COMPLEMENTATION OF AN ATCAM3 KNOCKOUT LINE RESTORES WILD TYPE ROOT GROWTH AND PROVIDES LOCALIZATION DATA THROUGH EXPRESSION OF A CHIMERIC GFP:ATCAM3 FUSION PROTEIN IN *ARABIDOPSIS THALIANA*

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Plant Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2011

Urbana, Illinois

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Abstract

Regulation of growth and acclimation to biotic or abiotic stressors involves a multitude of signal transduction events in higher eukaryotes, and oscillations in cytosolic Ca^{2+} are one highly conserved mechanism for transducing these signals. In *Arabidopsis thaliana* there are four main isoforms of Calmodulin (CaM), one group of proteins that bind Ca^{2+} ions and interact with a multitude of protein targets to modulate their activity. These four CaM isoforms are encoded in seven different genes. An additional fifty calmodulin-like proteins (CMLs) also are present in the *Arabidopsis* genome, allowing for rather complex signaling events to be communicated via Ca^{2+} signals during different developmental periods and in different tissues. Insertional knockout lines for AtCAM3, which produces an identical polypeptide sequence to AtCAM2 and AtCAM5, exhibit increased susceptibility to heat stress as well as reduction in root growth. This root growth phenotype was not impacted by varying osmotic stress of growth media relative to Columbia wild type plants. Transgenic lines created from AtCAM3 knockout plants expressing chimeric GFP:AtCaM3 exhibited restored root growth and provided localization data for AtCaM3 *in vivo.* AtCaM3 was shown to localize more highly in cytoplasmically dense cells such as meristematic regions of the root tip and sites of lateral root branching, as well as near cell membranes. Localization was also observed in cell nuclei where it could affect gene expression and explain differences in root growth rate and stress tolerance observed in AtCAM3 knockout plants.

Table of Contents

Chapter 1

Introduction

Higher organisms utilize several different mechanisms to generate cell to cell communication and coordinate cell activities of different tissue types or developmental phases. A range of molecules are employed, from complex and unique hormones to simple metabolites such as sucrose and Ca^{2+} ions. In plants Ca^{2+} signaling contributes significantly to normal development as well as mediating responses to biotic or abiotic stressors (Reddy, 2001.) During normal plant cell growth cytosolic Ca^{2+} is kept at levels in the 100-200nM range. Increases beyond this level can exhibit cytotoxic properties as protein aggregation occurs. Under normal conditions Ca^{2+} is largely located in sinks in the apoplastic space of cell walls, the endoplasmic reticulum, mitochondria and chloroplasts as well as vacuoles. Ca^{2+} tends to move through plants with transpirational water streams in the apoplastic cell wall space where it is mobile, whereas upon entering the vacuolar space it becomes more highly sequestered (Gilliham et. al, 2011). It is therefore possible that apoplastic Ca^{2+} reserves are a large contributor to cytosolic Ca^{2+} oscillations and that variation in the path water takes through the plant, apoplastic versus symplastic routes, could be integrated into plant developmental and stress responses.

One protein that has been identified as a major component in sensing cytosolic Ca^{2+} oscillations is calmodulin (CaM). In *Arabidopsis thaliana* seven calmodulin genes (CAM) have been identified which contribute to four unique protein isoforms; there are, in addition, fifty different CaM-like proteins (McCormack et. al, 2005). CaM is a relatively small and mobile protein at 16.7kD. It is composed of two globular domains, each of which contain two EF hand motifs, as well as a central linker domain. The helix-loop-helix structure of each EF hand motif is capable of binding one Ca^{2+} atom and upon binding results in a conformational change in the

central linker domain. Differences in CaM and CaM-like isoforms can lead to differences in binding affinity for Ca^{2+} as well as affinity for target proteins allowing for a very large and divergent number of target proteins in this Ca^{2+} signal transduction network (Zielinski 1998). This range of interactions allows Ca^{2+} signal transduction to regulate such diverse plant processes such as metabolism, growth and development and stress responses.

While the genes for AtCAM2, AtCAM3 and AtCAM5 all translate to identical polypeptides, AtCAM3 mRNA levels have been observed to increase at higher rates relative to the other two genes in response to heat stress (Xuan et. al. 2010). Higher mRNA levels may lead to increases in transiently elevated levels of CaM however protein pools appear to be regulated by trimethylation of lysine-115, and these transient spikes of CaM levels are likely targeted for degradation by ubiquitination pathways after a short period of time (Parag et. al, 1993 and Moon et. al, 2005). The relatively consistent regulation of CaM pools is in line with their roles as secondary Ca^{2+} signal transducers. Without consistent levels of CaM present in cells Ca^{2+} signaling would not be reproducible enough to make it useful for communication. Some Ca^{2+} oscillations and the linked responses can be quite complex (Evans et. al, 2001), allowing for the subtle changes necessary to regulate growth and other finely tuned plant responses. It is interesting that in this context the loss of one of three genes each encoding an identical polypeptide sequence would result in the range of phenotypes observed in AtCAM3 knockout plants with the amount of control over the CaM pool and the redundancy of genes encoding identical polypeptides.

Considering the complexity of Ca^{2+} signaling events and the wide range of protein interactions detected with recent protein microarray experiments involving plant CaMs (Popescu et. al, 2007) it is difficult to identify individual components of a plant response network that

contribute to observed phenotypes in AtCAM knockout plants. In AtCAM3 for example, relative amounts NO production after heat shock AtCAM3 knockout plants (Xuan et.al, 2010). NO production was identified as a downstream component of this signaling and response pathway, and likely overlaps in other signaling pathways as well. CaM proteins themselves are likely to target more than one protein, so whether an observed phenotype is a direct effect from reduction in one specific CaM-target protein interaction or the result of indirect downstream changes is difficult to determine. In the previously mentioned study AtCAM3 mRNA levels were shown to be more highly elevated after heat shock. While the increased levels of AtCaM3 may only exist for a short time due to ubiquitination they could still be present long enough to affect targeted proteins and alter physiology or the regulation of other genes that confer increased tolerance in response to the stress. Alternatively, breakdown products of the increased production of transcripts and gene products could later affect other CAM expression levels, thereby conferring increased tolerance to future recurrence of stress events through changes in the relative size of different CaM pools.

An alternative explanation for the phenotypes observed in CAM knockout lines is that loss of one gene, even in the presence of other genes encoding identical polypeptides and overlapping expression profiles, creates a disruption in the stochiometric ratios of these Ca^{2+} sensing proteins. Differences in these ratios would then in theory also alter how signature Ca^{2+} oscillations were sensed and the ensuing responses in future incidents of stress. While this change is presumably small the resulting subtle differences in Ca^{2+} sensing when integrated over several days in the case of a germinating seed and developing seeding could still materialize in the form of an altered phenotype such as the reduction in root growth observed in AtCAM3 knockout plants.

In order to determine where AtCaM3 was functioning in *Arabidopsis thaliana*, and to attempt to identify signaling networks and target proteins that may contribute to some of the phenotypic differences observed, transgenic plants expressing GFP:AtCaM3 under control of the native AtCAM3 promoter were created. These transgenic plants were then grown alongside both wild type Columbia plants as well as AtCAM3 knockout plants in order to determine if expression of the chimeric GFP tagged protein restored the original wild type phenotypic root growth. The results from this study allow for the design of additional experiments that rule out involvement of different response pathways in creating the observed knockout phenotypes.

Chapter 2

Materials and Methods

Primary plant lines, transgenic plant line generation and screening.

Plant lines. An AtCaM3 knockout line Salk_001357 was obtained from the ABRC, and screened for homozygous individuals. Growth of these individuals was compared to Columbia wild type plants for root phenotype analysis. This line of plants were then used for growth analysis and creation of transgenic GFP:AtCaM3 expressing plants.

Gene fusions. GFP:AtCaM3 driven with the native promoter was transferred into a binary vector (pPZP122) and then introduced into *Agrobacterium tumifaciens* GV3101 (pMP90) via electroporation. Colonies of this line of *A tumifaciens* were then used for transformation of knockout lines by floral dipping.

Trangenic plant generation. Knockout plants were transformed by floral dipping and recovered seed was screened on 0.5X MS media plates containing 75 ug/ml gentamycin. Seedlings that formed true leaves with trichomes and that did not show yellowing of cotyledons were transplanted onto soil and grown to maturity for collection of seed. This seed was then grown for a second generation to generate plant lines that were homozygous for the insert. Screening of second generation individual's seed was performed on identical plates to the original screen and plant lines that were homozygous for the transgenic insert.

Screening for GFP:AtCAM3-expressing plants. Initial screening of homozygous transgenic lines was performed using a Zeiss Axiovert 200M microscope equipped with an ApoTome structured illumination device using a 20X/0.8 N.A. Plan Apochromat objective. Fluorescence was observed using either Zeiss filter set 13 (BP 470/20 excitation, FT 495 beam splitter and BP 505-530 emission) for GFP. Additionally, as a check for false positives

generated by chlorophyll fluorescence that passed through this filter set, observations were also made using filter set 15 (BP 546/12 excitation, FT 580 beam splitter and LP 590 emission).

Root growth measurements and analysis.

Seed preparation.. Seed was surface sterilized for 5-7 minutes in a 70% ethanol solution followed by 5-10 minutes in a 50% bleach 0.02% Triton X-100 solution. Following these sterilizing steps seed was briefly centrifuged and rinsed 5 times in sterile water. After surface sterilization seed was stratified for 3 days at 4 ^{o}C in sterile water before being placed on media plates for root growth measurements.

Growth media. Square petri dishes with a 10cm x 10cm grid were prepared with 25mL of 0.5X MS media containing 0.5g MES/L, 0.8% agar and 1.0% sucrose. Seeds were placed along the top row of this grid spaced 0.5cm apart such that 11 plants could be visualized per plate. Seeded plates were partially sealed with surgical tape to reduce moisture loss and placed vertically under long day growth shelves with 18 hours of light per day at approximately 100 μmol m^{-2} s^{-1} PPF.

Root growth measurements. Images of plates were taken at the same time each morning in order to acquire 10 consecutive days of root growth. Main root length was measured using ImageJ software and manually tracing each root by hand. Root length was tracked for each plant for the duration of the growth period and later used for statistical analysis. Between January and March six separate replicates of plates were measured, yielding 135 measured Columbia plants, 153 measured AtCaM3 knockout plants, and 126, 119 and 96 transgenic plants from lines H64, 452 and H54 respectively. Root lengths were compared using a student's t-test.

Soluble Protein Analysis

Protein Extraction. Soluble proteins were extracted from 10-day-old Arabidopsis seedlings by homogenizing entire seedlings frozen with liquid nitrogen with liquid nitrogen chilled mortar and pestle. Ground tissues were then suspended in a buffer containing 50 mM Hepes-KOH pH 7.5, 100 mM KCl and 1 mM dithiothreitol. Extracts were clarified by centrifugation at 10,000 x g for 15 min and the soluble proteins quantified using a BCA assay kit (ThermoFisher Scientific).

Protein Identification. Ten µg of soluble protein extract from each transgenic line and a CAM3 knockout line control were separated by size on a NuPAGE Novex Bis-Tris 17 well mini gel (Invitrogen, Carlsbad, CA), transferred to PVDF membranes together with 30 ng of a purified 6X His:GFP standard. After blocking the membrane in TBST containing 2% gelatin, the blot was probed with a goat anti-GFP antibody (Rockland, Gilbertsville, PA) diluted 1:1,000 in the same buffer. Antibody-antigen complexes were detected by reacting the blot with IRDye 800CW-labeled donkey-anti goat IgG (LiCor, Lincoln, NE) and scanning the blot using an Odyssey IR scanner (LiCor, Lincoln, NE).

Localization with confocal microscopy.

Plant samples. Transgenic plants were grown in a fashion identical to those used for root growth measurements. In addition to the normal growth media for the square petri a larger amount of liquid media was prepared in order to create growth media for plates which contained agar as well as a nutrient solution without agar that would be otherwise identical to the media upon which the observed plants had grown. Immediately prior to imaging seedlings were

screened under a wide field microscope to confirm that all plants grown were expressing GFP, indicative of the homozygosity of the transgenic plant line, as well as to identify individual seedlings that showed the highest levels of expression. Seedlings were kept in a vertical orientation similar to conditions during initial growth and groups of plates were handled as delicately as possible.

Slide preparation. No 1 ½ cover glasses measuring 24 X 50 mm were used in place of a traditional slide. Seedlings were transplanted from the petri plates directly onto this cover slip and placed directly into 30ul of liquid nutrient media. A second No 1 ½ cover glass measuring 22 X 30mm was placed on top of the droplet of nutrient media just below the cotyledons, forcing the root to conform to a reference plane for further imaging.

Confocal settings. All confocal images presented in the figures section were captured using a Zeiss LSM 700 confocal microscope located at the Institute for Genomic Biology at the University of Illinois, Urbana-Champaign. Using a EC Plan-Neofluar 40x/1.30 Oil objective samples were screened for areas showing high levels of GFP signal. The MSB settings for the GFP channel, or track 1, were 405/488/555/639 with the DSB1 set at 551 nm. The excitation laser was set at 488 nm and 2.4 % relative intensity. As chlorophyll bleed through had been problematic with previous localization attempts a second track was also used with MSB settings of 405/488/555/639 and DSB1 of 630 nm and excitation at 639 nm and 5.5 % relative intensity. This channel and settings was also used to capture a DIC image. During imaging samples were periodically rehydrated with sterile water to avoid desiccation of the sample.

Image analysis and processing. In order to format the images for the figures presented in the figures section a version of Zeiss' imaging software, Zen light was used to manage the file formats generated at the time of image capture. This software was similar to that used in

conjunction with the LSM 700 yet lacked some of the higher end functionality which was not required for image processing. The images were processed to add scale bars and adjust the relative gain of some channels for image clarity. Gain adjustments were performed using the min/max function provided with the software and increased the gain setting from fifty to a range of values between 53 and 61 at most.

Chapter 3

Results

Root growth measurements of AtCaM3 knockout, Columbia and transgenic plant lines.

Knockout plants for AtCaM3 showed significantly slower growth of main roots compared to Columbia plants when measured for ten days starting at germination. Five out of the ten days average root lengths were significantly less in the AtCaM3 knockout line using a one-tailed t-test($p<0.05$), and when all data were compared the means were significantly different using a two-tailed analysis ($p <0.05$). One of the transgenic plant lines, H54, exhibited growth rates that were not statistically different from the Columbia plants, however there was a greater amount of variance in its root length measurements. The two other plant lines measured, H64 and 452, each showed statistically significant increases in root length using a two tailed students t-test ($p<0.01$).

The difference in root growth did not change significantly on subsequent days after germination. If the difference in root growth had become exacerbated with each consecutive day after germination or conversely had only been significant just after germination the possible underlying cause for the phenotype might have been easier to narrow down from a larger list. The general reduction in rate of growth suggested instead that the underlying cause may be something more general and pervasive during growth, such as ion transport necessary for nutrient uptake or metabolic regulation.

Root growth of Columbia and AtCAM3 knockout plants under varying concentrations of Mannitol.

In order to examine whether the decrease in root growth in AtCAM3 knockout plants was due to compromised water status in the roots Columbia and the knockout plants were grown on four separate growth media ranges, from a normal control media to media also containing 1.5%, 2.5% and 3.5% Mannitol. Growth was measured for ten days similar to the transgenic plant line data described in the previous section. As shown in Figures 5 and 6, mannitol repressed root growth in both the Columbia wild-type and AtCAM3 knockout plants. However, in order to look at relative differences in growth rate due to osmotic stress, root growth was normalized around the average root length for the control media. While the total root length data differed significantly between Columbia and AtCaM3 knockout plants consistent with the other measurements, after normalization this difference disappeared.

Analysis of plant soluble protein extracts.

In order to determine whether or not the GFP:AtCaM3 fusion protein was still intact after translation, and in order to rule out the excision of GFP from the protein through post translational modification or alteration, soluble protein extracts were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-GFP antibody as described in the Materials and Methods section. A His-tagged purified GFP was used as a control and was detected on western blots consistently with its size of approximately 27 kD. GFP was also detectable in extracts from the transgenic plants. These bands migrated consistent with the expected weight of the 16.7 kD AtCaM3 proteins fused to GFP to give a ~43-44kD fusion protein with no apparent unfused GFP as shown in Figure 7. These data in conjunction with the

root growth data suggest that the observed location of GFP in vivo is indicative of where AtCaM3 is localizing to function in its role in mediating root growth and development.

Visualization of GFP:AtCaM3 in transgenic plants.

Seven day old seedlings were imaged using a Zeiss LSM 700 confocal microscope as illustrated in Figures 8 through 10. Root samples were imaged using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective. MBS settings for the GFP channel were 405/488/555/639 and DBS1 was 551 nm. GFP was most visible in cytoplasmically dense cells and metabolically active cells. The meristematic region of the root tip and sites of lateral root branching also showed strong localization signals. The GFP signal was observed to be mobile at times with some cells localizing strongly at the cell membrane while nearby cells showed GFP localization primarily in the cytoplasm. At other times localization was observed more strongly in the cell nucleus, most visibly in leaf trichomes and root cells, exhibiting localization patterns that are consistent with results showing AtCAM3 knockout plants have reduced expression levels of some genes.

GFP signals were observed confocally in root and shoot epidermis cells as well as guard cells (Figure 10). While the primary region of localization appears to be in the root epidermis, strong signals were also observed in the cortext and endodermal initial cells as well as the epidermal and root cap initial cells. Some of the cells sloughed off into the root cap still showed residual GFP signals, and in general within five to six cells from the initials the GFP signal was primarily located in the root epidermal layer. Previous screens for GFP expressing plants using a Zeiss Axiovert 200M microscope equipped with an ApoTome structured illumination device also

showed a signal in the root pericycle and endodermis, however this was not observed when the LSM 700 confocal microscope was employed. Leaf protoplasts isolated from GFP:CaM3-expressing lines (Fig. 11) and examined by fluorescence microscopy revealed that expression of the fusion protein also occurred in leaf mesophyll cells. Because these cells are highly vacuolated and the cytoplasm is found in a very thin layer at the cell periphery and around the organelles, it is impossible to determine whether there is any localization of the protein to specific regions within the cytoplasm.

Chapter 4

Discussion

One of the potential difficulties in identifying gene function is functional redundancy among gene family members. However, in spite of the fact that AtCAM3 encodes a protein product identical to those encoded by AtCAM2 and AtCAM5, there here was a consistent and statistically significant reduction in root growth in the AtCAM3 knockout line. This difference was present early on and did not become more or less significant with consecutive days measurements after germination. The degree to which the reduction was consistent suggests that the underlying cause may be a fundamental process regulating growth in general, for example regulation of water loss through guard cells or of a basic metabolic impairment such as sucrose transport to the root or regulation of cell wall extensibility. Additionally the path water takes through the plant is guided not only by physical structures in the plant tissues themselves but also by ion channels and aquaporins, several of which contain putative CaM binding sites (Popescu et al., 2007). Regulation of aquaporins has been identified as a possible target for improved crop resistance to drought and other stresses (Rae et. al. 2011), and could easily affect basic phenotypes like root growth. Changes in the relative abundance of the different CaM isoforms could alter regulation of these important channels and the flow of water, thus impact the relative state of Ca^{2+} and other secondary signaling molecules or signaling events.

The strong localization of GFP:AtCaM3 near the cell membrane in fully expanded root cells places the majority of the AtCaM3 present in the cell near to a large contributor to Ca^{2+} oscillations from the apoplastic space. Additionally, many important hormone receptors line the cell membrane and this placement allows for modification of hormone sensing and responses to take place more efficiently or rapidly. One such cell membrane bound receptor, BAK1, has been

shown to be significant not only in eliciting stress response but also in normal growth of plants (Oh et. al. 2010).

Of the three transgenic lines measured for root growth only one was not statistically different from the Columbia wild type plants measured. This lack of significance was likely the result of poor germination rates as reflected by the lower number of plants measured in this line, H54, and of lower early growth rates. At the final day of measurements this line had reached an average root length which consistently exceeded that of the wild type. The two other transgenic lines each grew at consistently faster rates and to longer average lengths by the final day of measurements. Interestingly this difference was both more significant and represented a larger absolute change in root growth than was observed in AtCAM3 knockout plants. This could be explained if AtCAM3 was acting to relieve repression of growth, CaMs have been shown to bind autoinhibitory domains of proteins in order to activate the targeted protein function. If this were the case higher levels of AtCAM3 would be expected to relax the constraints put on growth under similar circumstances relative to a wild type plant and a smaller AtCAM3 pool.

The increased growth of the transgenic lines effectively reversed the stunted root growth phenotype of the AtCAM3 knockout line. Early screens for GFP expression in a larger pool of plant lines expressing transgenic GFP:AtCaM3 were used to select plants expressing the brightest GFP signal to aid in more precisely localizing expression of the protein. The assumptions in this approach are that: (1) the GFP:AtCaM3 fusion protein remains intact and not proteolytically processed into the two components of the fusion; and (2) that GFP does not greatly influence the distribution of CaM. . It is possible that the transgenic lines selected for the localization study expressed AtCAM3 at higher levels than the Columbia wild type plants, and the difference in expression rates underlies the reversal of the stunted root growth phenotype

observed in the knockout line. Whether or not the release of constraints on growth was the result of gene expression and other signaling cascades beyond the initial interaction of AtCAM3 and target proteins or the result of increased metabolic activity or sucrose transport would be possible to identify with further experiments described later. A second potential explanation for this reversal is that the construct used to generate the fusion protein expressing plants was created using a cDNA version of AtCAM3, and that the loss of the intronic regions may have either relieved some of the regulation of the expression levels or increased the rate of CaM mRNA accumulation and translation enough to increase its relative contribution to the CaM pool.

In order to rule out the cleaving of GFP from the chimeric GFP:AtCaM3 fusion protein extracts taken from the parental AtCAM3 knockout line, Columbia wild type plants and each of the three transgenic lines were probed for presence of GFP. Each of the transgenic lines exhibited antibody binding at a band location that corresponded to an intact GFP:AtCaM3 fusion protein at the expected size of approximately 44kD. These results verify that the visualized GFP signal from the transgenic plants did, in fact, correspond to location of transgenic CaM, and that the reversal of the stunted root growth rate in the parental knockout line could be implied to result from the protein distributions observed.

The localization of the GFP signal at the cell membrane could be due to the size of the vacuole in mature root cells where the cytoplasmic distribution is spatially kept in greater proximity to the cell membrane. While many of the tissues visualized showed GFP:AtCaM3 at the cell periphery relatively strong signals were also observed more diffusely in the cell cytoplasm of younger cells as well as highly localized in the cell nucleus in more mature root cells. Localization in older tissues showed more distinct patterns relative to younger dividing cells such as those at the root meristematic region, tending to be concentrated more closely to the

cell membrane or in the nucleus. Lateral root branches and root hairs also showed high amounts GFP localization at the cell membrane as well as in the cytoplasm. In young lateral root branches the pattern of distribution matched closely observations in root meristematic regions, with the cytoplasmically dense cells showing a more diffuse yet relatively strong GFP signal. These patterns of localization suggest that AtCaM3 can be mobile within the cell depending on the situation, and nuclear localization also allows for regulation of gene expression as observed in heat stress experiments (Xuan et. al. 2010).

In order for plants to meet the myriad of challenges presented to them as sessile organisms it is imperative that the root system be able to manage nutrient and water acquisition in the proper balance to maximize plant growth. This principle underlies the phenotypic plasticity observed in root architecture, in general roots growing further down are able to access larger water reserves while those closer to the surface often have access to more nutrients. Ultimately root architecture is the integrated response to environmental and physiological processes taking place in the plant and has a large role in its survival (Melamy 2005). The initiation and growth of lateral root branches is one of the key mediators in managing root architecture in Arabidopsis, and the localization of AtCaM3 near sites of lateral root branches suggests it may play a role in these processes. The previous observations of a GFP signal originating in the root pericycle, the site of lateral root branch initiation, also suggests that it could be playing a role in regulating the initiation or growth of lateral roots.

Chapter 5

Conclusions

While transgenic GFP:AtCaM3 plants did show recovered root growth, and in fact showed significantly higher growth rates than the Columbia wild type plants, the underlying cause for this difference is still unclear. Growing plants in media with varying water potential did not increase the relative reduction of root growth observed in the AtCAM3 knockout plants, suggesting the mechanism that is limiting root growth is not likely based solely on cell water potential and instead involves other pathways or mechanisms. One observation noted during growth trials which spanned January through March is that as average temperatures increased in the growth area the relative difference between the knockout line and Columbia wild type was decreased, although still maintaining statistically significant differences. The decrease in the relative difference of the AtCaM3 knockout plants and Columbia line as temperatures increase could indicate that the underlying mechanism generating the decreased root growth phenotype is metabolic.

Additional experiments which maintained consistent water potential between growth media yet contained varying levels of sucrose could test for differences in carbon metabolism that may result from differences in sucrose uptake or utilization. If this was part of the cause for the root growth phenotype knockout plants would be expected to exhibit even greater reductions in root growth relative to Columbia wild type plants. In addition to carbon metabolism, water use and transpirational regulation could represent an additional perturbation to Ca^{2+} signaling and developmental regulation. As GFP localization was observed in guard cells, experimental analysis of guard cell impact on root growth would also be worth addressing. A simple GFP:AtCaM3 construct similar to the one used in this study could be created with a guard cell

specific promoter. Transgenic plants expressing this construct could then be used for an additional root growth study. If plants exhibited normal root growth, or at least partially restored root growth relative to wild type plants, it would suggest guard cell regulation plays a role in regulation of root growth and the decreased root growth phenotype observed.

Assessing lateral root initiation and growth could provide another method of analysis to determine where primary root growth was being reduced. It is possible that AtCAM3 knockout plants have stronger allocation to lateral root branches versus primary roots. An analysis of lateral root formation relative to primary root length could illuminate another explanation for the observed differences in these plants. If lateral root initiation and growth were not different between the knockout line and wild type plants the underlying cause could be due to impaired sucrose or hormone transport which could be tested experimentally.

The main appeal of these additional studies is that they are relatively easy to achieve with the current materials and gene constructs available to the Zielinski lab. In combination with additional measurements of root growth under varying media water potential they would complement one another in such a way that the biochemical underpinnings of contributing factors could be narrowed down to a manageable size to allow for more precise identification of protein targets and networks of interest.

Figures

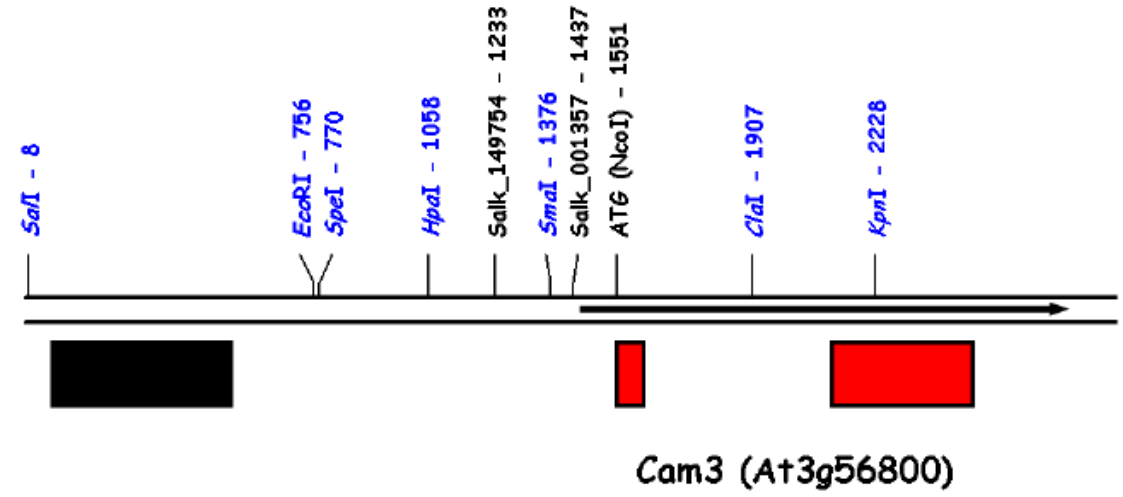

Figure 1. Physical map of AtCaM3 with restriction and T-DNA insertional sites.

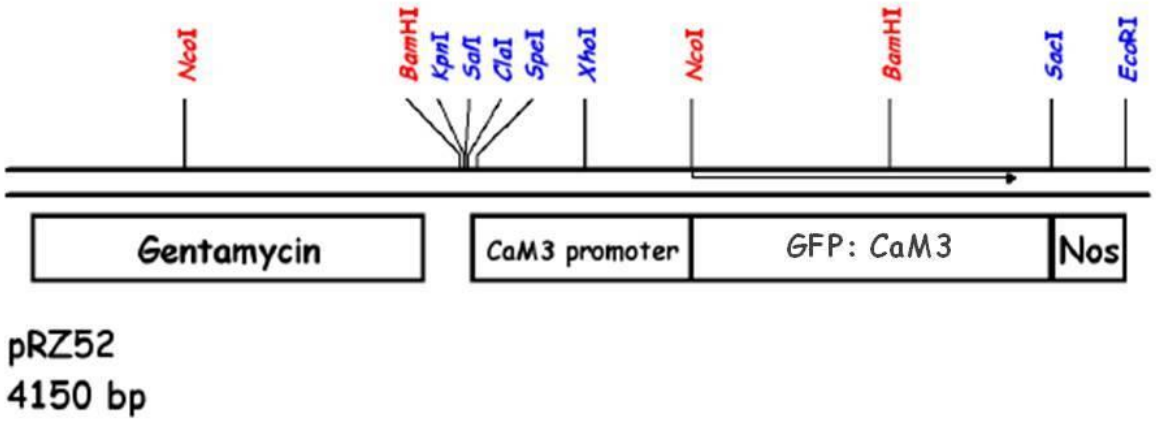

Figure 2. Physical map of the plasmid vector used to introduce GFP:AtCaM3 into knockout plants.

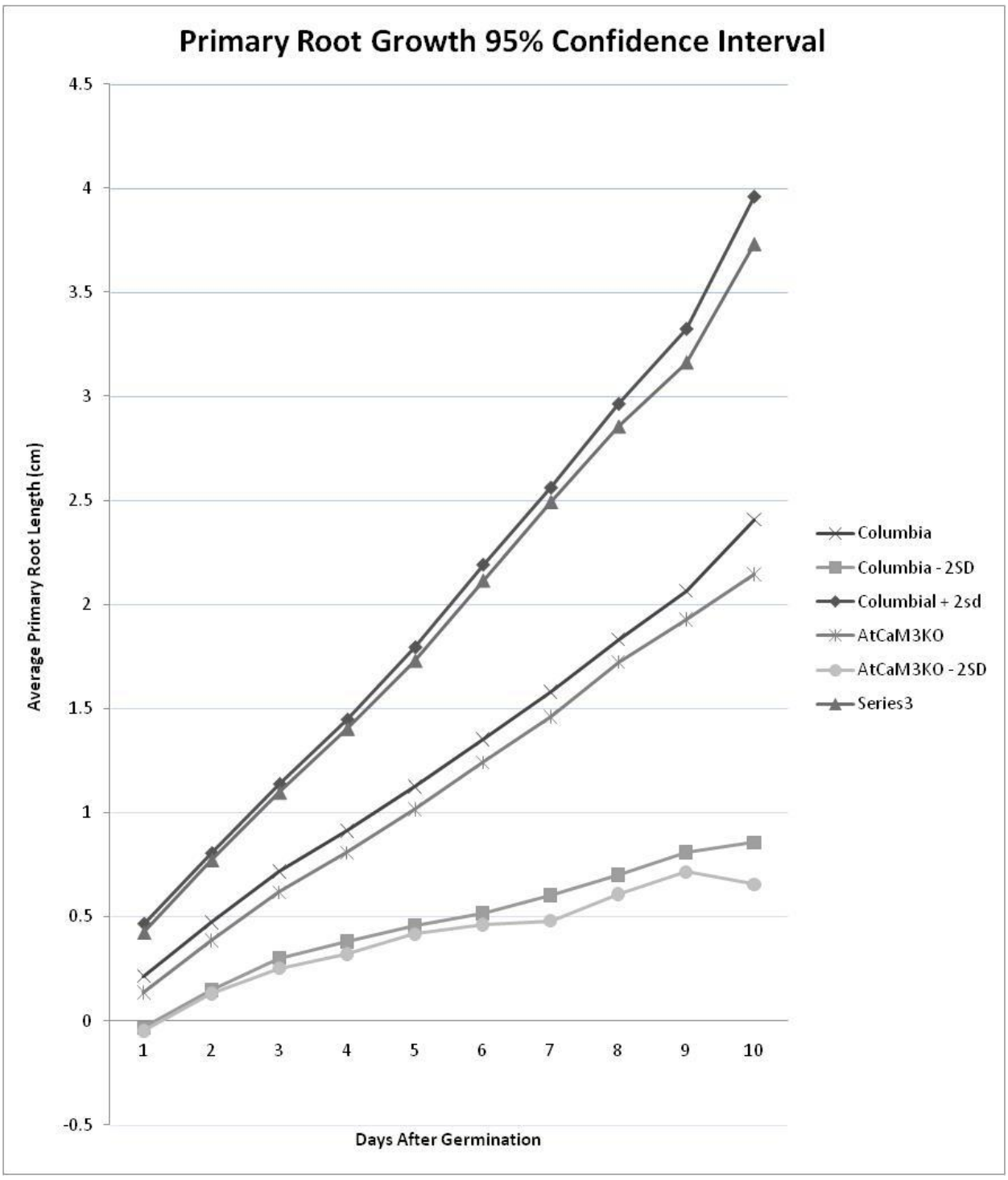

Figure 3. Columbia wild type root average root growth compared to AtCaM3 knockout plants shown with 95% confidence intervals.

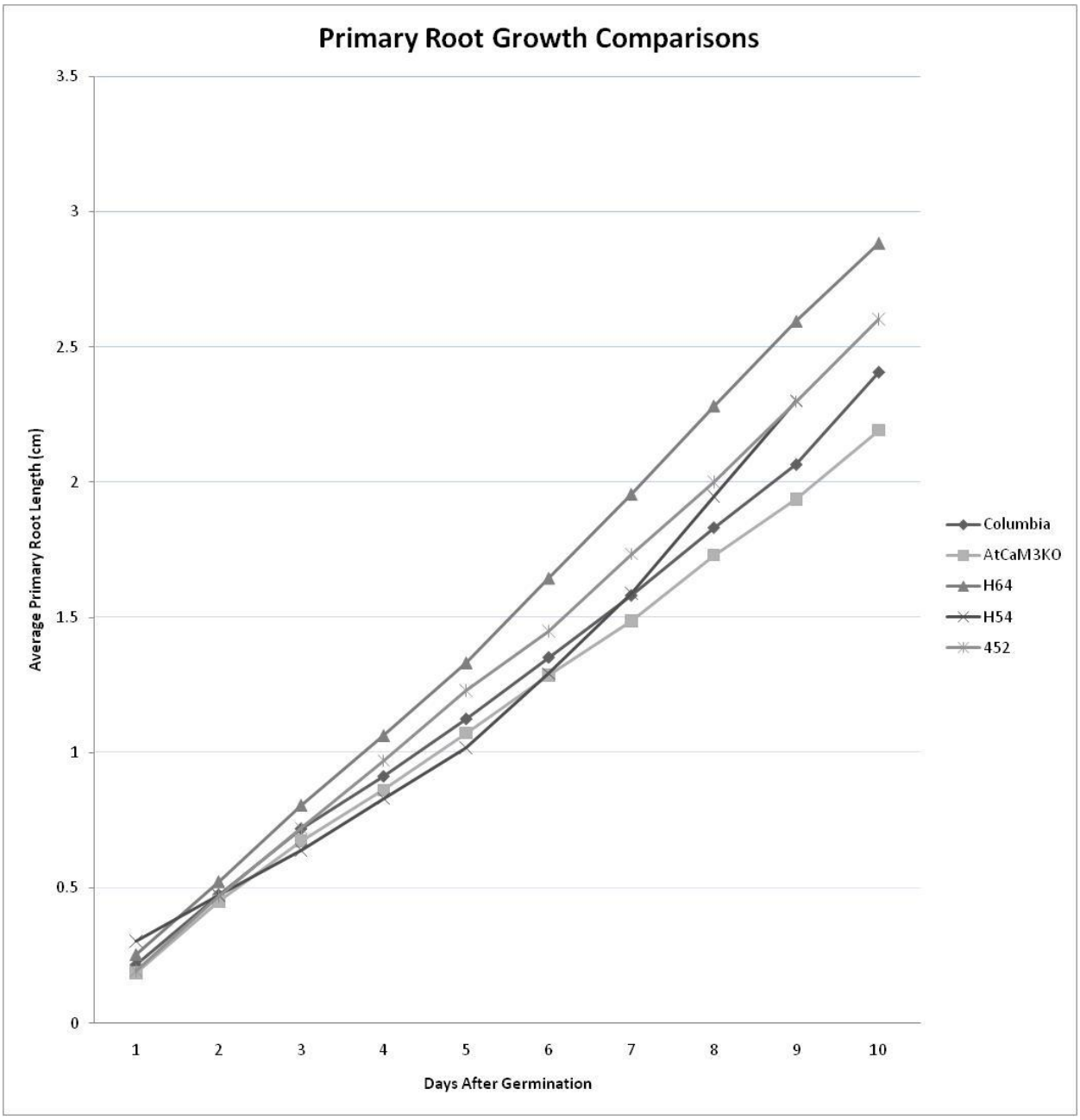

Figure 4. Average root length for Columbia wild type, AtCaM3 knockout plants and AtCaM3:GFP transgenic lines H64, H54 and 452 by day after germination

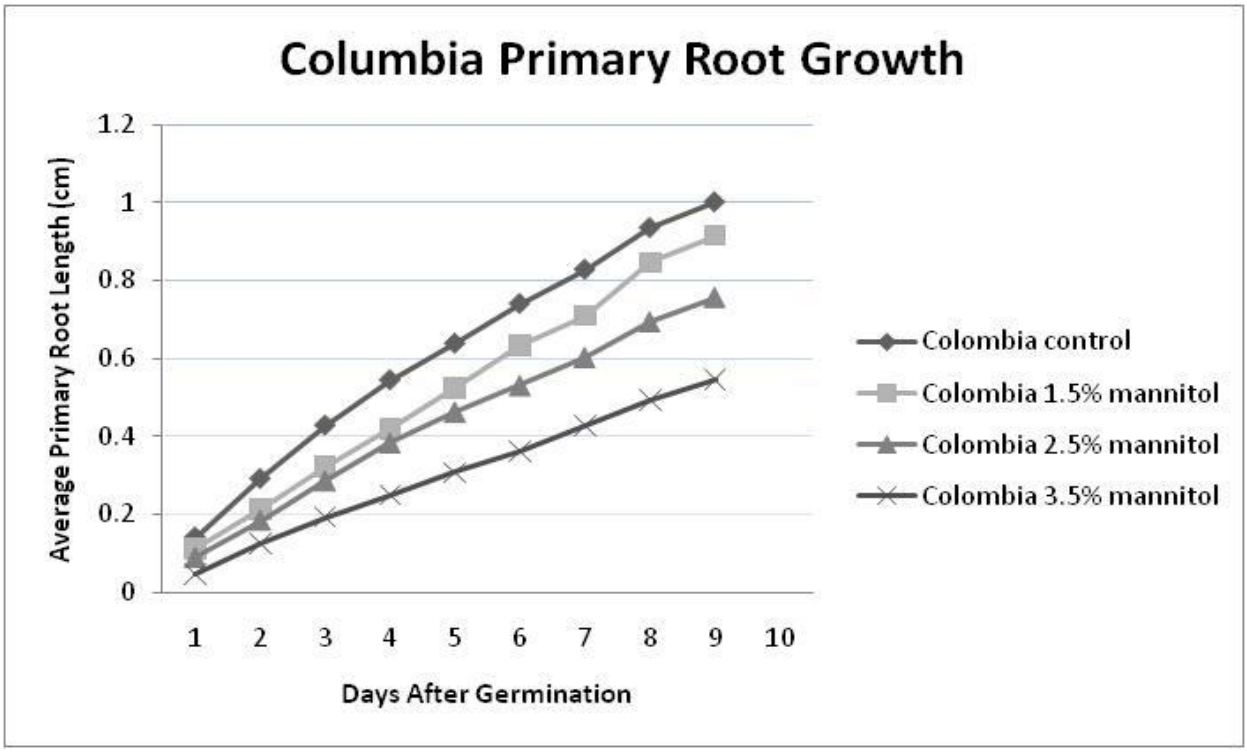

Figure 5. Columbia wild type root growth under varying concentrations of mannitol. Growth was normalized by the average growth on control media to allow for comparison to AtCAM3 knockout plants.

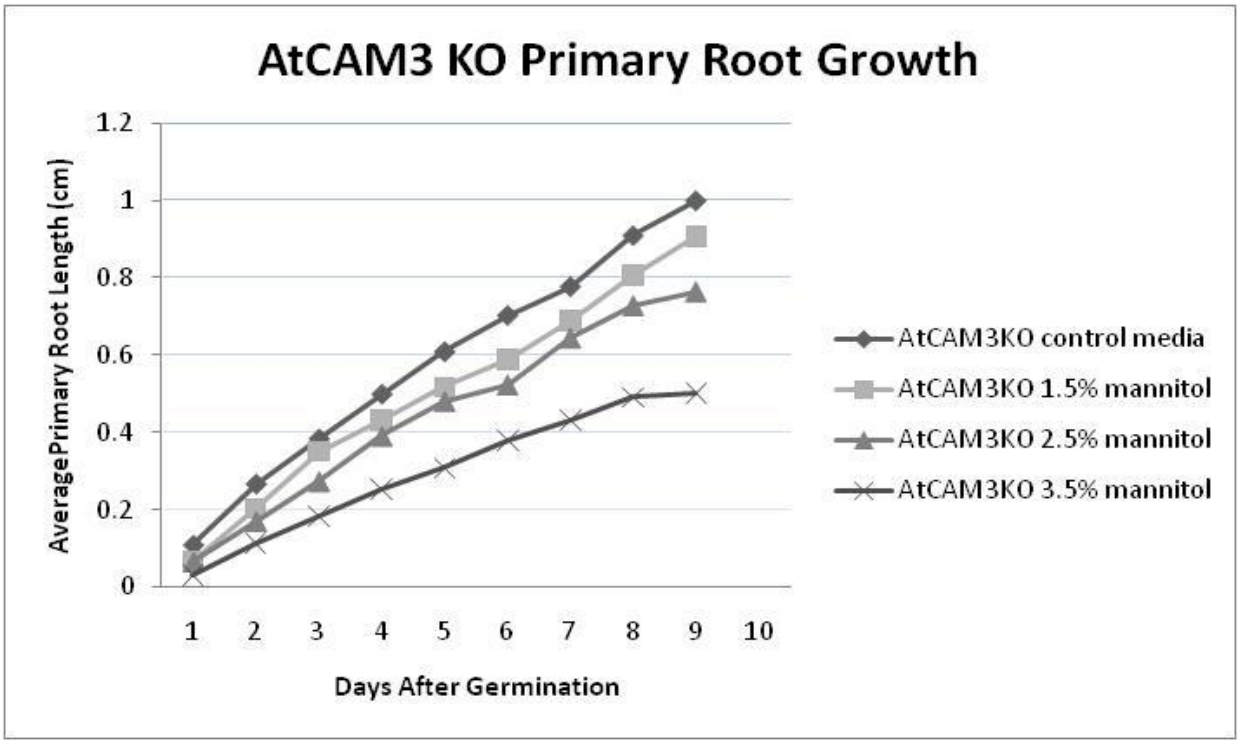

Figure 6. AtCaM3 knockout plant normalized primary root growth with varying concentrations of mannitol.

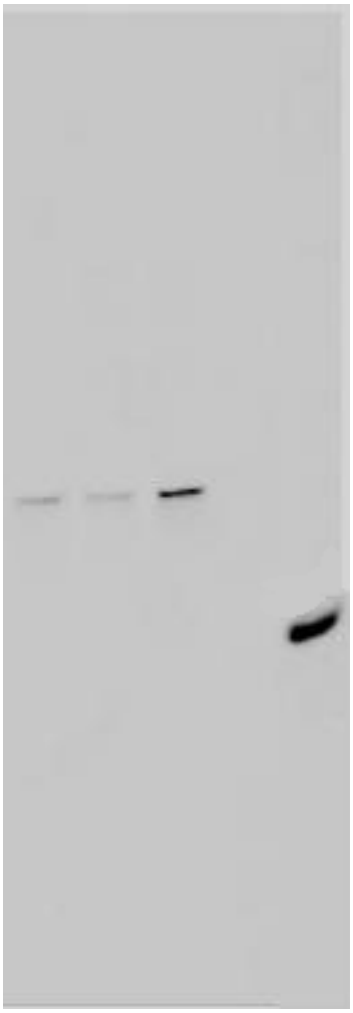

Figure 7. Soluble whole plant protein extracts taken from 10 day old seedlings. Bands shown from left to right are 452, H54, H64, AtCaM3 knockout and a 30ng standard of His-tagged GFP.

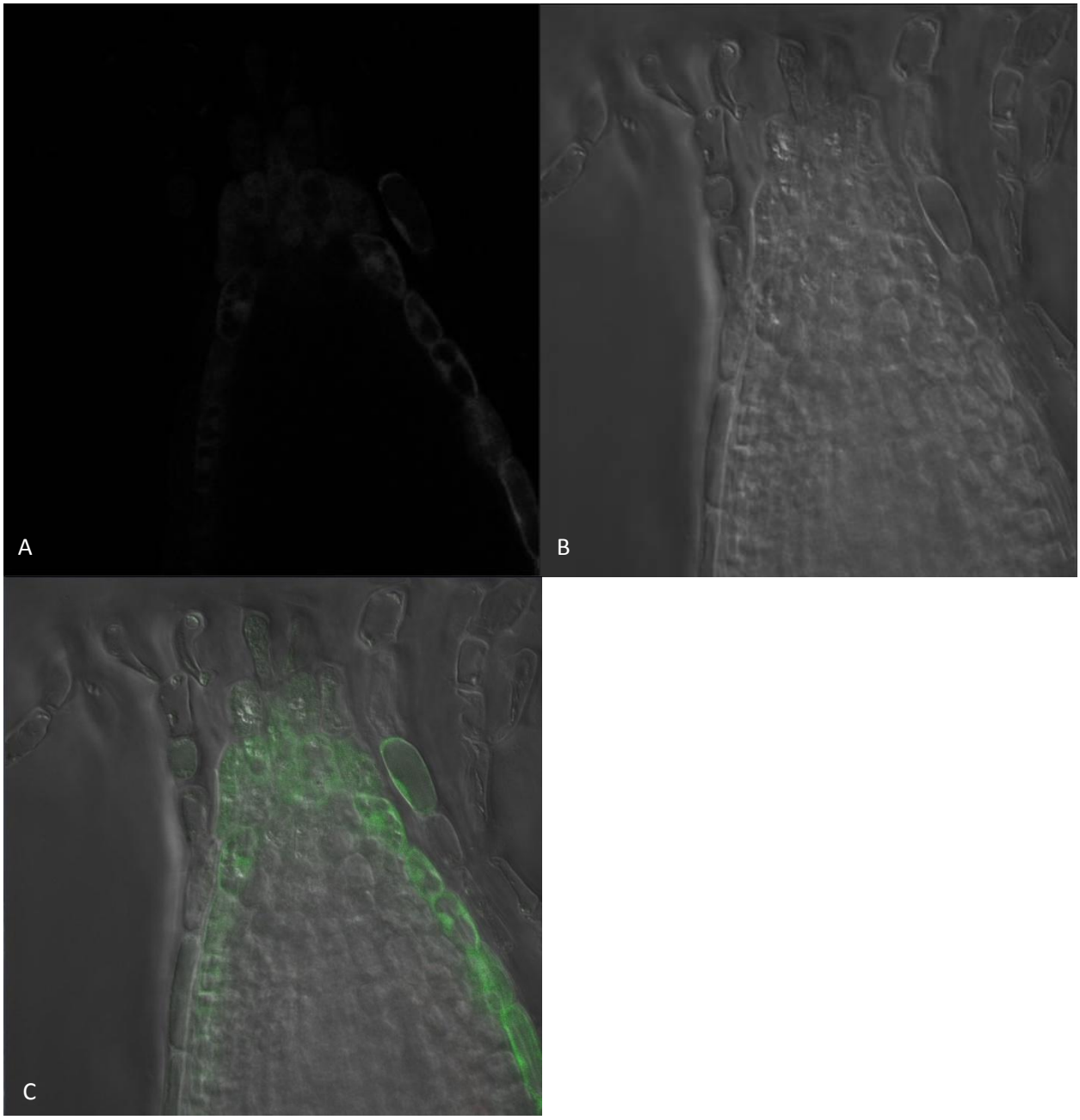

Figure 8. AtCaM3:GFP line H64 root tip. **A.** GFP filter set. **B**. DIC image. **C.** Combined color image.

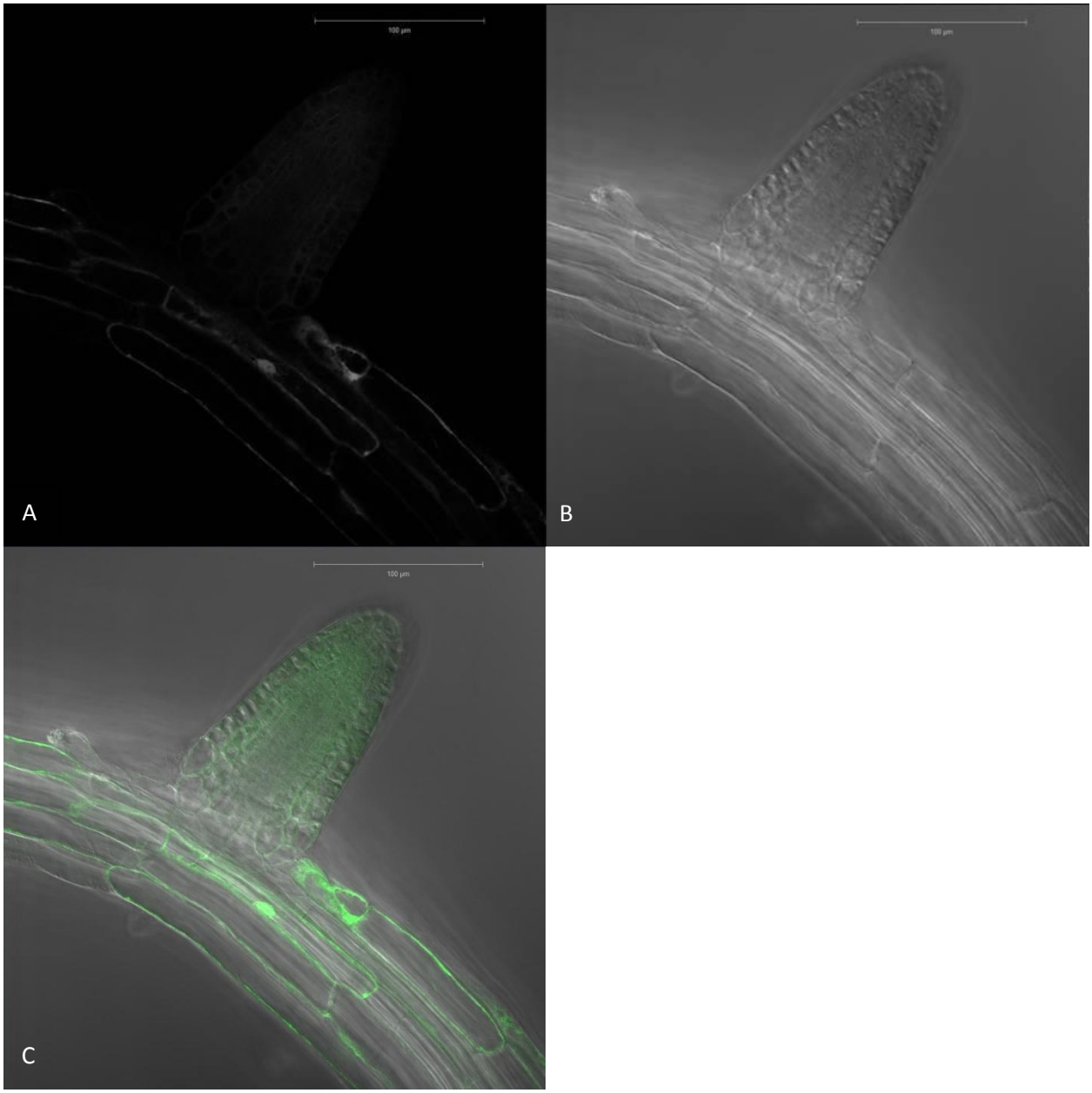

Figure 9. GFP:AtCaM3 line H64 root showing initiation of a lateral root branch. **A**. GFP filter set. **B.** DIC image. **C.** Combined color image.

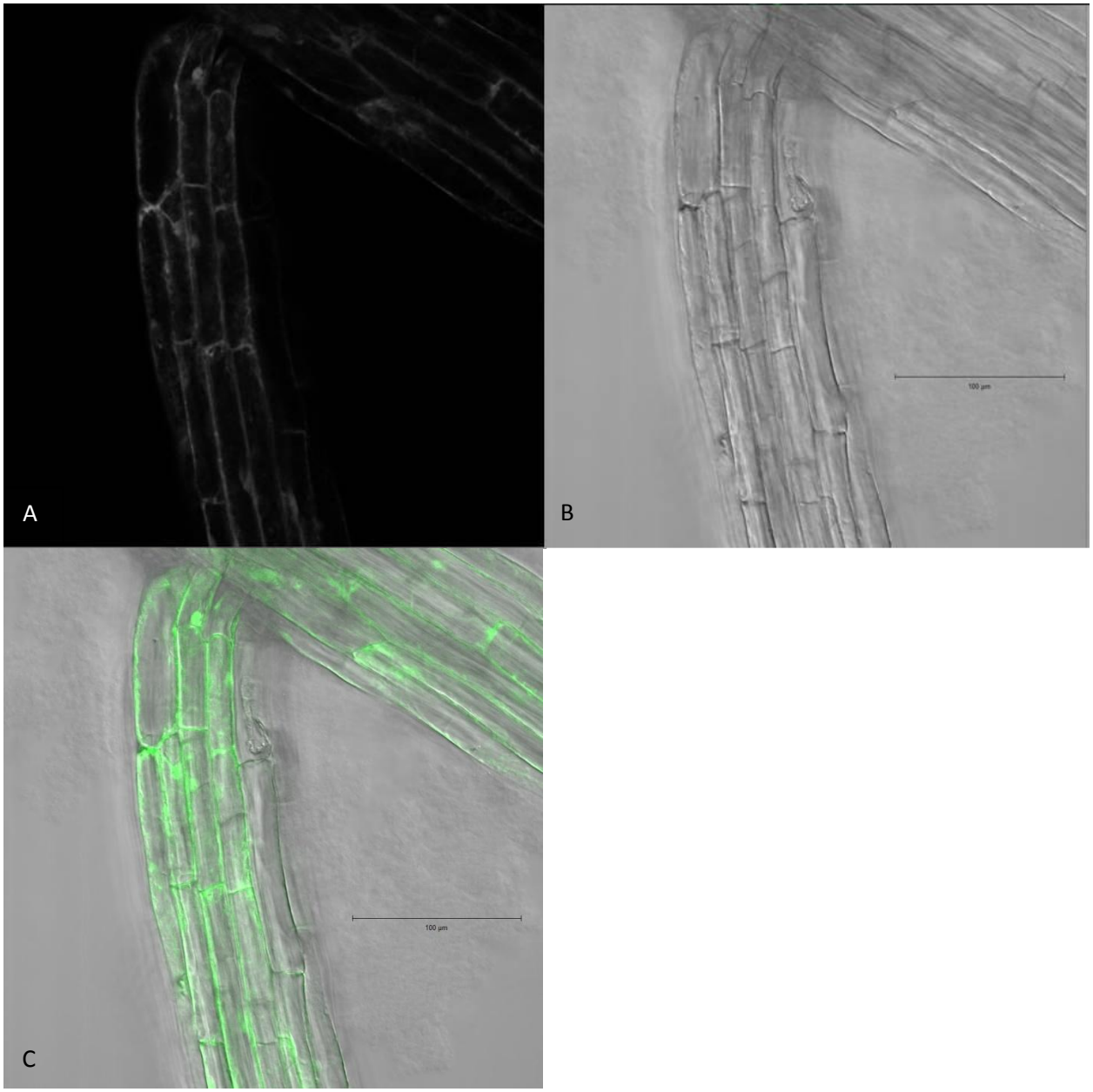

Figure 10. GFP:AtCaM3 line H64 lateral root branch. **A**. GFP filter set. **B.** DIC image. **C.** Combined color image.

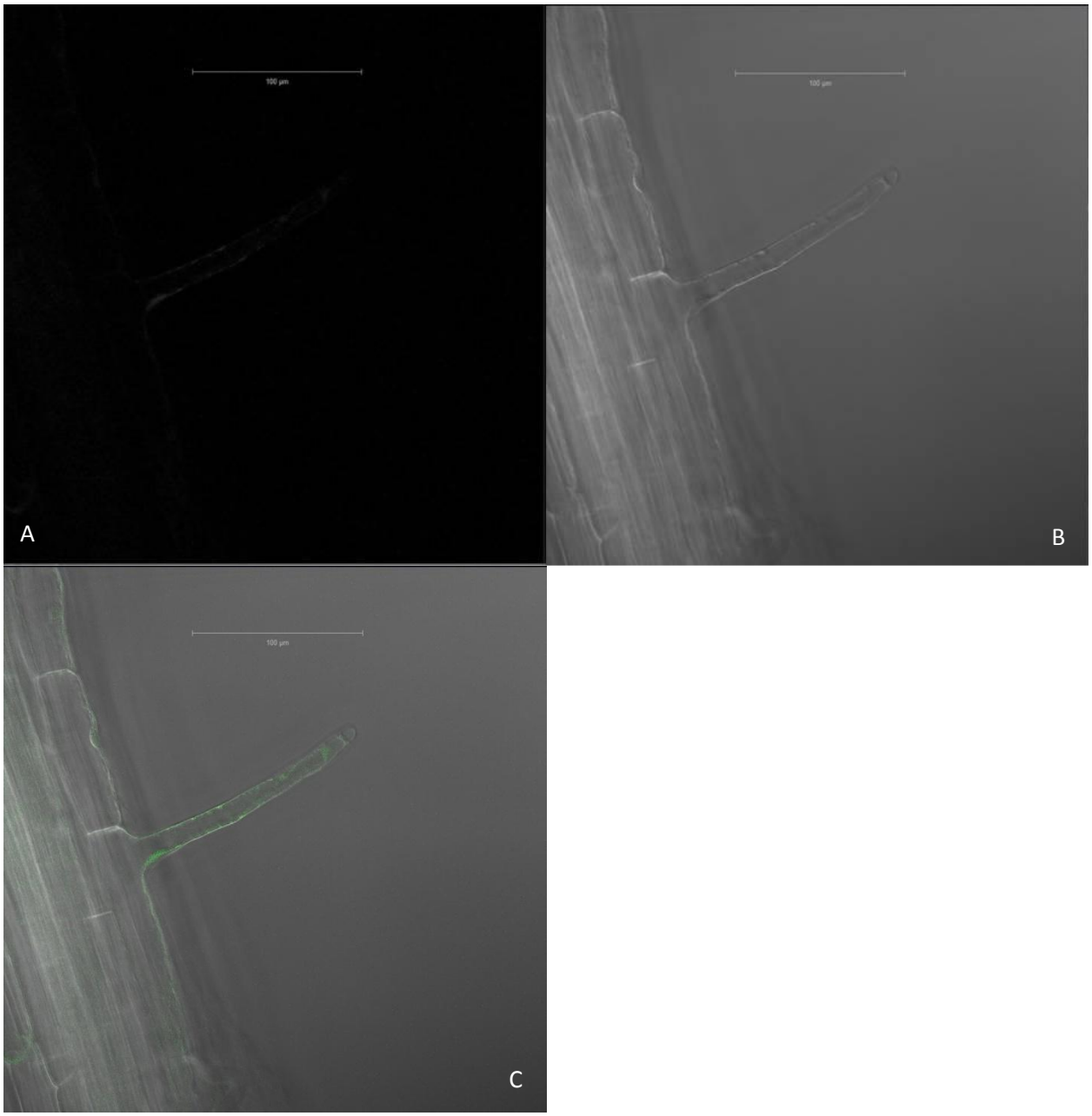

Figure 11. Mature emerging root hair from GFP:AtCaM3 line H64. **A.** GFP filter set. **B.** DIC image. **C.** Combined color image.

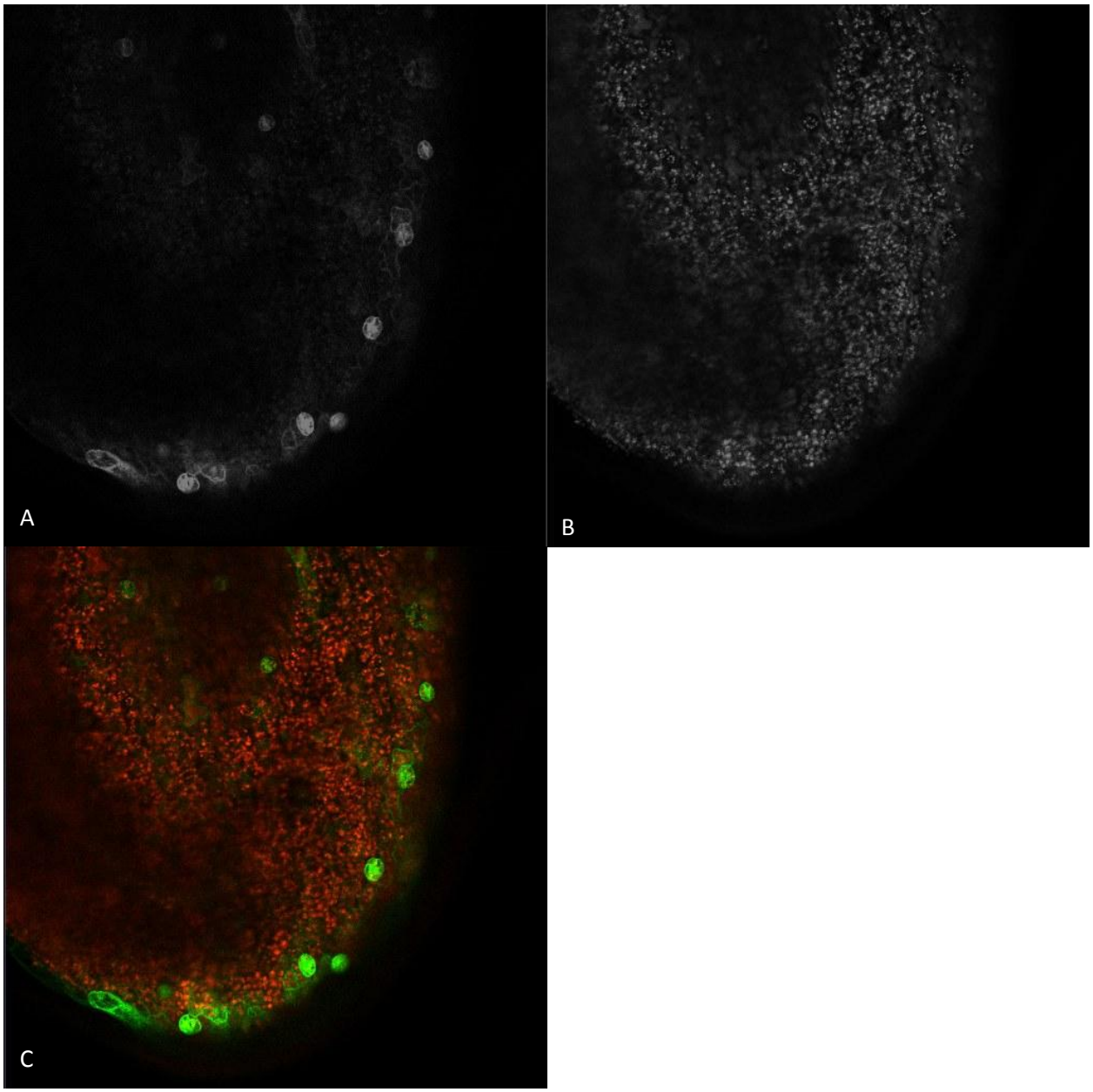

Figure 12. GFP:AtCaM3 line 452 cotyledon showing GFP localization in epidermis and guard cells. **A.** GFP filter set. **B.** Chlorophyll filter set. **C.** Combined color image.

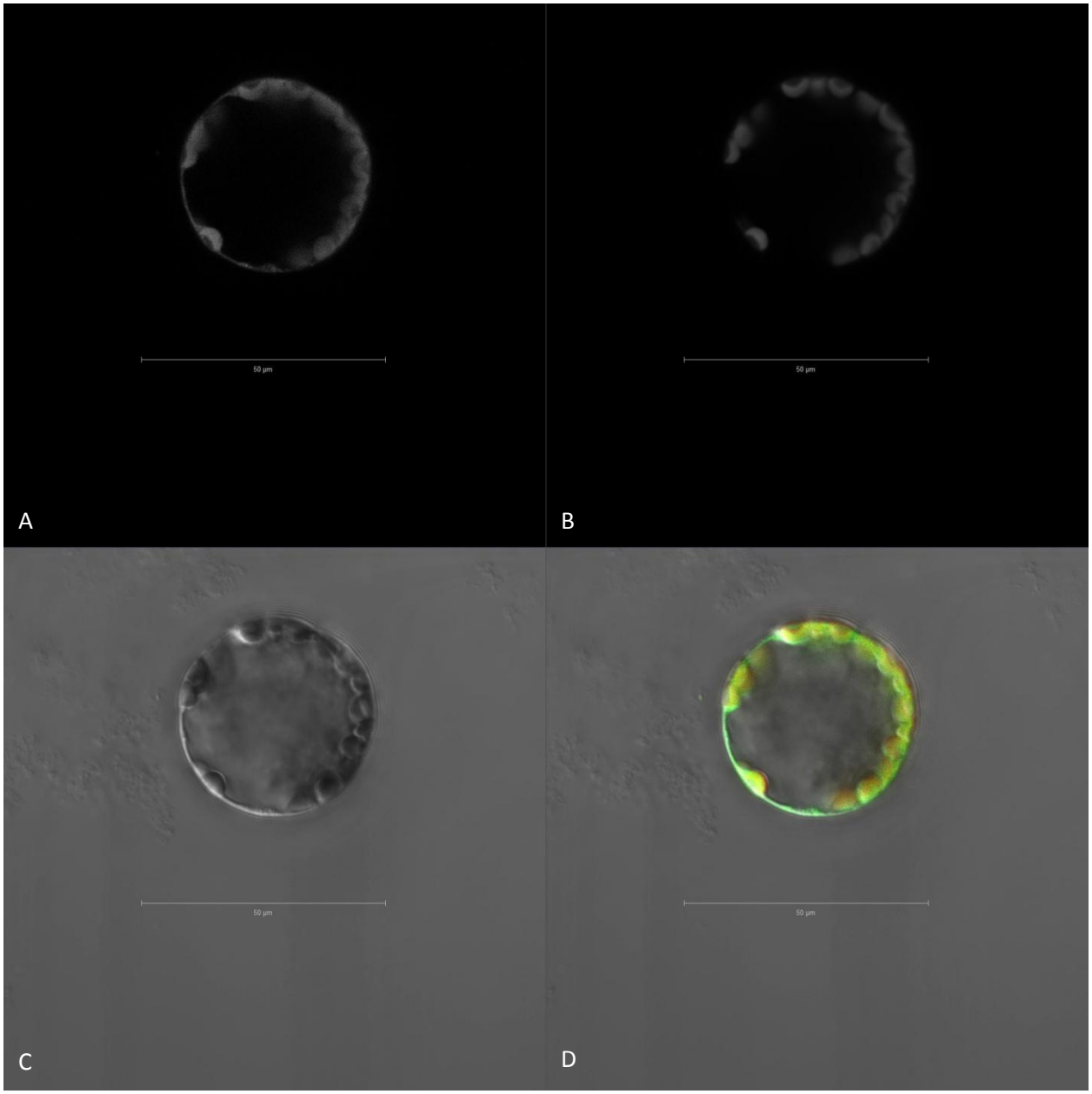

Figure 13. GFP:AtCaM3 line 452 leaf mesophyll protoplast. **A.** GFP filter set. **B.** Chlorophyll filter set. **C.** DIC image. **D**. Combined color image.

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