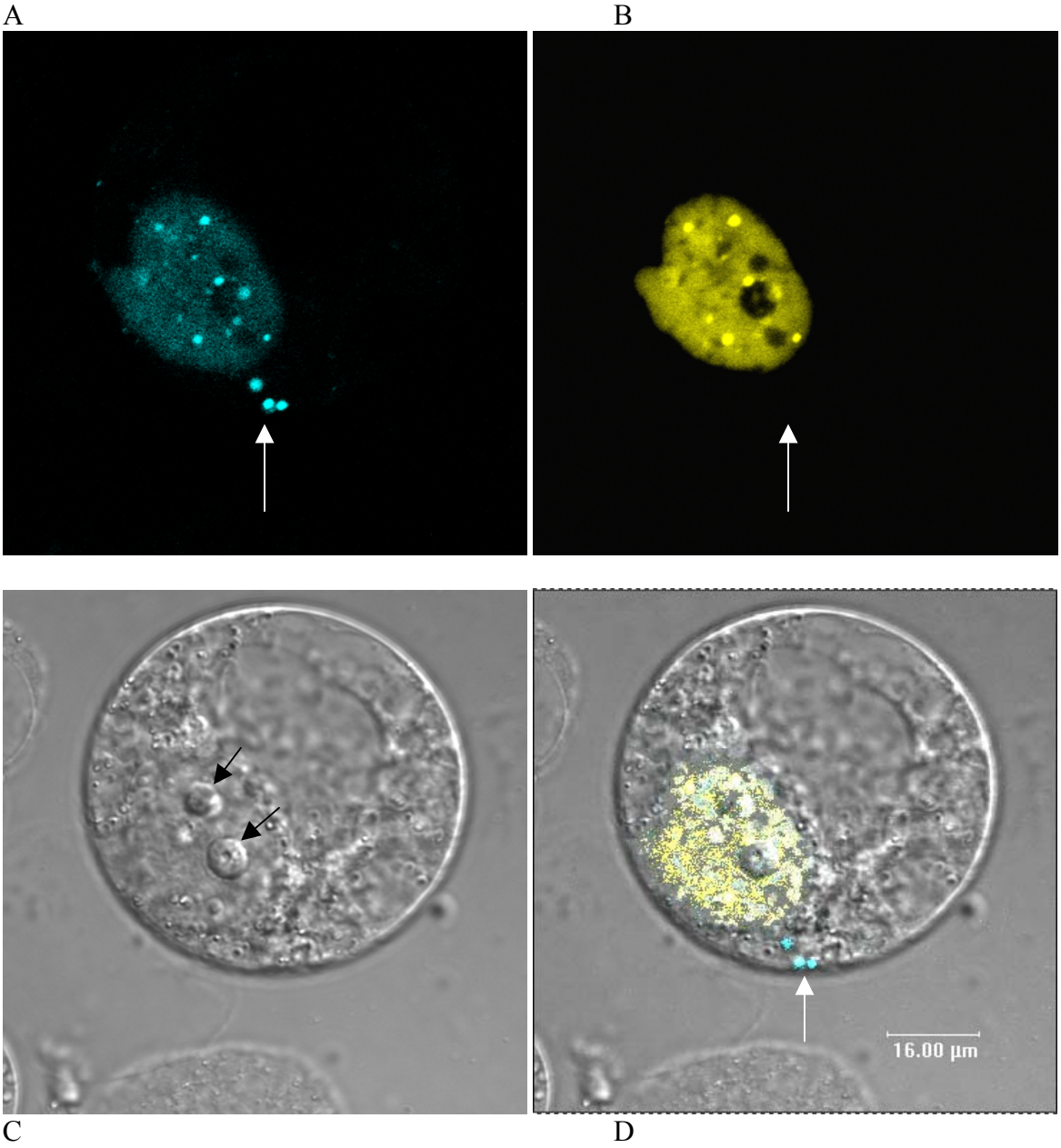


Figure 3-16. Co-localization of ARF1 and *axr3-1* protein in transiently transfected carrot protoplasts.

A. Fluorescent protein expression pattern of ARF1. White arrow indicates ARF1 localization outside the nucleus.
B. *axr3-1* fluorescent protein expression pattern. White arrow indicates a lack of *axr3-1* protein outside of the nucleus
C. Transmitted image of cell. Black arrows indicate nucleoli.
D. Overlay of A, B, and C. White arrow indicates localization of ARF1 without *axr3-1* outside of the nucleus.

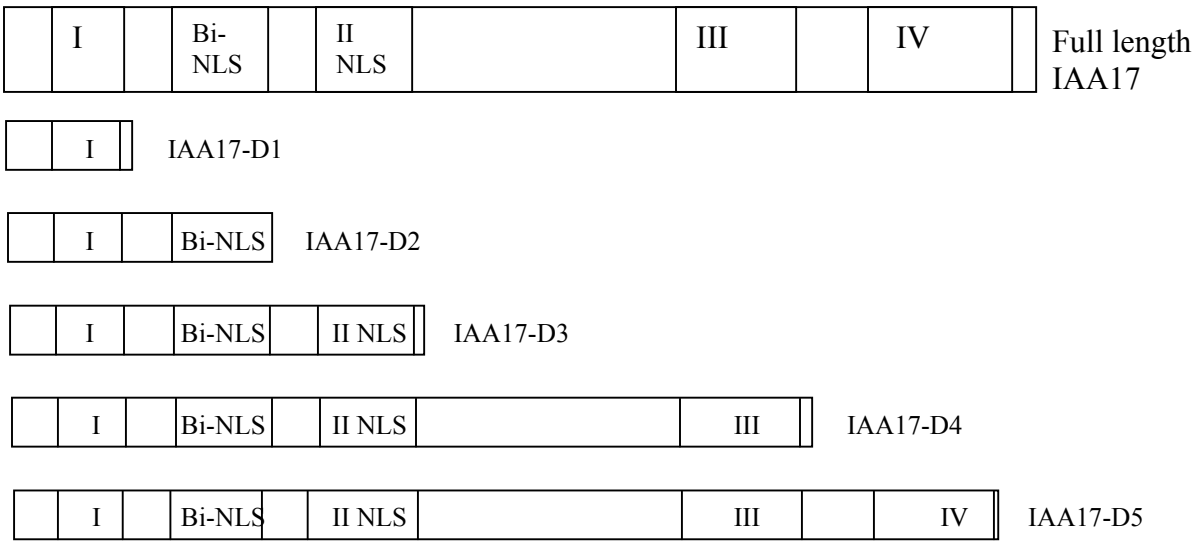


Figure 3-17. Schematic diagram of Aux/IAA 17 deletion constructs. Diagram includes putative domains and putative bi-partite nuclear localization signal and NLS in domain II.

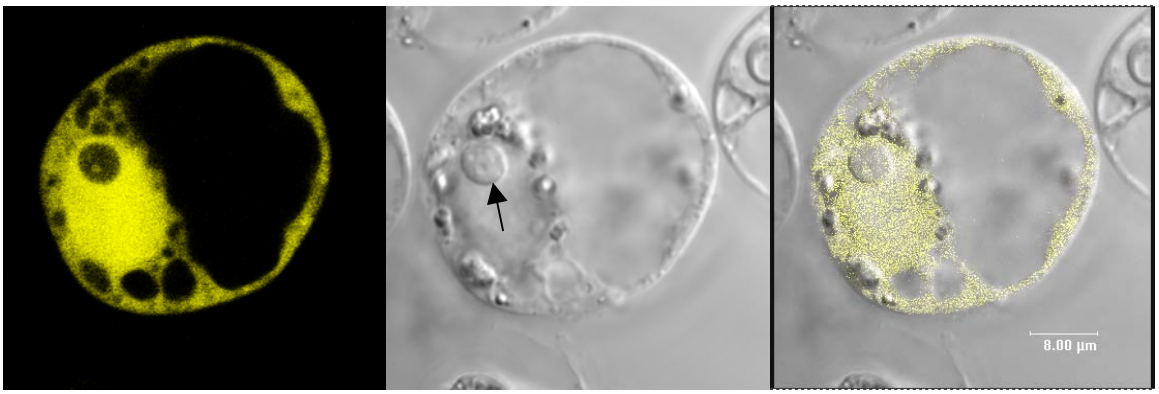
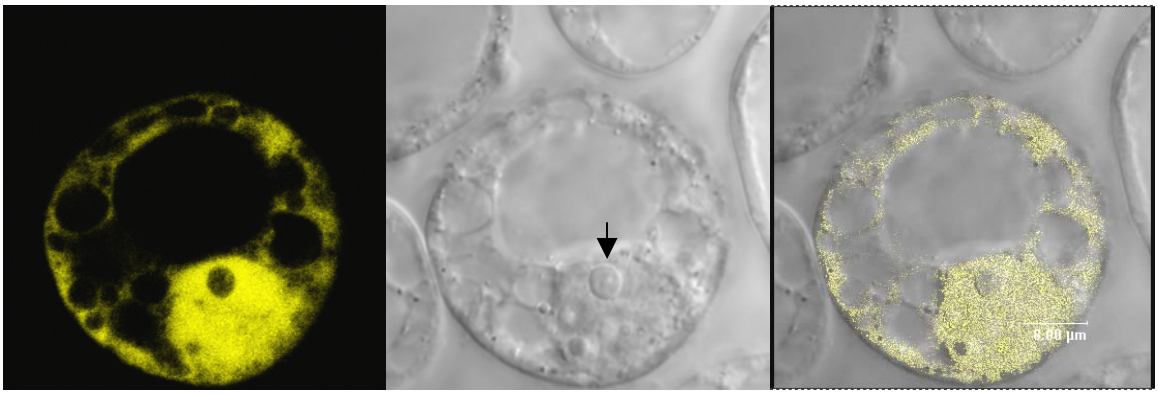


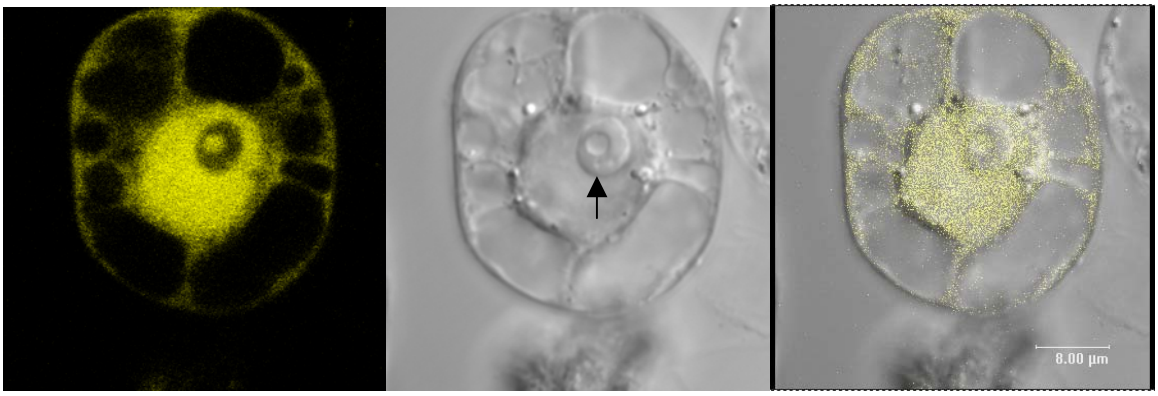
Figure 3-18. Localization of IAA17-D1 protein in transiently transfected carrot protoplasts.

A. Localization pattern of IAA17-D1.
B. Transmitted image of cell. Arrow indicates nucleolus
C. Overlay image of A and B.



A B C

Figure 3-19. Localization of IAA17-D2 protein in transiently transfected carrot protoplasts.
A. Fluorescent localization of IAA17-D2.
B. Transmitted image of cell. Arrow indicates nucleolus.
C. Overlay image of A and B.



A B C

Figure 3-20. Localization of IAA17-D3 protein in transiently transfected carrot protoplasts.
A. IAA17-D3 fluorescent protein localization.
B. Transmitted image of cell. Arrow indicates nucleolus.
C. Overlay image of A and B.

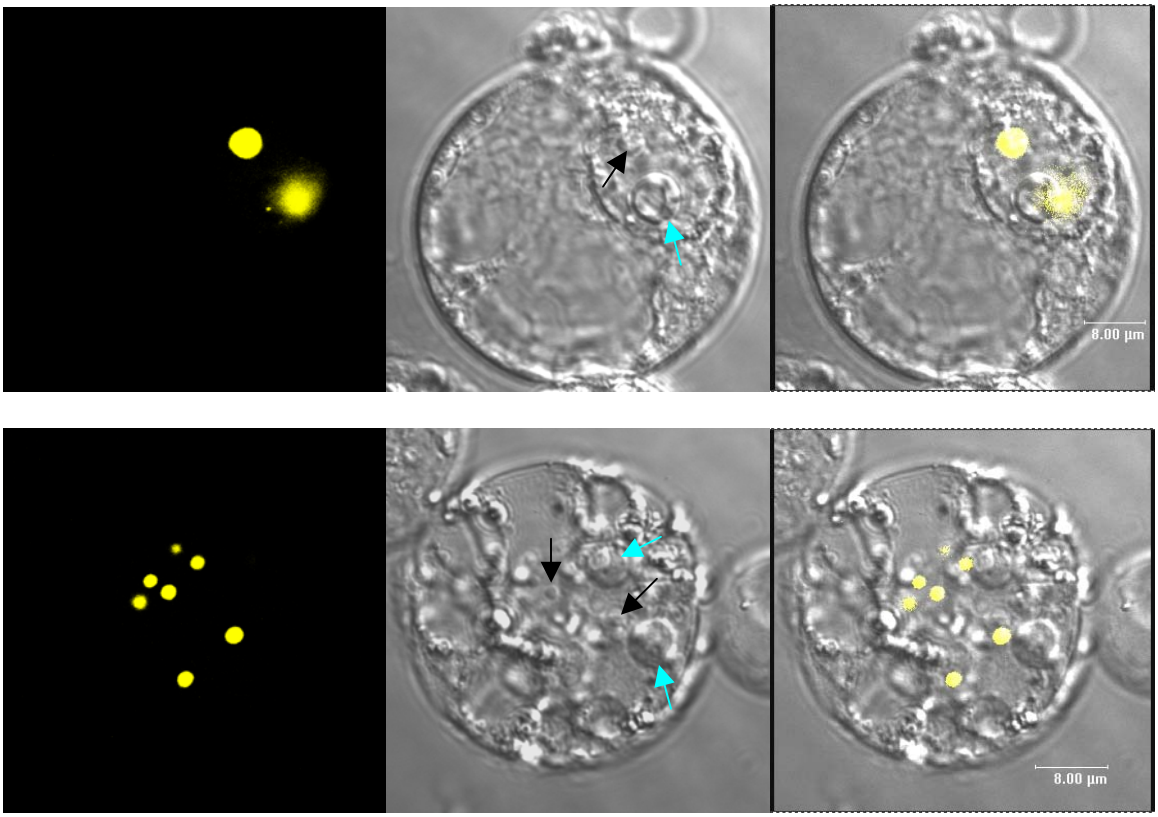


Figure 3-21. Localization of IAA17-D4 protein in transiently transfected carrot protoplasts.
A. Fluorescent localization of IAA17-D4.
B. Transmitted image of cell. Black arrow indicates structure with IAA17-D4 protein. Blue arrow indicates nucleolus.
C. Overlay image of A and B.

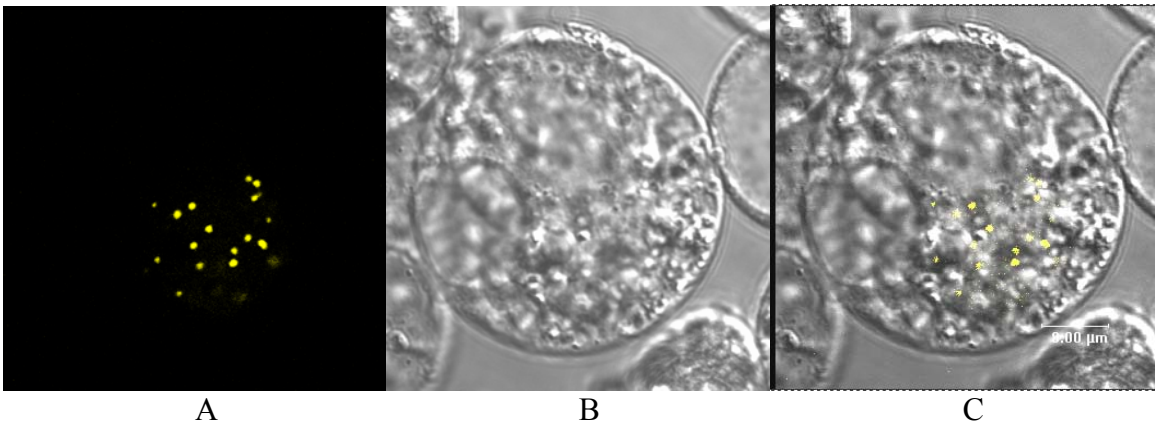
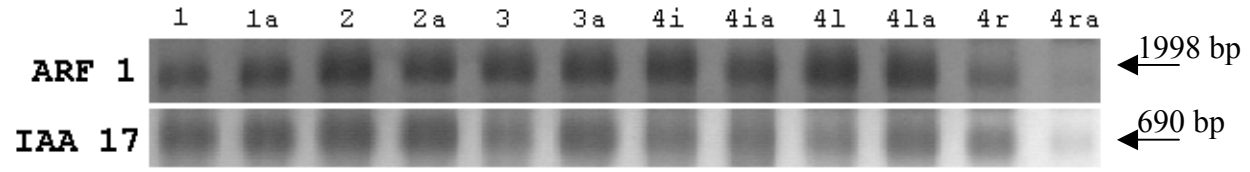
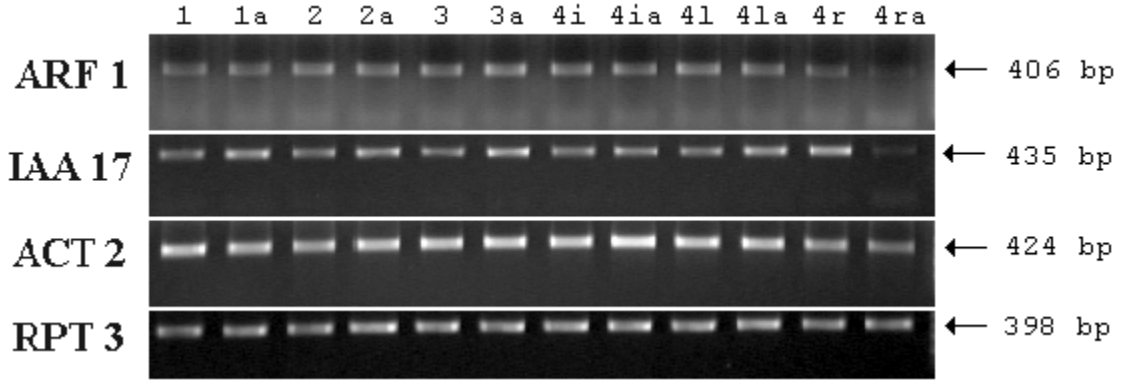


Figure 3-22. Localization of IAA17-D5 protein in transiently transfected carrot protoplasts.
A. Fluorescent protein localization of IAA17-D5.
B. Transmitted image of cell.
C. Overlay image of A and B.



(a)



(b)

Figure 3-25. (a) Northern blot analysis of mRNA isolated from *A. thaliana* total RNA probed with full length ARF1 and IAA17 genes. Film was exposed 5 minutes. (b) Semi-quantitative RT PCR of mRNA isolated from *A. thaliana* total RNA amplified using sequence specific primers for ARF1, IAA17, ACT 2 and RPT 3. Number above lane indicates age of seedlings when harvested in weeks. Plants were treated with auxin (a) or water. Four week-old seedlings were dissected into inflorescence stalks (i), leaves (l), and roots (r).

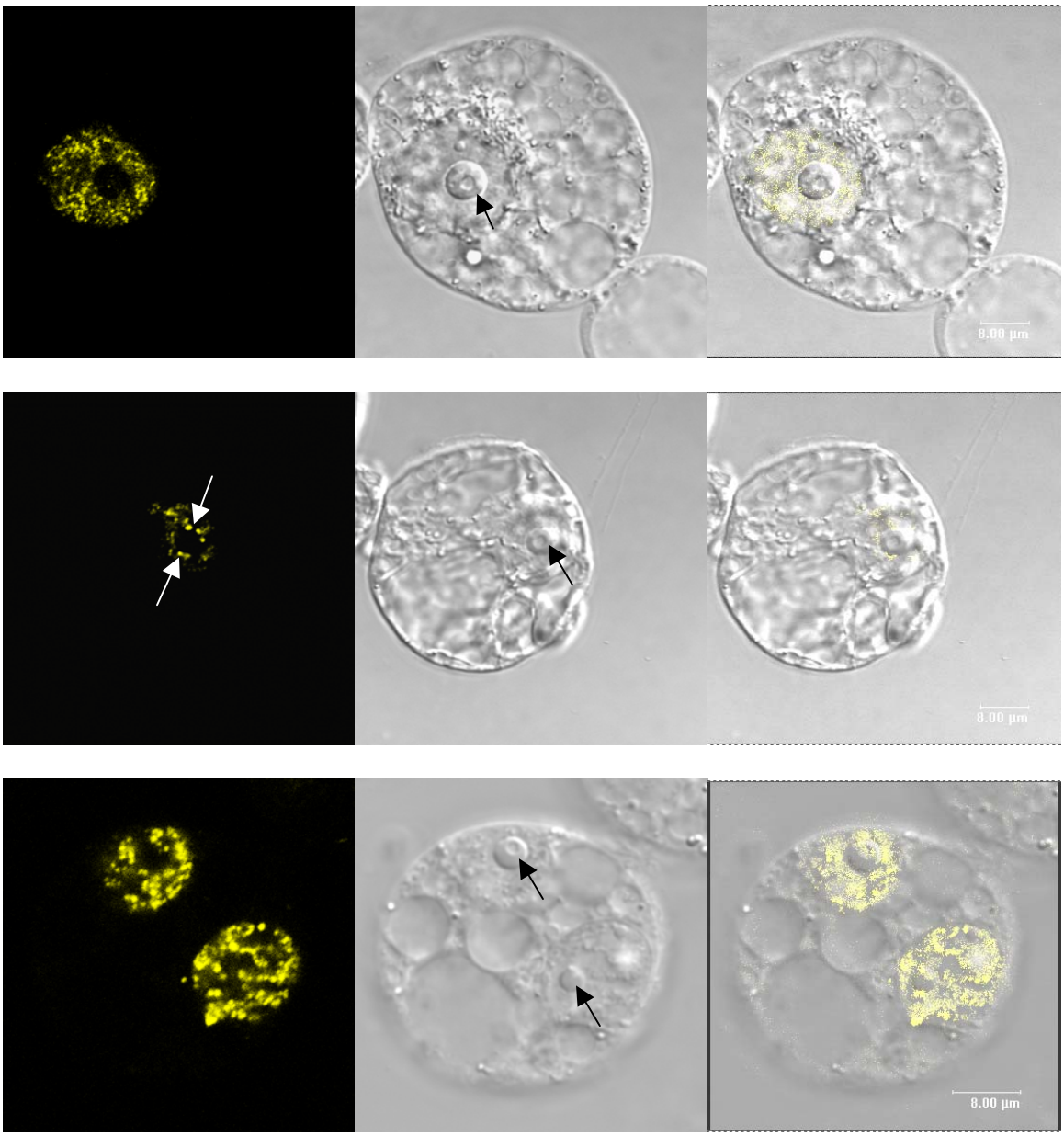


Figure 3-24. Localization of ARF6 protein in transiently transfected carrot protoplasts. Fluorescent images are on the left, transmitted images center, and overlay images on the right. The middle set shows ARF6 localizing to the peri-nucleolar region. Black arrows indicate nucleolus. White arrows indicate peri-nucleolar localization.

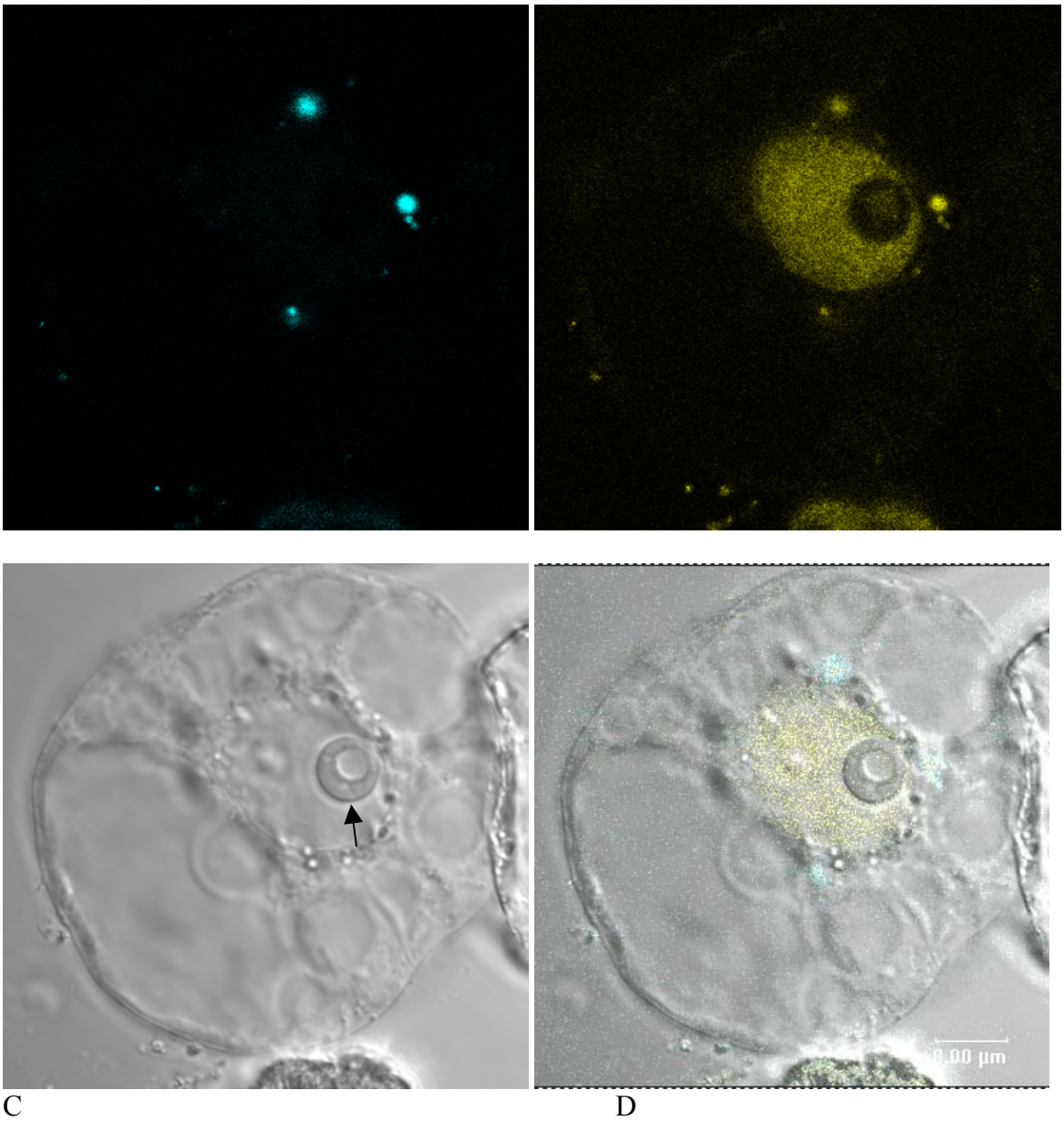


Figure 3-25. Co-localization of ARF1 and ARF6 protein in transiently transfected carrot protoplasts.

A. ARF1 localization pattern.
B. ARF6 localization pattern.
C. Transmitted image of cell. Arrow indicates nucleolus.
D. Overlay of A, B and C.

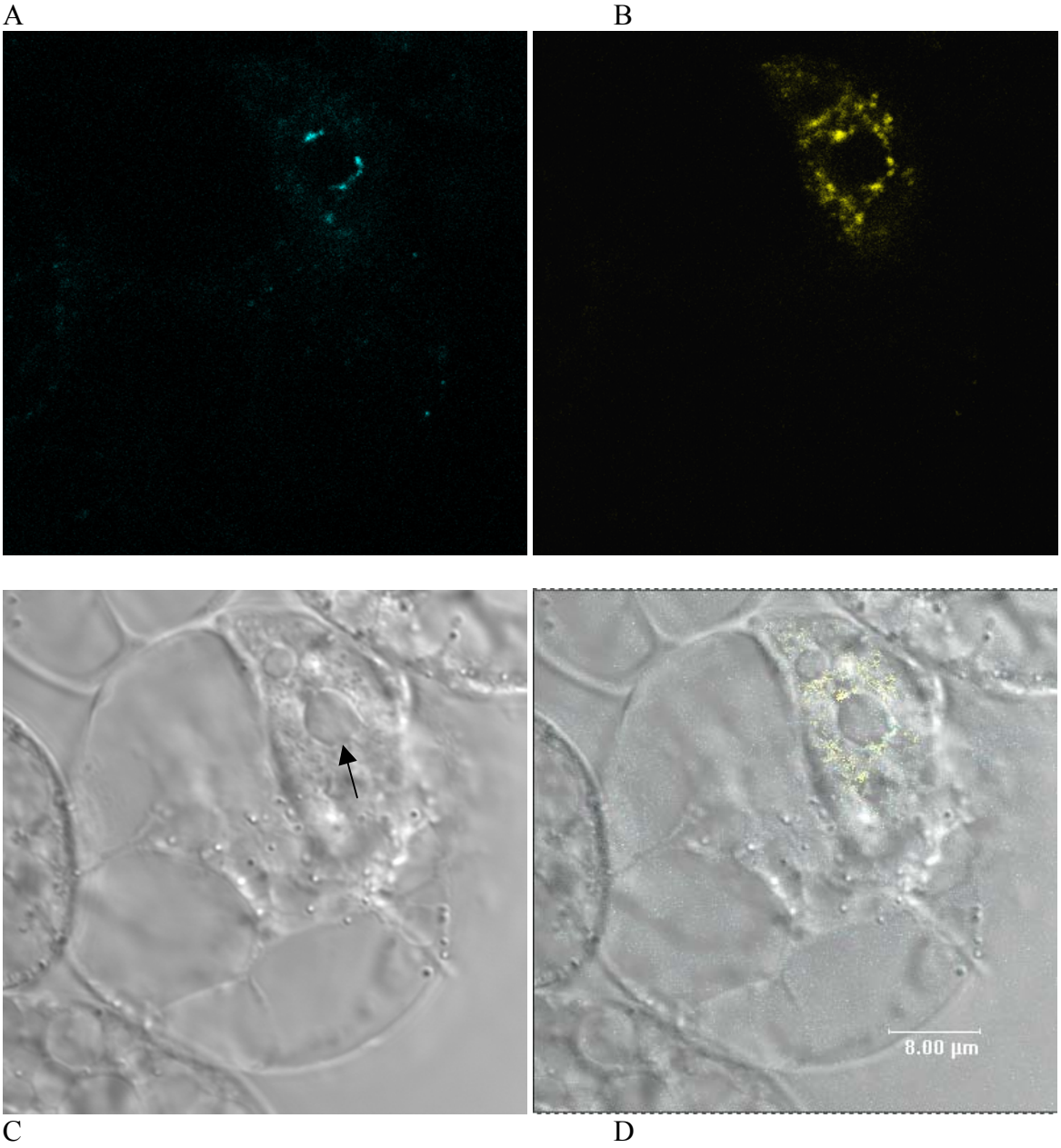


Figure 3-26. Co-localization of ARF1 and ARF6 protein in transiently transfected carrot protoplasts.

A. ARF 1 localization pattern.
B. ARF 6 localization pattern.
C. Transmitted image of cell. Arrow indicates nucleolus.
D. Overlay image of A, B, and C.

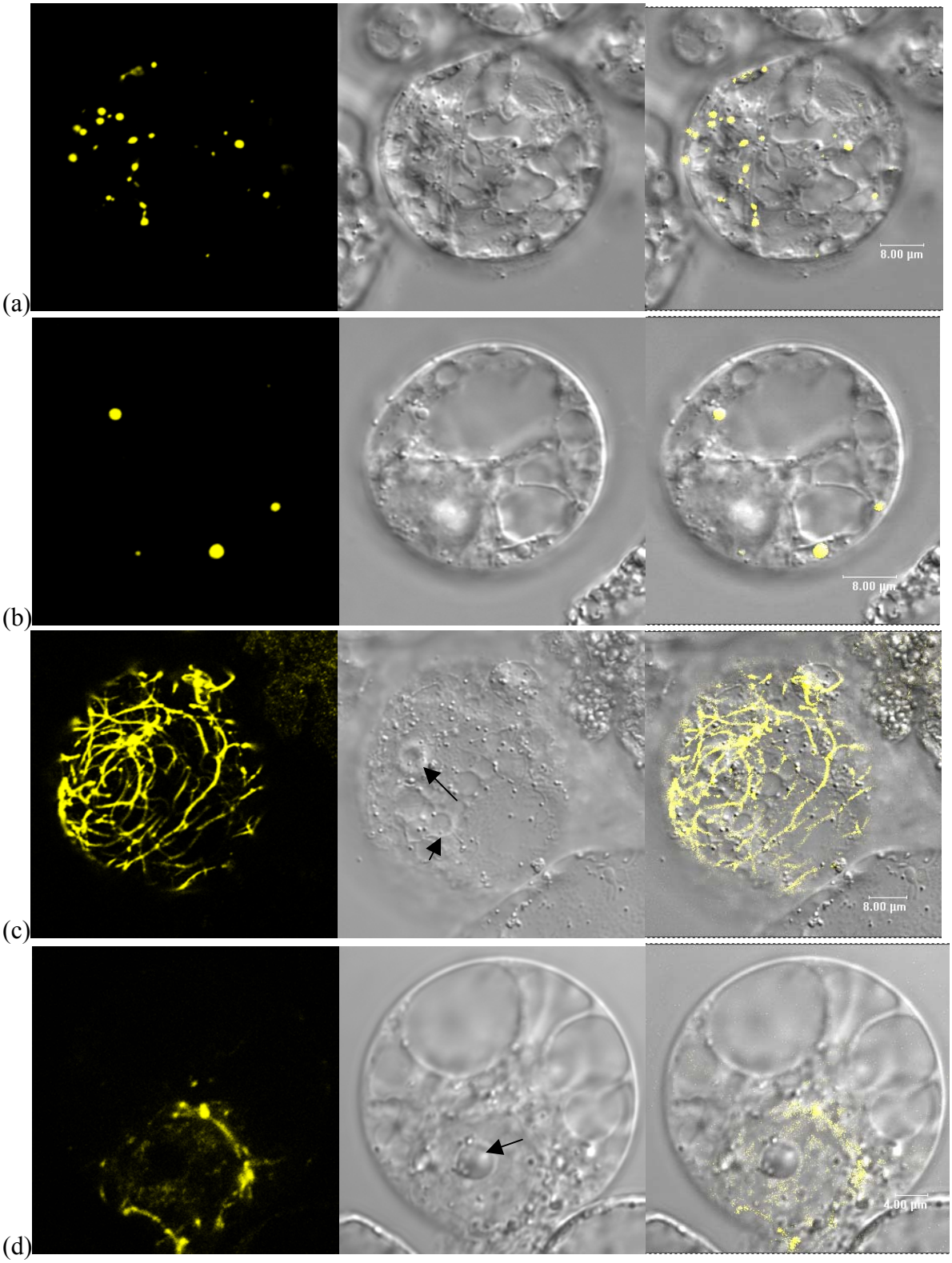


Figure 3-27 a-d. Localization of ARF7 protein in transiently transfected carrot protoplasts. Images on the left are fluorescent images, while those in the center are transmitted and overlay images are on the right. Arrows indicate nucleoli.

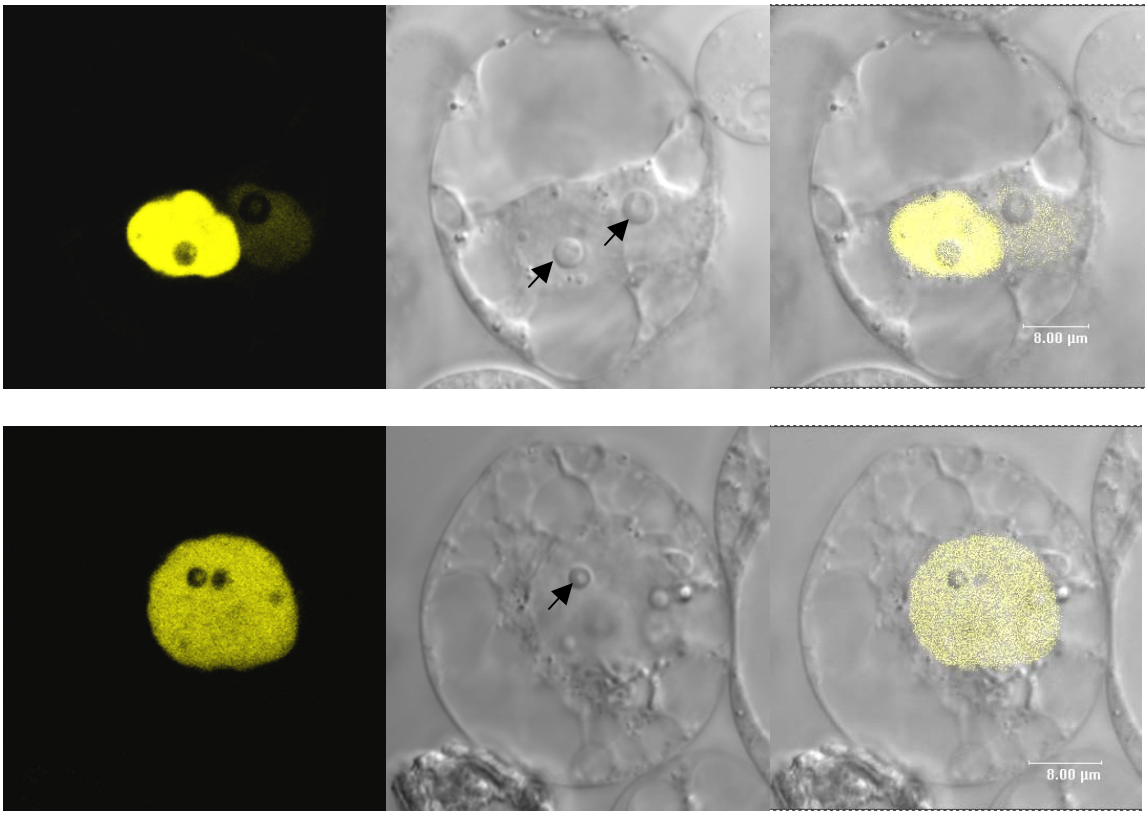


Figure 3-28. Localization of IAA2 protein in transiently transfected carrot protoplasts. Images on the left are fluorescent expression, in the middle are the transmitted images and the overlay images are on the right. Arrows indicate location of nucleoli.

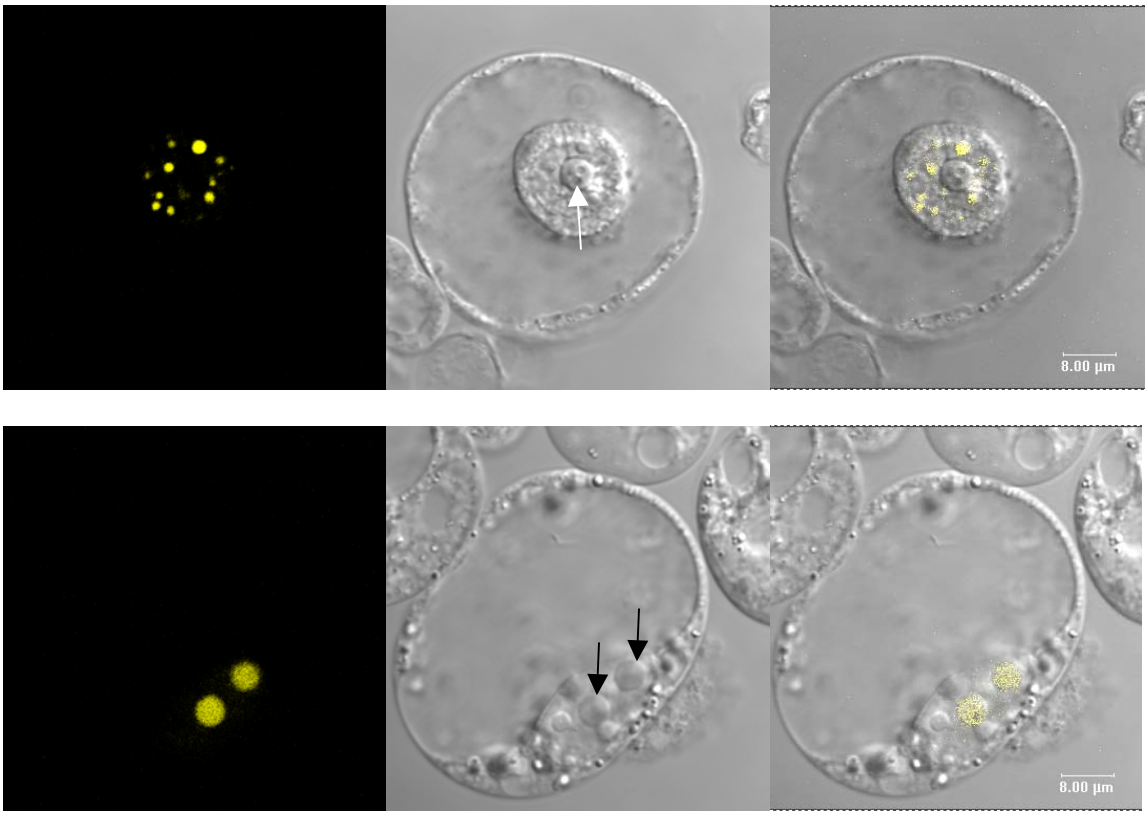


Figure 3-29. Localization of IAA3 protein in transiently transfected carrot protoplasts. IAA3 exhibits punctate expression exclusively inside the nucleus. Images on the left are fluorescent protein, in the middle are the transmitted images and the overlay images are on the right. Black arrows indicate structures with IAA3 protein. White arrow indicates nucleolus.

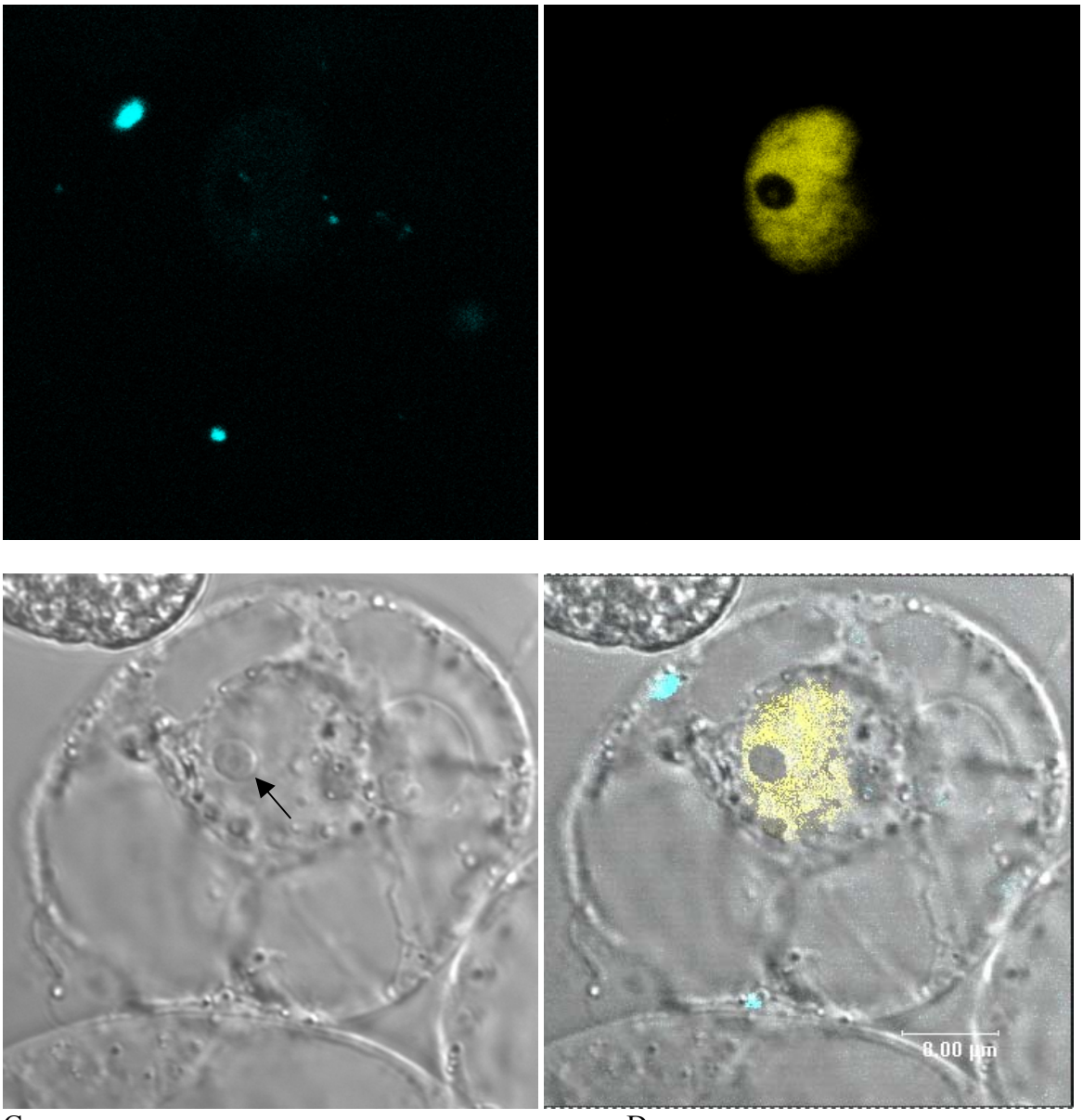


Figure 3-30. Co-localization of ARF1 and IAA3 protein in transiently transfected carrot protoplasts.

A. ARF1 localization.
B. IAA3 localization.
C. Transmitted image of cell. Arrow indicates nucleolus
D. Overlay image of A, B, and C.

Chapter 4 Discussion

ARF1

The ARF and Aux/IAA gene families encode putative transcription factor proteins that regulate gene transcription and ultimately plant growth and development. Spatial and temporal expression of different members of each gene family is thought to limit the number of possible interactions that occur in a given cell. The focus of this study was on the sub-cellular localization and *in vivo* interactions of specific members of these two families. Prior research has shown that one IAA from pea (*Pisum sativum*) w*as* localized to the nucleus using a GUS assay (Abel and Theologis, 1995). From this information, and putative localization signals predicted in sequences by computational methods, it was hypothesized that Aux/IAA proteins are nuclear localized. The DNA binding domain of *monopteros*/ARF5 was experimentally localized using a GUS reporter, and it was found that the 5 putative NLS had varying efficiencies for localizing the DBD to the nucleus (Hardke and Berleth, 1998).

All functional ARF and Aux/IAA proteins theoretically have the ability to interact with themselves, and other members of both gene families, but this interaction has not been examined *in vivo*. This interaction has been shown for many members of both gene families *in vitro* (Guilfoyle et al., 1998b). ARF1 and IAA17 were the main focus of this research and were selected because of the extensive characterization and mutant phenotypes that were available. This was the first reported study to experimentally examine the sub-nuclear targeting of an ARF or Aux/IAA protein. This was also the first report to examine co-localization of members of these two gene families *in vivo*.

Utilization of fluorescent protein fusions and laser confocal microscopy permits visualization of protein in living cells and provides a higher level of resolution than that obtained with GUS fusions. Expression of the ARF1 and IAA17 genes was examined to determine whether the two potentially interacting proteins were co-expressed in tissues of developing *Arabidopsis* plants and therefore have the potential to interact *in vivo* during plant development.

ARF1 had a specific repeatable expression pattern. ARF1 was localized to sub-nuclear foci in the peri-nucleolar region, as well as punctate structures or compartments inside and outside the nucleus (Figure 3-1). When multiple nuclei were present in the cell, ARF1 protein could be seen localized to both nuclei and the peri-nucleolar region. Images are representative of the localization pattern observed for ARF1. It is possible that the peri-nucleolar localization of ARF1 were active sites of gene regulation.

There appeared to be a possible correlation between the localization of ARF1 and the cell cycle. This is a possible explanation of the different localization patterns seen, with some cells having no peri-nucleolar localization and others exhibiting nuclear localization. It is also possible that the cells that exhibited different patterns were differentially localized due to differences in gene regulation. Accumulation inside of the nucleus as well as to specific structures argues for a nuclear and sub-nuclear localization signal in the ARF1 protein. The protein that localized to discrete sub-nuclear structures may regulate the transcription of Aux/IAA genes.

Control vectors containing the fluorescent protein coding region were also expressed in carrot protoplasts. Fluorescent protein was distributed throughout the nucleus and cytoplasm, with no punctate accumulations of protein observed in the cells (Figure 3-2). Fluorescent protein was never seen inside the nucleolus in any of the constructs that were visualized. Structures seen

in non-transformed cells resemble those seen in transformed cells (Figure 3-3). Structures present in transformed cells were not artifacts of protein over expression, or sequestering of the protein by the cells.

Aux/IAA4 from a pea (*Pisum sativum*) was shown to be localized to the nucleus using a GUS fusion (Abel and Theologis, 1995). However, GUS substrate readily diffuses in fixed tissue, making it difficult to resolve sub-cellular targeting. Fluorescent protein fusion to genes of interest in living cells allowed resolution at the sub-cellular and sub-nuclear level. To begin determining if putative localization signals were accurately predicted, deletion constructs were created. Starting from the N-terminus, increasingly larger fragments of ARF1 (Figure 3-4) and IAA17 (Figure 3-17) were ligated into the C-terminal fluorescent protein expression vector, to begin defining the nuclear and sub-nuclear localization signals.

ARF1 deletion constructs

ARF1-D1 (Figure 3-6) and ARF1-D2 (Figure 3-7) show no specific nuclear localization. These proteins were small enough to enter the nucleus by passive diffusion. In cells with a reduced number and size of vacuoles, the cytoplasm had similar levels of the ARF1 fusion protein accumulation compared to that observed in the nucleus (data not shown). The ARF1-D1 construct did not contain either of the two putative NLS, and no localization was expected. ARF1-D2 contained the putative bi-partite NLS, but lacked the second putative NLS. It might be possible for this construct to have some localization pattern because one putative localization signal was present, but no localization pattern was seen. These constructs exhibited localization patterns that were indistinguishable from controls expressing fluorescent protein alone (Figure 3-2).

ARF1-D3 (Figure 3-8) and ARF1-D4 (Figure 3-9) protein expression exhibited restored nuclear and sub-nuclear localization. These constructs showed the same pattern of protein localization as the full-length ARF1 and therefore contained all localization signals that were required, and were sufficient for proper localization. These constructs contained both the putative bipartite nuclear localization signal and the second putative NLS. There was no visible difference between deletion constructs ARF1-D3, ARF1-D4 and the full-length ARF1. ARF1-D3 contained both of the putative NLS whereas the ARF1-D2 construct lacked the second putative NLS. These data suggest that either both NLS motifs, or at least a motif present in ARF1-D3, was necessary and sufficient to target the protein to the nucleus and to sub-nuclear foci.

There are a number of possible explanations for the regulation of targeting of ARF1. It is possible that both NLS motifs, and possibly yet unidentified motifs, are necessary for targeting to the nucleus and sub-nuclear foci. Another possibility is that one NLS is responsible for targeting to the nucleus and a second NLS directs targeting to sub-nuclear structures or regions. This mechanism has been reported for the COP1 transcription factor (Osterlund et al., 1999). It is also possible that a single signal is responsible for both nuclear and sub-nuclear targeting. Constructs assembled for this study were not sufficient to test these possible models. Additional deletion and fusion constructs may aid in defining the specific motifs necessary for ARF1 targeting *in vivo*.

IAA17

Fusions of a fluorescent protein coding region to the N-terminus and the C-terminus of IAA17 were made and expressed in carrot protoplasts (Figure 2-1). The same patterns of localization were observed for these two constructs (data not shown). Aux/IAA proteins were reported to be induced by adding exogenous auxin (Hagen and Guilfoyle, 2002). Carrot cells

transformed with IAA17 plasmid were incubated overnight with and without auxin. No difference in localization was observed in the two treatments (data not shown). Diffuse fluorescence was observed throughout the nucleus, excluding the nucleolus, with much higher levels of fluorescence concentrated in sub-nuclear foci. Protein was not seen outside of the nucleus. Structures or compartments were observed in transmitted images of the cell that correlated to the sites of protein accumulation (Figure 3-10). These structures were also observed in nuclei of un-transformed cells suggesting that they do not represent an artifact created by over-expression of a heterologous protein in the cell (Figure 3-3). The pattern of localization for the IAA17 fusion proteins was distinct from that observed for the ARF1 fusion protein. IAA17 was not observed in the peri-nucleolar region where ARF1 accumulates and ARF1 was not associated with the sub-nuclear structures that were the sites of IAA17 accumulation.

***axr3-1*/IAA17**

The *Arabidopsis* line, *axr3-1*/IAA17 has been previously described (Rouse et al., 1998). This mutant is caused by a point mutation, which produces a predicted P88L amino acid change in domain II immediately before the putative NLS (Rouse et al., 1998). The mutant phenotype includes short highly agravitrophic roots, increased number of adventitious roots, reduced number of root hairs, a short hypocotyl, upcurled leaves and leaves present in the dark (Rouse et al., 1998). Domain II is believed to be involved in protein turnover, and this mutant exhibits increased protein stabilization by a decrease in degradation by the ubiquitin/proteasome degradation pathway (Park et al., 2002).

The *axr3-1* coding region was fused to the fluorescent protein expression vector. This experiment was performed to determine if the mutation affected protein localization. Repeated localizations showed that the localization pattern of this construct (Figure 3-11) was

indistinguishable from IAA17 (Figure 3-10). This mutation did not affect the targeting of protein, so incorrect targeting is probably not involved in generating the mutant phenotype. Based on deletion analysis of IAA17 reported here, domain II was not found to be exclusively responsible for nuclear targeting and localization. Although this mutant shows major phenotypic effects in plants, there was no observable difference in the pattern of localization when compared to the wild-type protein in this system.

Co-expression of ARF1 with IAA17 and ARF1 with *axr3-1*

Members of the ARF and IAA gene family have been shown to interact *in vitro* through shared domains III and IV. ARF1 and IAA17 were used to examine putative protein-protein interactions, and localization *in vivo*. The cyan and yellow color variants of the green fluorescent protein (GFP) have different emission spectra, allowing discrete visualization of the two proteins when viewed through the proper filters. This allowed for positive distinction between fusion proteins made with these color variants.

There are several possible outcomes of co-expression of ARF1 and IAA17. If the ARF1 and IAA17 proteins do not interact in the cell, co-localization should show localization patterns similar to their individual localization patterns. If interaction occurs, the pattern of localization could be like IAA17 when localized alone, ARF1 alone, or some overlap of the two. Another possibility would be that co-expression and resulting protein-protein interaction would result in altered targeting to sub-nuclear foci not observed for either of the proteins when expressed alone.

Co-localization of ARF1 and IAA17 suggested that an interaction was taking place between these two proteins. ARF1 and IAA17 both exhibited overlapping patterns of expression that were not seen when individually localized (Figure 3-2, Figure 3-13). ARF1 was observed associated with structures that IAA17 localized to when localized alone and IAA17 was

observed in the peri-nucleolar region where ARF1 was observed when localized alone. The data suggests putative protein-protein interactions may facilitate altered targeting and/or recruitment of these proteins to regions or structures where individual proteins were not normally localized. The hypothesis that ARF1 and IAA17 are able to dimerize *in vivo* was strongly supported by these experiments. Therefore, it is possible that these two proteins dimerize inside the nucleus of the cells, and recruit each other to their respective foci.

ARF1 and *axr3-1* co-localization was similar to co-localization of ARF1 and IAA17 (Figure 3-14,15,16). When localized together, both proteins showed localization to distinct sub-nuclear foci (Figure 3-14). Like IAA17, *axr3-1* was recruited to the peri-nucleolar region when expressed with ARF1. ARF1 was also localized to the small or large structures where *axr3-1* was normally found. This recruitment may be due to protein-protein interactions between these two constructs. Recruitment may also be due to other unknown factors that were able to interact with both proteins to affect localization, or through an unknown mechanism. It was also observed that when ARF1 localized outside of the nucleus it did not have *axr3-1* associated with it (Figure 3-16), which was also consistent with ARF1 and IAA17 co-localizations (Figure 3-12). One possible reason for this could be that ARF1 has a nuclear localization signal, sub-nuclear localization signal, and a cytosolic localization signal, whereas IAA17 only has a nuclear and sub-nuclear localization signal. Some of the ARF1 protein may be recruited to foci outside of the nucleus before being imported into the nucleus and interacting with the IAA17 protein that was recruited exclusively to the nucleus.

It is logical to hypothesize that *axr3-1* has the same localization and co-localization pattern as the wild-type IAA17 protein. The only difference between these two proteins is a single base substitution in domain II of the mutant. This domain has been reported to play a key

role in proteasome-mediated degradation. Although IAA17 does have a putative NLS in domain II, the single base mutation is just before the putative NLS, and does not appear to have an effect on localization.

ARF and Aux/IAA proteins can form homodimers and heterodimers with all members that have been examined (Kim et al., 1997). However, spatial and/or temporal regulation of individual genes may limit possible interactions *in planta* (Hardtke and Berleth, 1998). Northern Blot analysis and semi-quantitative RT PCR, indicated that ARF1 and IAA17 were co-expressed at all stages of growth examined. Expression patterns confirm that ARF1 and IAA17 are co-expressed and therefore protein-protein interaction is biologically possible *in planta* (Figure 3-23).

IAA17 deletion constructs

IAA17-D1 (Figure 3-18), IAA17-D2 (Figure 3-19), and IAA17-D3 (Figure 3-20) exhibited no nuclear or sub-nuclear localization. IAA17-D3 contained both the putative bi-partite NLS and the NLS, but had an expression pattern similar to controls (Figure 3-17). The nuclear localization signal, sub-nuclear localization signal or both were not present in this construct. Therefore, it appeared that additional, unidentified motifs were also required for proper localization.

The localization patterns of the IAA17 deletions indicate that the putative bi-partite NLS in the amino terminal portion of the protein and the putative NLS in domain II are not sufficient for proper localization of the fusion protein. IAA17-D4 contained both theoretical nuclear localization signals, like IAA17-D3, but also contained the region between domain II and III, and domain III (Figure 3-5). IAA17-D4 exhibited a localization pattern like full length IAA17 (Figure 3-21). Therefore, some sequence element(s) between domain II and III or in domain III

was necessary to direct nuclear localization. It may be that all that was required for nuclear and sub-nuclear localization was present in domain III. This suggests that some as yet unidentified motif was also required for proper localization of the IAA17 protein. It is not clear if the nuclear and sub-nuclear localization signals are in the same motif, or if they are present as separate motifs. A more thorough investigation is required to answer this question.

ARF6, ARF7, IAA2 and IAA3 localization

ARF6 is reported to be an activator of transcription, while ARF1 is reported to be a repressor of transcription (Ulmasov et al., 1999a). ARF6 (Figure 3-24) exhibited a pattern of localization that was similar to ARF1 (Figure 3-1). There was a nuclear localized expression pattern with higher levels of fluorescence localized to sub-nuclear structures. In some cells, possibly at different times in the cell cycle, there was also a peri-nucleolar localization pattern, like that seen in ARF1. Unlike ARF1, ARF6 was not observed in structures outside of the nucleus.

Co-localization of ARF1 and ARF6 yielded unexpected results. It might be hypothesized that activators and repressors may have different distributions inside the cell, due to their opposite functions (Ulmasov et al., 1999a). Overlapping and non-overlapping patterns of localization for these two protein fusions were observed inside the cell. In some co-transformed cells, ARF1 was excluded from the nucleus, while ARF6 was observed exclusively inside the nucleus (Figure 3-25). This pattern was never observed when ARF1 was expressed by itself. Research in this field does not indicate a mechanism for exclusion of ARF1 protein from the nucleus. It was also observed in other cells, that ARF1 was not only found inside the nucleus, but also co-localized with ARF6 in the peri-nucleolar region (Figure 3-26). When ARF6 was localized alone, it was not observed outside of the nucleus. With co-localization, ARF1 and

ARF6 were co-localized outside of the nucleus. ARF6 could always be localized with ARF1 but the opposite was not always true (Figure 3-25).

ARF7 protein had two localization patterns in transformed carrot cells. Some cells had a pattern of expression like that of other constructs, with protein localized to specific visible structures inside and outside of the nucleus (Figure 3-27 a-b). In this respect, ARF7 is similar to the other ARF constructs localized. Unlike the other ARF constructs, ARF7 had an expression pattern in some cells that appeared to be localized to the cytoskeleton (Figure 3-27 c). ARF7 is the only protein to have this localization pattern. ARF7 was also found associated with the nuclear and cellular membranes, although less frequently than either punctate or cytoskeletal localization (Figure 3-27d). ARF7 is reported to be an activator of transcription like ARF6, but was never observed in the peri-nucleolar region where other ARF proteins were localized.

In addition to looking at other ARF proteins, other Aux/IAA proteins were also visualized. IAA2 was cloned into a fluorescent protein vector and transformed into carrot cells. This construct was found exclusively in the nucleus (Figure 3-8). The localization of the IAA2 protein did differ from the fluorescent protein control because IAA2 was found only in the nucleus, indicating that a functional nuclear localization signal was present.

IAA3 was found associated with either a few large or many small structures inside the nucleus like IAA17 (Figure 3-29). The identity of these structures could not be determined, but based on the information known about ARF and Aux/IAA gene function, it is possible that these localization sites were somehow activating or repressing gene transcription. It may be that these proteins were made inactive when associated with these structure, or that they were actively regulating transcription when associated with these structures.

When ARF1 and IAA3 were co-expressed ARF1 was localized to discrete structures exclusively outside the nucleus, while IAA3 was observed inside of the nucleus either punctate or diffused (Figure 3-30). IAA3 or some unknown factors may be able to somehow exclude ARF1 from the nucleus, by an unknown mechanism.

Localization markers

Not much is known about the structures present inside the nucleus of plant cells. Several known markers were used to try and determine the structures that ARF1 and IAA17 localize to. None of the markers tested exhibited a localization pattern like the ARF and IAA protein tested (data not shown).

COP1 is reported to be part of the light-signaling pathway for photomorphogenesis (Osterlund et al., 1999). It has been reported that COP1 might interact with transcription factors like the Aux/IAA gene family to control the transition from skotomorphogenesis to photomorphogenesis when plants are exposed to light. COP1 was also reported to be nuclear localized to punctate structures in onion epidermal cells (Stacey and von Arnim, 1999). Based on this evidence the COP1 coding region was amplified from *Arabidopsis* and cloned into a fluorescent protein expression vector to be used as a possible co-localization marker for IAA17. COP1 protein expression did not differ from that of controls (data not shown). No interaction was seen when IAA17 and COP1 were co-localized in carrot protoplasts (data not shown). One possible reason for a lack of protein localization and interaction is that the carrot cells are not a functional plant, and therefore do not undergo skoto- or photomorphogenesis. It is possible that the carrot cells do not have the components necessary for proper COP1 targeting.

A putative coiled body marker U2B snRNP was tested for possible co-localization with ARF1. In BY-2 cells, a potato U2B was localized to sub-nuclear foci resembling those of ARF1

(Boudonck et al., 1999). It was reported that this construct localized to the peri-nucleolus. The protein reported here showed nuclear membrane localization without peri-nucleolar localization (data not shown). Primers were designed to amplify the *Arabidopsis* gene that had the highest sequence similarity to the potato U2B. The coding region amplified may not be the *Arabidopsis* ortholog of the U2B gene reported by Boudonck et al. (1999). This may explain the difference observed in the localization of these two proteins.

Conclusions

This research provided a preliminary investigation into the localization and possible interactions between the ARF and Aux/IAA gene families inside living cells. The carrot protoplast fluorescent protein system provided an efficient system to visualize proteins inside living cells. This research provided information about the sub-nuclear localizations of several ARF and Aux/IAA proteins *in vivo*. This research also strongly supported previous work indicating that ARF and Aux/IAA proteins are able to dimerize and interact inside living cells. Expression data also suggested that ARF1 and IAA17 are expressed in the same tissues studied so the putative interactions seen when these proteins were co-localized is biologically possible. Deletion analysis, although preliminary, indicated that the putative ARF1 NLSs appeared to be sufficient to restore nuclear targeting. Putative IAA17 NLSs did not appear to be sufficient to restore proper targeting. An as unidentified motif is also required for proper localization to be restored.

Future research

Based on the information gathered in these experiments, there are many exciting directions for this research. Because of the limited number of ARF and Aux/IAA genes localized in this study, and the variety of localization patterns seen, one of the first steps would

be to localize other members of the ARF and IAA gene families from *Arabidopsis* in carrot protoplasts using fluorescent protein vectors. Genes from the Aux/IAA family should be localized both with and without auxin to determine if auxin has any effect on localization. Co-localization of selected members of both families might prove interesting, considering the data presented here. Co-localization of different ARF repressors and activators with other IAA genes would also prove useful.

Another area still in need of further investigation is defining nuclear localization motifs in both families. Further deletion analysis, as well as site directed mutagenesis studies would help define precise motifs that are necessary and sufficient for proper nuclear and sub-nuclear targeting. Data presented here indicated that the putative nuclear localization signals in IAA17 are not sufficient for proper targeting. Other Aux/IAA genes could also be tested to determine where the localization signals are for this family.

Northern Blot analysis and semi-quantitative RT PCR showed ARF1 and IAA17 were expressed in the same tissues, but these methods cannot determine expression at the cellular level. Research has shown that expression of ARF and Aux/IAA genes are very specific, perhaps only a few cells in a given tissue. Based on this information, *in situ* hybridization would be a valuable tool to look at gene expression at the cellular level. Work in this area has shown very specific expression patterns for those genes that have been investigated (Hardtke and Berleth, 1998). Localization and co-localization experiments can be designed based on the results of *in situ* hybridization.

The carrot protoplast fluorescent protein system used in this research, although sufficient for looking at accumulation of protein, could not differentiate between protein that was interacting and protein that was associated with the same structures. For example, if one of the

co-localization markers was used to determine the structures that an ARF or IAA protein localized to, there would be no way of determining if those proteins were interacting, or if they were just in the same physical space. Fluoresence Resonance Energy Transfer (FRET) analysis allows for the distinction between proteins that are interacting, such as a protein-protein dimer, and proteins that are not, like a co-localization marker. Developing FRET analysis for these proteins would allow positive identification of proteins that are interacting.

Developing a stable transgenic *Arabidopsis* line of selected fluorescent protein vectors with ARF, IAA and mutant genes would be another valuable tool to look at gene regulation and protein interaction. Stably transfected plants would allow researchers to look at protein targeting and interactions in different types of plant cells, bringing researchers closer to the mechanisms involved in gene regulation.

Lastly, developing co-localization markers would be useful for defining the structures that these proteins localize to. Markers would allow positive identification of structures that contain the protein of interest. Defining those structures would bring researchers closer to determining what processes are associated with those foci.

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APPENDICES

Appendix 1. Primer sequences

Primer sequences and restriction sites that were used to amplify and clone gene coding regions into expression vectors. Fluorescent proteins were fused to either the C-terminus (C) or N-terminus (N) of the gene of interest (Figure 2-1).

Primer Name	Primer Sequence 5'-3'	Cloning Site
ARF1-C5'	TCGCCTAGGTCGCCACCATGGCAGCTTCAATCATTCATC	Avr II
ARF1-C3'	TCGACGCGTTCTTGATCCCGCCTAAGATG	Mlu I
ARF1-D1-C5'	TCGCCTAGGTCGCCACCATGGCAGCTTCAATCATTCATC	Avr II
ARF1-D1-C3'	TCGACGCGTGTGCCTAAAATGCCATTCACTATTGTG	Mlu I
ARF1-D2-C5'	TCGCCTAGGTCGCCACCATGGCAGCTTCAATCATTCATC	Avr II
ARF1-D2-C3'	TCGACGCGTCTCTTCATTCTCACCCCTTAAG	Mlu I
ARF1-D3-C5'	TCGCCTAGGTCGCCACCATGGCAGCTTCAATCATTCATC	Avr II
ARF1-D3-C3'	TCGACGCGTAACAGGACCAGATGGACCAG	Mlu I
ARF1-D4-C5'	TCGCCTAGGTCGCCACCATGGCAGCTTCAATCATTCATC	Avr II
ARF1-D4-C3'	TCGACGCGTTATCTGTCTACTTTGTGATTGAG	Mlu I
ARF6-C5'	TCGCCTAGGTCGCCACCATGAGATTATCTTCAGCTGGG	Avr II
ARF6-C3'	TCGACGCGTGTAGTTGAATGAACCCCCAAC	Mlu I
ARF7-C5'	TCGCCTAGGTCGCCACCATGAAAGCTCCTTCATCAAATGG	Avr II
ARF7-C3'	TCGACGCGTCCGGTTAAACGAAGTGGCTG	Mlu I
IAA2-C5'	TCGCCTAGGTCGCCACCATGGCGTACGAGAAAGTCAAC	Avr II
IAA2-C3'	TCGACGCGTTAAGGAAGAGTCTAGAGCAGG	Mlu I
IAA3-C5'	TCGCCTAGGTCGCCACCATGGATGAGTTTGTTAACCTCAAG	Avr II
IAA3-C3'	TCGACGCGTTACACCACAGCCTAAACCTTTG	Mlu I
IAA17-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-C3'	TCGACGCGTAGCTCTGCTCTTGCACTTCTC	Mlu I
IAA17-D1-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-D1-C3'	TCGACGCGTGGTTACCGGAGCCACT	Mlu I
IAA17-D2-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-D2-C3'	TCGACGCGTGACGTCATGAGTCGTAGATC	Mlu I
IAA17-D3-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-D3-C3'	TCGACGCGTGCCACCGCTTGATTTTTGG	Mlu I
IAA17-D4-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-D4-C3'	TCGACGCGTGAACATGTTGGACAAAGCATTAG	Mlu I
IAA17-D5-C5'	TCGCCTAGGTCGCCACCATGATGGGCAGTGTCGAGC	Avr II
IAA17-D5-C3'	TCGACGCGTGAACATTGGCCAAGGAACG	Mlu I
COP1-C5'	TCGCCTAGGTCGCCACCATGGAAGAGATTTCGACGGATC	Avr II
COP1-C3'	TCGACGCGTCGCAGCGAGTACCAGAACTT	Mlu I
U2B2-C5'	TCGCCTAGGTCGCCACCATGTTAACGGCAGATATACCACC	Avr II
U2B2-C3'	TCGACGCGTTTTCTTGGCGAAAGAGATGACC	Mlu I
ARF1-N5'	TCGACGCGTATGGCAGCTTCCAATCATTCATC	Mlu I
ARF1-N3'	TCGGACGTCTCATCTTGATCCCGCCTAAG	Aat II
IAA17-N5'	TCGACGCGTATGATGGGCAGTGTCGAGC	Mlu I
IAA17-N5'	TCGCTCGAGTCAAGCTCTGCTCTTGCACTTC	XbaI

Primer sequences for semi-quantitative RT PCR analysis of gene expression.

Primer Name	Primer Sequence 5'-3'
ARF1 Q 5'	ACGTTTGGTCATGGTGGGAACA
ARF1 Q 3'	TGCCTTGCATGTGCACCTTTGT
IAA17 Q 5'	TGAGCCTGCAAACAAGGAAGGA
IAA17 Q 3'	ACGAGCATCCAATCACCGTCTT
ACT2 Q 5'	ATGTCGCCATCCAAGCTGTTCT
ACT2 Q 5'	ATTCCAGCAGCTTCCATTCCCA
RPT3 Q 5'	TGATGCTCAAACAGGAGCCGAT
RPT3 Q 3'	TAACATTTGCGCGGTAGCCCTT

Appendix 2. Solutions for maintaining and digesting carrot cell cultures

Protoplast solutions

W5 solution 5x conc. (500ml)

Chemical Formula	Weight (g/mol)	1x conc.	g/500ml
NaCl	58.44	154 mM	22.5
KCl	74.56	5 mM	0.932
CaCl2-2H20	147.02	125 mM	45.944
Glucose	180.2	5 mM	2.2525

pH to 6.0 with HCl bring up to 500 ml and sterile filter with .22 um filter. Store at 4°C. Expires after 6 months.

MC solution 1x 500 ml

Chemical Formula	Weight (g/mol)	1x conc.		g/500ml
MES	195.24	5 mM		0.4881
CaCl2-2H2O	147.02	20 mM	1	0.4702
Mannitol	182.18	0.5 M		45.55

pH to 5.7 with NaOH bring up to 500 ml and sterile filter with .22 um filter. Store at 4°C. Expires after 6 months.

CM solution 1x 100ml

Chemical Formula	Weight (g/mol)	1x conc.	g/500ml
Ca(NO3)2•4H2O	236.15 g/mol.	0.1M	2.3615 g
Mannitol	182.18 g/mol	0.4M	7.2872 g

Bring up to 100ml with dd$H_2$0.

PEG CM solution

To 70 ml CM solution (from above) add 40g PEG 4000 (40%w/v).
Dissolve by stirring and heating (up to 50 deg C).
No need to bring up to 100 ml.
pH to 10.0 with dilute KOH.
Filter sterilize through .22 um filter.
Make 2 ml aliquots and store at –20°C. Expires after 3 months.

Media solutions

Carrot cell media for 1 liter:
Gibco BRL MS salt mix (cat. # 11117) was used for macro and micronutrients and Fe-EDTA

Dissolve in about 800 mL ddH_20

MS salt	4.3 g
Sucrose	40 g
Kinetin	80 ul

pH to 5.8 with KOH and bring up to 1 L. Autoclave 15 min liquid cycle. Place in tissue culture hood for ~8hrs until solution returns to room temperature, then add:

1 ml B5 vitamin stock
1 ml NAA stock
1.5 ml mefoxin (200 mg/mL)

NAA stock
Dissolve 2mg per ml ddH_20. pH to 6.0 with KOH. NAA dissolves only with the addition of KOH. .22 um filter sterilize. Store at –20°C. Expires after 3 months.

Kinetin stock
Dissolve 5mg Kinetin per ml ddH_20 with 10% HCl. Filter sterilize through .22 um filter. Store at 4°C. Expires after 1 month.

B5 Stock 50 mL

Thiamine HCl	50 mg
Pyridoxine HCl	50 mg
Nicotinic Acid	50 mg
Glycine	200 mg

Dissolve in ddH_20 and heat, if necessary, to 42°C for a short time. Long exposure to heat will break vitamin down.
Sterilize through .22 um filer freeze 1ml aliquots –20°C. Expires after 3 months.

Mefoxin
Resuspend mefoxin to a final concentration of 200 mg/ml. Freeze 1.5 ml aliquots at –20°C.

Driselase solution 250 ml

Dissolve in about 200 ml ddH_20

Chemical Formula	Weight (g/mol)	1x conc.	g/500ml

MES	195.24 g/mol	5mM	0.245
Sorbitol	182.17 g/mol	400mM	18.3
Driselase		2%	5

pH to 5.7 with KOH. Bring up to 250 ml and stir for at least 3 hours at room temperature.
Centrifuge at 5000 x g for 1hr at 4°C
Sterilize through a .22 um filter. Freeze 25-50 ml aliquots at –80°C.