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Differential Sub-localization of Actin Variants within the Nucleus

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Abstract

Conventional actin has been implicated in various nuclear processes including chromatin remodeling, transcription, nuclear transport, and overall nuclear structure. Moreover, actin has been identified as a component of several chromatin remodeling complexes present in the nucleus. In animal cells, nuclear actin exists as a dynamic equilibrium of monomers and polymers. Actin binding proteins (ABPs) such as ADF/Cofilin and profilin play a role in actin import and export, respectively. However, very little is known about the localization and roles of nuclear actin in plants. In multicellular plants and animals, actin is comprised of an ancient and divergent family of protein variants. Here, we have investigated the presence and localization of two ancient subclasses of actin in isolated *Arabidopsis* nuclei. Although the subclass 1 variants ACT2 and ACT8 and subclass 2 variant ACT7 were found distributed throughout the nucleoplasm, ACT7 was often found more concentrated in nuclear speckles than subclass 1 variants. The nuclei from the *act2-1/act8-2* double null mutant and the *act7-5* null mutant lacked their corresponding actin variants. In addition, serial sectioning of several independent nuclei revealed that ACT7 was notably more abundant in the nucleolus than the subclass 1 actins. Profilin and ADF proteins were also found in significant levels in plant nuclei. The possible functions of differentially localized nuclear actin variants are discussed.

Keywords

Arabidopsis; nuclear actin; chromatin remodeling; ABPs; profilin; ADF/cofilin

INTRODUCTION

In vertebrates and angiosperms, an ancient actin gene family encodes several moderately divergent protein variants that are differentially expressed in various organs, tissues and/or cell types. Based on their phylogeny and expression pattern, these actin variants are grouped into two or more major classes: cytoplasmic and muscle actins in cordate animals and vegetative and reproductive actins in higher plants (Herman 1993; Kandasamy et al. 2007; Meagher et al. 2000; Tondeleir et al. 2009). Aside from some specialized functions for muscle actins, actins in most eukaryotic cells share a number of common cytoplasmic functions that collectively include roles in the movement of organelles like chloroplasts, mitochondria, peroxisomes and Golgi vesicles, wound plugging, cytokinesis, cell motility, cell signaling, and maintenance of cell-to-cell junctions, cell polarity, cell shape, and cell visco-elasticity (Hussey et al. 2006; McCurdy et al. 2001; Pollard and Cooper 2009; Staiger and Blanchoin 2006).

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There are early reports of presence of actin in the nuclei from the frog *Xenopus laevis* and slime mould *Physarum polycephalum* (Clark and Merriam 1977; Jockusch et al. 1974). Nuclear actin in both these organisms was found in high concentrations. While 75% of the *Xenopus* nuclear actin is extractable in a soluble form, the remainder forms a gel and stays stably associated with chromosomes and nucleoli, and hence is presumed filamentous (Clark and Merriam 1978; Clark and Rosenbaum 1979). Moreover, actin appears to be essential to the structural integrity of the giant frog nuclei (Bohnsack et al. 2006; Gall 2006; Morozova and Kiseleva 2008). Plant and animal nuclear actin is distributed both in a diffuse manner throughout the nucleus and is concentrated in speckles (Cruz and Moreno Diaz de la Espina 2009; Gieni and Hendzel 2009; Schoenenberger et al. 2005). Other than that found in giant frog nuclei, there are few and conflicting data supporting the presence of *bona fide* filamentous F-actin in most nuclei that have been examined. However, there is strong support from inhibitor studies and F-actin-binding protein interactions that some nuclear actin (e.g., speckles) exists in a polymeric form (Gieni and Hendzel 2009; McDonald et al. 2006; Pederson and Aebi 2005). The list of well-characterized monomeric and polymeric actin binding proteins once thought to be “cytoplasmic” but found more recently in the nucleus includes ADF/cofilin, profilin, RhoGap, spectrin, filamin, tropomyosin, actinin, myosin and paxillin, further implicating a spectrum of actin forms and functions in the nucleus (Abe et al. 1993; Dingova et al. 2009; Gieni and Hendzel 2009; Kandasamy et al. 2002; Ohta et al. 1989; Ruzicka et al. 2007).

Based on sub-localization, protein-protein interactions, and mechanistic studies, a few essential functions have been ascribed to nuclear actin (Bettinger et al. 2004; de Lanerolle et al. 2005; Gettemans et al. 2005; Miralles and Visa 2006; Visa 2005). Nuclear actin is a stoichiometric component of a majority of chromatin remodeling complexes via its binding together with a nuclear actin-related protein (e.g., ARP4, Baf53) to the helicase-SANT-Associated (HSA) domain of DNA dependent ATPase subunit of chromatin remodelers including SWI-SNF, SWR1 and INO80 or the HSA domain in the Vid21-related helicase subunits of chromatin modifiers like NuA4 HAT (Meagher et al. 2009; Szerlong et al. 2008). Actin is essential for the assembly of these large chromatin-altering machines. Actin appears to participate in transcriptional initiation both via direct interactions with RNA polymerase (Pol) I, II, and III and via activity in pre-messenger RNA complexes that prepare chromatin for transcription (Gieni and Hendzel 2009; Grummt 2006; Louvet and Percipalle 2009; Miralles and Visa 2006; Percipalle and Visa 2006). Actin is found in nucleoli and is suggested to participate in the translocation of ribosomal subunits from their site of assembly in the nucleolus through nuclear pores to the cytoplasm (Cisterna et al. 2006). In addition, actin is localized to plant and animal Cajal bodies, where it may position target genes like *U2* and play a role in the export of ribonuclear particles (RNPs) and small RNAs (Cruz and Moreno Diaz de la Espina 2009; Dundr et al. 2007; Gedge et al. 2005). The isolation of a nuclear actin conformation-specific monoclonal antibody 2G2 that reacts with nuclear, but not cytoplasmic actin in paraformadehyde fixed tissue, supported the idea that nuclear actin may be functionally and structurally distinct from cytoplasmic actin (Schoenenberger et al. 2005). Finally, nuclear actin appears to be dynamic in its activity(s), being concentrated in nuclei and condensed chromatin in resting human lymphocytes, and moving to the nucleoplasm and decondensed chromatin, when cells are transcriptionally activated (Kysela et al. 2005).

The model plant *Arabidopsis thaliana* encodes eight functional actins that based on their sequence phylogeny and expression patterns are grouped into two major classes, vegetative and reproductive, and into five subclasses as shown in Fig. 1A. The vegetative actins are strongly expressed in shoots, roots, inflorescences, seeds, sepals, microspores, and petals, whereas reproductive actins are expressed in mature pollen and/or ovules (An et al. 1996;McDowell et al. 1996a;Meagher et al. 1999). The *Arabidopsis* vegetative class has two

ancient and differentially expressed subclasses of actins, subclass 1 and 2. Subclass I ACT2 and ACT8 actins are more strongly expressed in older tissues and subclass 2 ACT7 protein is more strongly expressed in young and developing tissues and organs, and is regulated by hormones and environmental signals (An et al. 1996;Kandasamy et al. 2001;McDowell et al. 1996a;Meagher et al. 2000). The subclass 1 actins, ACT2 and ACT8 differ from each other by only a single amino acid, but the genes encoding them are saturated with silent nucleotide substitutions, since their divergence from a common ancestral sequence 30 to 40 million years ago (McDowell et al. 1996b). However, these two subclass 1 actins differ from the subclass 2 actin, ACT7, by 7% at the amino acid level, as much as either subclass differs from the reproductive class actins.

Null mutant alleles deficient in ACT7 (*act7-4, act7-5*) or a double mutant lacking ACT2 and ACT8 (*act2-1/act8-2*) have relatively normal aboveground plant stature. However, the ACT2/ACT8 double deficient plants are severely defective in root hair tip growth, because they cannot extend the bulges on root trichoblast cells into root hairs, and the ACT7-deficient plants have extreme dwarf root architecture with disordered root cell files and cellular polarity. Further, the aberrant ACT7 deficient roots do not initiate normal number of trichoblast cells (Gilliland et al. 2003; Kandasamy et al. 2009b). The root cells of both mutants show an altered pattern of F-actin distribution relative to wild type. Neither subclass 1 nor subclass 2 vegetative plant actins are known, hitherto, to be localized to the nucleus. Indirect evidence that plant actins make their way to the nucleus comes from the fact that the ACT7 polypeptide contains a sequence that is strongly homologous to the bipartite nuclear export signal (NES) in mammalian alpha actin, differing in only two of 24 amino acids (Wada et al. 1998), while the NESs in ACT2 and ACT8 differ by 5 amino acids from the animal sequence as shown in Fig. 1B. All three vegetative actins contain an extra leucine in the first leucine rich repeat relative to the mammalian sequence, which may enhance the nuclear export of plant actins.

The ancient divergence of the subclass 1 and 2 actins, the divergence between their NESs, and their distinct mutant phenotypes suggested that they might be differentially localized to the nucleus and/or have different nuclear functions. Published studies on the localization of plant actin in the nuclei are limited to examination of the giant nuclei of *Allium cepa* roots using broadly reactive anti-actin antibodies including the nuclear actin-specific antibody 2G2 (Cruz and Moreno Diaz de la Espina 2009). Herein, we have employed genetic and cell biological approaches as well as several general and actin subclass specific antibodies to examine the presence and differential distribution of the vegetative actin variants in the small leaf and root cell nuclei of *Arabidopsis*. To further demonstrate that the nuclear actin examined is not due to cytoplasmic actin contamination, we have prefixed the tissue with paraformaldehyde before isolating nuclei for immunolocalization. We report here that a number of novel anti-actin monoclonal antibodies identified actin in the nucleus, and the specificity of these reagents was further confirmed using nuclei isolated from actin-deficient mutant plants. We also show that the two ancient vegetative actin subclasses (subclass 1 and 2) have different nuclear localization phenotypes. However, when we target high levels of actin appended with a putative NLS (e.g. ACT7-NLS) into the nucleus, it forms intranuclear rods resembling cofilins-actin rods seen in aberrant animal cells. Finally, using plant profilin and ADF/cofilin subclass specific antibodies we have also found subsets of these ABPs in the nucleus.

MATERIAL AND METHODS

Antibodies and Reagents

To detect nuclear actin, the following anti-actin monoclonal antibodies were used: 2G2, an antibody that was raised against the profilin-actin complex and that specifically recognizes a

nuclear-specific epitope of actin observed in paraformaldehyde fixed samples (Gonsior et al. 1999; Schoenenberger et al. 2005). 2G2 serum (Antibody Facility, Braunschweig, Germany) may have weak affinity for plant actins, because it revealed weak reactivity on Western blots and poor immunolabeling of nuclear actin in whole cells; MAbGEa, a general antibody that was raised against *Arabidopsis* recombinant ACT1 and that reacts with all eukaryotic actins [(Fig. 1A) (Kandasamy et al. 1999) (#MA1-744, Thermo Scientific, Rockford, IL)]; MAbGPa, a general plant-actin specific antibody that was raised against purified *Arabidopsis* ACT8 and reacts with all plant actin variants [(Kandasamy et al. 1999) (#A0480, Sigma, St. Louis, MO)]; MAb13a, an actin-subclass specific antibody that was raised against purified *Arabidopsis* ACT2 and that reacts with subclass 1 and 3 actins [(Kandasamy et al. 2001) (#A0605, Sigma, St. Louis, MO)]; MAb2345a, an antibody raised against purified *Arabidopsis* ACT11 and that reacts with subclass 2, 3, 4 and 5 actins, but not subclass 1 actins (Kandasamy et al. 2001). The Threonine at amino acid position number 43, which is present in subclass 2, 3, 4, and 5 actins, but replaced with Histidine in subclass 1 actins, confers specificity to MAb2345a. Thus, in vegetative tissue, MAb2345a recognizes only ACT7 (Fig. 1A). Likewise, MAb13a reacts only with ACT2 and ACT8 in vegetative tissue. As positive control, MAbARP4a and MAbARP7a were used to localize the nuclear ARPs ARP4 and ARP7, respectively (Kandasamy et al. 2003). Two antibodies were used to examine either the nuclear distribution of profilin by immunocytochemistry or its level of expression by Western blot analysis: MAbPRF1, a monoclonal antibody, which recognizes specifically the constitutively expressed PRF1 variant and PAbPRF, a rabbit polyclonal antibody detecting all expressed *Arabidopsis* profilins (Kandasamy et al. 2002). An ADF antibody MAbADF4a, which reacts with seven of the eleven *Arabidopsis* ADF/cofilins representing subclass I and subclass II (ADF1, ADF2, ADF3, ADF4, ADF7, ADF10 and ADF11), was used to localize nuclear ADF proteins. In the nuclei isolated from leaf tissue, MAbADF4a can react only with the subclass I ADFs (ADF1-4; (Ruzicka et al. 2007). An FITC-conjugated anti-mouse antibody raised in sheep (#F6257, Sigma, St. Louis, MO) was used as the secondary antibody in immunolabeling experiments. Similarly, Horseradish Peroxidase linked anti-mouse whole antibody raised in sheep (#NA931V, GE Healthcare, UK) and anti-rabbit whole antibody raised in donkey (#NA934V, GE Healthcare, UK) were used as secondary antibodies for Western blotting.

Actin Mutants

To further validate the nuclear localization of specific actin variants, we used two *Arabidopsis* actin mutants: *act7-5*, a single mutant and *act2-1/act8-2*, a double mutant. *act7-5* is a newly characterized null mutant allele that has a T-DNA insertion in third exon of *ACT7* gene (Fig. 2A) and has no expression of ACT7 protein variant (Fig. 2B, upper panel). Moreover, the mutant plants have reduced levels of total actin compared to wild-type (Fig. 2B, lower panel). Similar to the previously characterized *act7-4* allele (Gilliland et al. 2003), *act7-5* seedlings are smaller with shorter roots than wild-type (Fig. 2C). The *act2-1/act8-2* plants fully lack in the expression of subclass 1 actins ACT2 and ACT8 (Fig. 2D, upper panel), but have same level of total actin as wild-type (Fig. 2D, lower panel), due to an up-regulation in the expression of ACT7 (Kandasamy et al. 2009b). The double mutant plants do not develop root hairs (Fig. 2E); otherwise they were identical to wild-type.

Generation of Plants Expressing the *A7P:ACT7-NLS* clone

To construct *A7P:ACT7-NLS* clone, the *ACT7* cDNA was amplified from a custom made flower library using the following primers: ACT7-NcoIS: 5′ tag tga acc ATG GCC GAT GGT GAG GAT ATT CAG CCA CTT 3′ and ACT7nlsBamHIA: 5′ gtc tag gga tcc TCA AAC CTT TCT CTT CTT CTT AGG ATG AAC AAC AGA *AGA ACC TGA TCC* GAA GCA TTT CCT GTG AAC AAT CGA 3′ (lower case letters correspond to the clamp, underlined represent NLS and italics represent the spacer). The NLS amino acid sequence

(Kim et al. 2005) is shown in Fig. 1C. The 1220 bp PCR product was then cloned into the *Nco*I and *Bam*HI sites of the *ACT7pt* vector in Bluescript. The new *A7P:A7-NLS* construct contains 1208 bp of *ACT7* promoter, a multi-linker and 375 bp of *ACT7* terminator sequence. This construct was moved from the Bluescript to pCambia-hyg vector with the restriction enzymes *Kpn*I and *Sac*I. The expression plasmid was mobilized into the *Agrobacterium tumefaciens* strain C58C1 and transformed into *act7-5* mutant plants by dipping floral buds in the infiltration medium (pH 5.7) containing the *Agrobacterium*, MS salt, 5% sucrose and 0.02% Silwet L-77. Mutant plants were used for transformation to avoid interference from endogenous ACT7, while visualizing nuclear ACT7-NLS localization by fluorescence microscopy.

Protein Gel Blot Analysis

Profilin and actin proteins from wild-type and different mutant (*act7-5, act2-1/act8-2 and prf1-3*) seedling shoot samples were detected on Western blots using the methods described previously (Kandasamy et al. 2007). 10% and 12% SDS-polyacrylamide gels were used to resolve actin and profilin proteins, respectively. Proteins on immunoblots were detected with suitable primary and secondary antibodies (see above).

Isolation of Nuclei

Shoot and root tissue (~1 g) from two- to three-week-old wild-type, single mutant (*act7-5, prf1-3*) or double mutant (*act2/act8*) seedlings, which were grown on soil or on MS medium at 21 °C with 16 h light and 8 h dark periods, were used for isolation of the nuclei. To avoid contamination with cytoplasmic actins, the tissue samples were pre-fixed with 0.5% or 4% paraformaldehyde in 50 mM PIPES buffer (pH 7.0) containing 5 mM EGTA, 1 mM MgSO4, 0.5% casein and 0.05% Triton X-100 for 1 h. Following washing with phosphate buffered saline (PBS: 10 mM sodium phosphate, pH 7.0, 150 mM NaCl) three times for 10 min, the samples were thoroughly blotted and frozen in liquid nitrogen. The frozen tissue was ground into a fine powder and mixed with 5 ml of 25 mM sodium phosphate buffer (pH 7.5) containing 0.44 M sucrose, 10 mM magnesium chloride, 0.1% Triton X-100 and the protease inhibitor cocktail (Roche, Germany). After a brief homogenization (3 × 30 sec bursts with a Polytron probe), the sample was first filtered through two layers of Miracloth and then through 50 μm nylon mesh. The filtrate in a 15 ml falcon tube was then centrifuged at 2000g for 10 min. The supernatant and a small soft layer of chloroplasts were carefully removed from the top of the firm gray nuclear pellet. The nuclear pellet was then resuspended in 1 ml of the same nuclear isolation buffer and transferred to an Eppendorf tube. This sample was centrifuged at 200g for 2 min to further get rid of the smaller organelles. After gently removing the supernatant, the nuclear pellet was resuspended in 1 ml of the same phosphate buffer mix. Finally, after centrifuging at 200g for 2 min, the pellet was re-suspended in 200 μl of PBS containing the protease inhibitor cocktail. All the above steps were carried out at 4 °C. The crude nuclear prep was then transferred to chrom-alum coated slides, air dried and stored at 4 °C or used immediately for immunolocalization. For Western blot analysis of actin in the nuclear fraction, the final pellet was resuspended in sample buffer (30 μl for nuclei from 1g tissue), boiled for 5 min and loaded onto the gel. Similarly, actin in the cytoplasmic fraction was assayed by mixing equal volume of the supernatant from the first step with 2x sample buffer and the mixture was boiled and loaded.

Immunocytochemistry

The prefixed nuclei on slides were permeabilized with 0.5% Triton X-100 in PBS for 30 min, washed in PBS twice for 10 min, and incubated with 0.1M Glycine in PBS to quench the aldehyde groups remaining from formaldehyde fixative. After washing again in PBS, the nuclei were blocked in TBST-BSA (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20 and 2.5% BSA) for 30 min and labeled with different primary antibodies in the blocking

solution for 3 h. Following washing with PBS (3 × 10 min), the slides were incubated with FITC conjugated anti-mouse secondary antibody in TBST-BSA (1:100 dilutions) for 2 to 3 h. The nuclei on slides were washed twice for 5 min, and then incubated with DAPI (4′-6 diamidino-2-phenylindole) at a concentration of 0.2 μg/ml of PBS for 10 min. After washing again in PBS twice for 5 min, the slides were mounted in 80% glycerol in PBS containing 1 mg/ml p-phenylenediamine and then examined under a Leica DM6000B model fluorescence microscope using the 100× oil immersion lens. Images from serial or single median optical sections were recorded using suitable filters and with SimplePCI Software (Compix Inc., Sewickley, PA). For comparison, nuclear samples were also observed under a Leica SP2 confocal microscope using a 60× water immersion objective lens. Although both methods revealed clear localization and differential distribution of nuclear actin variants, we consistently obtained good signal with the Leica fluorescence microscope and hence used it throughout the study. At least five different nuclei from two independent preparations were utilized during serial sectioning. For the images collected in each experiment, the same gain, offset, exposure and contrast parameters were used. The Z-series images were taken at 0.2 μm intervals from the bottom to the top of each nucleus and without deconvolution the images were then transferred to a Macintosh computer, identically cropped, and equally adjusted for contrast using Adobe Photoshop (Adobe System, San Jose, CA). For negative control, slides were processed as described above except lacking the primary antibody and labeled only with the secondary antibody. Slides containing nuclei isolated from mutant plants served as other negative controls for immunolabeling of the two subclasses of vegetative actins and PRF1.

Immunocytochemical localization of actin, ARPs and profilin in whole cells was carried out using paraformaldehyde fixed samples as described previously (Kandasamy et al., 2003). To reveal the diverse shapes of the *Arabidopsis* plant nuclei in different tissue (see Supplemental Fig. 1), cryofixed and freeze substituted or chemically fixed tissues were stained with DAPI and observed under the Leica fluorescence microscope.

RESULTS

Immunolabeling Identifies Actin in Isolated *Arabidopsis* Nuclei

Despite growing evidence that actin is involved in multiple functions in the vertebrate nucleus and the demonstrated proof that actin is an essential component of various complexes, which control chromatin remodeling in yeast and mammals, there is still very little evidence for the presence or activity of nuclear actin in plants. The only clear example where actin has been shown to be localized to the plant nucleus is in isolated giant onion nuclei (Cruz and Moreno Diaz de la Espina 2009). On the other hand, several divergent ARPs, which are the other cytoskeletal proteins that serve as integral components of chromatin remodeling complexes, have clearly been shown to be concentrated in the nucleus of *Arabidopsis* and other dicot plants (Deal et al. 2005; Kandasamy et al. 2009a; Kandasamy et al. 2003). To examine the presence of actin in the nucleus of *Arabidopsis* cells, we tested various monoclonal antibodies that were raised against plant actin variants. We used the nuclear actin conformation specific antibody 2G2 as a positive control. We employed isolated nuclei for immunolocalization, because labeling of whole leaf cells with the plant actin antibodies revealed such intense cytoplasmic actin staining that it usually obscured staining in nuclei (Fig. 3A and B). Moreover, even labeling with 2G2 antibody did not resolve nuclear actin staining in intact plant cells (Fig. 3C and D). To avoid cytoplasmic actin contamination of nuclei during their isolation, we prefixed the tissue with paraformaldehyde before disrupting the cells. Thus, cross-linking by the fixative should exclude diffusion of any cytoplasmic actin into the nucleus during nuclear preparation. DAPI staining of the nuclei prepared from seedling tissue revealed various nuclear morphologies ranging from circular to sickle shaped nuclei (Supplemental Fig. 1H). This is

not due to fixation artifact, because cryofixed or paraformaldehyde fixed whole cells also showed the same range and distribution of nuclear morphologies depending upon the type of the tissue (Supplemental Fig. 1A-G).

Labeling of nuclei isolated from *Arabidopsis* leaf tissue with the general plant actin specific MAbGPa revealed positive staining for actin throughout nucleoplasm (Fig. 3H-J). The staining pattern with MAbGPa was very similar to that with 2G2 antibody, which is supposed to recognize only the nuclear form of actin in fixed samples (Fig. 3E-G). Moreover, MAbGEa, an antibody that reacts with all eukaryotic actins, also recognized actin in the isolated nuclei (Fig. 3K-M). The intensity of nuclear actin staining with all these three antibodies was almost identical, however, considerably less compared to nuclear ARP4 staining with MAbARP4a (Fig. 3N-P). This weaker actin staining was not surprising, because nearly all the APR4 protein or other nuclear ARP proteins (e.g. ARP7) present in an interphase cell is concentrated in the nucleus (Fig. 3T and U). Labeling with the secondary antibody alone revealed no significant nuclear staining (Fig. 3Q-S), which suggests that the positive staining observed with all these anti-actin antibodies represents actin protein present in the nucleus.

To further demonstrate that actin is present in the nucleus, we performed Western blot analysis of the nuclear fraction from wild-type as well as the *act2-1 act8-2* double mutant with the nuclear actin-specific 2G2 antibody, which identified a distinct ~45 kD band in the nuclear, but not cytoplasmic fraction (Fig. 4, top panel). The actin band identified in the nuclear fraction of the double mutant represents ACT7. The general anti-plant actin antibody MAbGPa, however, detected actin in both fractions (Fig. 4, middle panel). Coomassie Brilliant Blue staining of an identical gel revealed that the cytoplasmic and nuclear fraction lanes contained adequate and similar levels of total protein (Fig. 4, bottom panel). Based on the total recovery of protein in both the samples from a specific amount of tissue and the intensity of the protein bands as detected with MAbGPa, the nuclear protein represents less than approximately 1/250 of the total cytoplasmic actin.

Subclass 1 and Subclass 2 Actins are Differentially Distributed in the Nucleus

Immunolabeling and the Western blot analysis of isolated leaf nuclei with the conformation specific, nuclear actin antibody 2G2 and the general actin antibody MAbGPa (or MAbGEa) clearly suggested that actin is present in *Arabidopsis* nuclei. Next, we wanted to examine whether the seedling leaf cells that express only subclass 1 and subclass 2 vegetative actins contain one or both subclasses of actin variants in their nuclei. For this experiment, we employed antibodies that were actin variant specific and nuclei that were isolated from mutant plants deficient in the expression of one or the other of the two subclasses of actins. For instance, we immunolabeled nuclei from *act7-5* mutant seedlings (see Materials and Methods) that are null for the expression of ACT7 (subclass 2) protein with MAb2345a antibody recognizing ACT7, but not ACT2 or ACT8, in seedling leaf tissue. The *act7-5* nuclei labeled with this antibody showed no significant staining above background (Fig. 5D-F) demonstrating that there is no ACT7 in the mutant nuclei. However, this MAb2345a antibody showed clear staining for ACT7 in wild-type nuclei labeled in parallel experiments (Fig. 5A-C). In contrast, the *act7-5* nuclei labeled with MAb13a, an antibody that recognizes ACT2 and ACT8 (subclass 1), but not ACT7 in vegetative tissues, revealed obvious actin staining (Fig. 5G-I). We found actin labeling in interphase nuclei, but not in dividing nuclei containing condensed chromosomes (see the arrows in Fig. 5G-I). This distribution was very similar to the distribution of nuclear ARPs in dividing cells lacking a nuclear membrane (Kandasamy et al. 2003). Thus, the *act7-5* interphase nuclei have ACT2 and ACT8 variants, but no ACT7 protein.

To demonstrate the presence of ACT7 variant in the seedling nuclei, we isolated nuclei from *act2/act8* double mutant plants that are deficient for the expression of ACT2 and ACT8 proteins. When nuclei from these double mutant plants were labeled with MAb13a recognizing ACT2 and ACT8 in vegetative tissue, there was no staining (Fig. 5M-O). However, when the *act2/act8* nuclei from the same preparation were labeled with MAb2345a that recognizes only ACT7 in vegetative tissue, there was distinct positive staining (Fig. 5P-R). Thus, the *act2/act8* double mutant nuclei contain ACT7, but no ACT2 or ACT8 variant. On the other hand, the wild-type nuclei labeled with MAb13a revealed nuclear staining (Fig. 5J-L) suggesting the normal presence of ACT2 and ACT8 variants in the seedling leaf nuclei.

Our immunolabeling of wild-type nuclei with either MAb13a (Fig. 5J-L) or MAb2345a (Fig. 5A-C) suggested that both subclasses of vegetative actins (ACT2 and ACT8 of subclass 1, and ACT7 of subclass 2) were detected in seedling leaf nuclei. This result was corroborated by evidence from labeling experiments with mutant nuclei deficient in the expression either one of the two subclasses of actin variants. However, surprisingly, we observed distinct differences between the nuclear labeling of ACT2 and ACT8 with MAb13a and ACT7 with MAb2345a. Although labeling for both antibodies was observed evenly distributed throughout the leaf nuclei, the subclass specific staining of ACT7 (Fig. 6D-F) showed a superimposed punctate pattern not seen for ACT2 and ACT8 (Fig. 6A-C). The ACT2 and ACT8 staining was diffuse, while ACT7 was more frequently concentrated in nuclear speckles. Moreover, ACT7 was evenly distributed throughout the leaf nucleus, including the nucleolus, whereas ACT2 or ACT8 staining in the nucleolus was very weak (Fig. 6A-F).

Moreover, in the nuclei isolated from root tissue, we observed clear staining with the general actin antibody MAbGEa (Supplemental Fig. 2A and B) and no staining with secondary antibody alone (Supplemental Fig. 2G and H), again suggesting that actin is present in *Arabidopsis* nuclei. In addition, immunolabeling of root nuclei also supported the possible difference in the distribution of the two subclasses of actins: ACT7 showing more punctate and nucleolar staining (Supplemental Fig. 2E and F; also see Fig. 7Y-A5), while ACT2 and ACT8 showing clear diffuse nucleoplasmic and poor nucleolar staining (Supplemental Fig. 2C and D; also see Supplemental Fig. 3V-A1). Thus, examination of nuclei from leaf or root tissue suggests the universal presence of both classes of vegetative actin variants in the nucleus, but in a different distribution pattern.

In order to prove that the ACT7 rich speckles were present throughout the nucleoplasm, we performed serial optical sectioning of the nucleus from leaf (Fig. 7A-X) and root (Fig. 7Y-A5) samples. The speckles were present in all optical sections, thus confirming their presence even deep into the nucleus. Also, the nucleolar staining of ACT7 is clearly evident through the optical sections, especially in the root cells that contain a large nucleolus (Fig. 7Y-A5). The intranuclear actin speckles were clearly distinguished from the remnants of the nuclear basket still attached to the nuclear membrane (Fig. 7A6). Usually in plant cells, the nuclei and other large organelles such as chloroplasts are surrounded by a tight basket of actin microfilaments (Kandasamy and Meagher, 1999). Due to the presence of actin filaments attached to the surface of significant number of isolated fixed nuclei (25 ± 5% in 0.5% paraformaldehyde fixed sample and 35 ± 5% in 4% paraformaldehyde fixed sample), it was hard to avoid low levels of contamination from cytoplasmic F-actin during Western blot analysis of nuclear extracts. This was true, when using all actin antibodies other than 2G2. However, to keep actin filament contamination in the nuclear preparations to the minimum, we generally fixed tissue samples with low levels of paraformaldehyde (0.5%).

Moreover, we performed serial sectioning of leaf (Supplemental Fig. 3A-U) and root (Supplemental Fig. 3V-A1) nuclei stained with MAb13a for ACT2 and ACT8. The results of this experiment confirmed the diffuse and less punctate staining pattern for subclass 1 actin proteins throughout the nucleus and their poor staining in the nucleolus compared to ACT7. The two subclasses of vegetative actins, therefore, revealed distinct subnuclear distribution.

Localization of Profilins and ADFs to the Nucleus

The presence of actin in the nucleus was long considered a contamination from the cytoplasm due to the lack of phalloidin F-actin staining. However, now it is very clear from studies in animals (Gonsior et al. 1999; Pendleton et al. 2003; Rando et al. 2000) and recent research from plants (Cruz and Moreno Diaz de la Espina 2009) and the present study that the nucleus contains actin in monomeric and perhaps polymeric (speckles) forms. G-actin is on the edge of the exclusion limit of the nuclear pore complex, but can enter the nucleoplasm by diffusion, as actin does not have any distinct nuclear localization signals (Gieni and Hendzel 2009). However, actin contains *bona fide* nuclear export signals (see Fig. 1B) suggesting it requires active export from the nucleus. In addition to the possible role of diffusion, actin binding proteins such as ADF/Cofilin and profilin have also been shown to play a role in the import and export of animal cell nuclear actin, respectively (Abe et al. 1993; Gieni and Hendzel 2009; Pendleton et al. 2003). Profilin mediated actin export requires exportin-6, which recognizes actin/profilin complexes (Stuven et al. 2003). Thus, localization of ADFs and profilins to the plant nucleus is anticipated, as has been shown earlier with whole cells (Kandasamy et al. 2002; Ruzicka et al. 2007). We previously localized the two strongly expressed constitutive profilins PRF1 and PRF2 together in whole *Arabidopsis* root cells using MAbPRF12a that recognizes both variants (Kandasamy et al. 2002). Herein we specifically examined the distribution of the PRF1 variant in dissociated root apical cells and isolated leaf nuclei using the PRF1 specific antibody MAbPRF1a (Kandasamy et al. 2002) and a new mutant allele of *PRF1*, *prf1-3*. The *prf1-3* null mutant allele contains a T-DNA insertion in the protein coding sequence of first exon of *PRF1* (Fig. 8A) and qRT-PCR analysis revealed that the mutant plants contained less than 10% of *PRF1* transcripts (not shown). Western blot analysis demonstrated that this allele is completely lacking in the expression of PRF1 protein (Fig. 8B). However, the mutant plants were morphologically similar to the wild-type (Fig. 8C).

Immunolabeling of wild-type root cap cells showed that PRF1 protein is diffuse, but highly concentrated in the nucleus and thus the nuclear staining is significantly above the level of cytoplasmic staining (Fig. 8D-F). However, in the files of root cells isolated from the apex, the level of nuclear PRF1 staining is the same or only marginally above the cytoplasmic staining (Fig 8G). As expected, the *prf1-3* mutant root cells showed no staining in the cytoplasm or nucleus (Fig. 8H). To further confirm the presence of PRF1 in the nucleus, we isolated leaf nuclei from wild type and mutant plants and labeled them with the PRF1 variant specific antibody. As shown in Fig 8I-K, the MAbPRF1a-labeled wild-type nuclei revealed positive staining for PRF1 protein. Similar to the *prf1-3* root cells (Fig. 8H), the nuclei isolated from *prf1-3* mutant leaves also revealed no detectable staining with MAbPRF1a (Fig. 8L-N).

Moreover, we wanted to see whether the isolated leaf nuclei also contain ADF proteins. Therefore, we labeled them with the ADF antibody MAbADF4a, which reacts only with the subclass I ADFs (e.g., ADF1, ADF2, ADF3 and ADF4) in leaf tissue, and recognized the nuclear form of ADFs in leaf cells (Ruzicka et al. 2007). We observed strong staining of the nuclei with this antibody(Fig. 9D-F). However, unlike total actin staining (Fig. 9A-C), which was highly punctate, the ADF staining was diffuse throughout the nucleus. Thus,

actins and the ABPs (profilin and ADF), which may be involved in nuclear actin dynamics and translocation in and out of the nucleus, are all present in *Arabidopsis* nuclei.

Cells Expressing High Levels of ACT7-NLS Form Intranuclear Rods

Although actin was detected easily in isolated *Arabidopsis* nuclei with different antibodies, it was still hard to visualize nuclear actin in intact cells, unlike the nuclear ARPs. This may be due the presence of significantly higher levels of actin in the cytoplasm and lower levels actin in the nucleus. Alternatively, a large portion of the nuclear actin may be in a different conformation (e.g., 2G2 reactive actin) such that we can't detect it with general actin antibodies. In fact our rough estimation of total actin in the nuclear and cytoplasmic fractions, based on western blot analysis, suggested that the nuclear to cytoplasmic ratio of actin is less than 1: 250. The ratio of volumes of the nucleus to cytoplasm is similarly 1:250 to 1:500, suggesting the nuclear concentration of actin is not particularly low. The overwhelming amount of cytoplasmic actin staining may simply obscure the small amount of nuclear actin staining in intact cells (Fig. 3A and B). Even the nuclear actin specific 2G2 antibody did not show clear nuclear staining over background in intact plant cells (Fig. 3C and D).

To determine whether expression of higher levels of actin in the nucleus can improve the nuclear staining in intact cells, we appended ACT7 with a C-terminal NLS tag (see Fig. 1C) and expressed the *A7P:A7-NLS* construct in the *act7-5* mutant background. Interestingly, we were able to detect nuclear ACT7-NLS in the intact cells of mutant plants expressing the transgene (Fig. 10A-G). The untransformed mutant cells revealed no nuclear staining (Fig. 10H and I). However, expression of high levels of ACT7-NLS resulted in the formation of intranuclear actin rods both in the leaf (Fig. 10A-E) and root cells (Fig. 10F and G). Some transformed cells with lower ACT7-NLS levels still did not reveal clear nuclear staining (see Fig. 10D). Also, overexpression of ACT7 alone without the NLS tag (*A7P:A7*) in the mutant background did not improve nuclear staining of actin in whole cells (not shown). Thus, actin is normally expressed and maintained at low levels in nucleus of wild-type cells.

DISCUSSION

Actin is one of the most abundant and highly conserved proteins in eukaryotic cells. Although actin has long been recognized for its multiple prominent roles in the cytoplasm (Staiger and Blanchoin 2006), only very recently its involvement in many aspects of genetic and epigenetic regulation including mRNA processing, chromatin remodeling and global gene regulation and other nuclear functions has become evident (Gieni and Hendzel 2009; Pederson 2008). The notion of actin occurring as a constant component of the nucleus was first apparent in animals and now from the present study on *Arabidopsis* nuclei and the recent work on onion nuclei (Cruz and Moreno Diaz de la Espina 2009), it is very clear that the plant nuclei also contain actin as an integral component. We confirmed the nuclear localization of plant actin with the conformation specific nuclear actin monoclonal antibody 2G2 (Schoenenberger et al. 2005) as well as several monoclonal antibodies with different specificities that we raised against plant actins. The functional diversity of actins in multicellular eukaryotes is often reflected in the diversity of the gene family that encodes them, with the most divergent family members being differentially expressed in different tissues and organs. Thus, for example, the eight *Arabidopsis* actins are grouped into vegetative and reproductive classes (Kandasamy et al. 2007; Meagher et al. 2000). The vegetative class is further divided into two ancient and highly divergent subclasses encoding three actin variants. In this study, we have shown that the relatively divergent subclasses of vegetative actin variants are localized throughout the nucleoplasm. But, we observed some difference between the two subclasses with regard to their intranuclear distribution by using actin subclass specific antibodies. ACT7, which constitutes subclass 2, is more concentrated

in speckles in the nucleoplasm and in addition is abundant in the nucleolus. On the other hand, subclass 1, which includes ACT2 and ACT8, is in small particles or more diffuse throughout the nucleoplasm, and is relatively less abundant in the nucleolus.

The two vegetative actin subclasses encoding the three different variants (ACT2 and ACT8 of subclass 1 and ACT7 of subclass 2) are the only actins expressed in all organs and tissue of the predominant sporophytic phase of the plant life cycle. While ACT2 and ACT8 are strongly expressed in mature organs and fully differentiated cell types, ACT7 is predominant in young organs and differentiating tissue and in addition is regulated in response to hormones and several environmental signals. Mutation in *ACT7* affects cell diffuse growth and polarity, whereas lack of *ACT2* and *ACT8* expression in *act2/act8* double mutants completely blocked tip growth in root hairs (Kandasamy et al. 2009b). Moreover, the two ancient subclasses are phylogenetically highly divergent differing by more that 7% at the amino acid level. For comparison, human cytoplasmic beta actin is 6% divergent from human smooth muscle or cardiac muscle actins and the cytoplasmic and muscle actins appear not to have shared common ancestry for several hundred million years. Thus, it is not surprising that the two subclasses of plant vegetative actins might reveal some difference in their sub-nuclear distribution. These two subclasses may thus be involved in different nuclear functions. For example, they may be constituent of different chromatin remodeling complexes that epigenetically control global gene regulation or different subsets of transcriptional complexes. Also, ACT7, but not ACT2 or ACT8, may be more involved in nucleolar actin functions such as remodeling rRNA encoding chromatin or the transport of ribosomal protein precursors for ribosomal subunit assembly. In particular, the nucleolar organizer remodeling complex, NoRC, is actin dependent and contains the Swi/Snf related protein Smarca5 (Santoro et al. 2002). ACT7 may serve as the actin subunit for NoRC assembly binding to the HSA domain along with the plant nucleolar specific actin-related protein ARP8 (Kandasamy et al. 2008).

For the first time, actin subclass-specific antibodies were used to examine mutants deficient in distinct actin variants to prove the existence of different vegetative actin variants in the nucleus. Also, our use of mutants and subclass specific antibodies eliminated any possibility of artifacts from the cross reactivity of the antibody with any protein other than the target actin. The high concentrations of G-actin in the cytoplasm of most eukaryotic cells create the very real possibility of its diffusion into nuclei during the disruption of cells and isolation of nuclei. The pre-fixation of plants with paraformaldehyde before the nuclear isolation essentially eliminated the possibility of cytoplasmic actin contamination and thus substantiated the existence of actin in plant nucleus.

Moreover, our study confirms previous reports on the presence of profilins and ADF/cofilins in plant nuclei (Kandasamy et al. 2002; Ruzicka et al. 2007). The actin monomer binding protein profilin and the actin monomer and polymer binding protein ADF/cofilin are essential for the regulation of actin dynamics in the cytoplasm (Staiger and Blanchoin 2006) and because actin occurs in dynamic equilibrium of monomeric, dimeric, trimeric, and polymeric forms (McDonald et al. 2006), theses two ABPs may also be involved in the nuclear actin dynamics. Moreover, profilin is utilized as a cofactor in nuclear export of actin (Stuven et al. 2003) and is also found in the nuclear interior specifically associated with nuclear compartments related to transcription, including nuclear speckles and Cajal bodies (Skare et al. 2003). The localization of profilin to areas of active transcription could create a local pool of activated G-actin required for efficient polymer formation (Goldschmidt-Clermont et al. 1992) and thereby have a role in transcriptional regulation (Wu et al. 2006; Yoo et al. 2007). ADF/cofilin appears to be at the heart of plant actin filament turnover in the cytoplasm by mediating filament severing (Staiger et al. 2009), but it is sometimes present in the nucleoplasm at levels even higher than in the cytoplasm. ADF/cofilin also can

act as a transporter molecule and help in the import of actin into the nucleus (Chhabra and dos Remedios 2005; Pendleton et al. 2003). This nuclear role for ADF/cofilin is supported by the fact that large amounts of actin accumulate in the form of rods in the nucleus when cultured muscle cells are disrupted with dimethyl sulfoxide, and cofilin, which has a nuclear localization signal, is present in a high ratio in the actin rods (Ono et al. 1993). Actin by itself is not known to contain a nuclear localization signal. Moreover, treatment of non-muscle cells with latrunculin or ATP depletion also leads to cofilin-mediated translocation of ß-actin into the nucleus (Pendleton et al. 2003). Finally, pull-down experiments using tagged ADF/coflin and profilin in human cells identified Brg1 and Baf170 Swi/Snf-related subunits of SWI/SNF BAF chromatin remodeling complex as interacting proteins (Zhao et al. 1998). These results suggest a possible direct but unknown role for ADF/cofilins and profilins in controlling chromatin structure in addition to binding monomeric actin.

We observed strong immunostaining of actin in the form of intranuclear rods, when we expressed high levels of ACT7 appended with an NLS. Therefore, the difficulty in visualizing actin in the nuclei of intact cells was not likely due to the absence of actin or altered actin conformation, but due to the presence of low levels of actin within a strongly staining cytoplasm, which became easily detectable once we isolated the nuclei. We assume that the rod-like structures are formed due to the lack of sufficient levels of ABPs in the nucleus to sequester higher levels of actin. On the other hand, the addition of NLS to the C-terminus of actin might have interfered with its function, resulting in the aggregation of non-functional actin into rods. Various stress conditions and mutant forms of actin are also known to produce nuclear actin rods in animal cells, via the enhanced import of cofilin-actin complexes (Abe et al. 1993; Domazetovska et al. 2007; Nishida et al. 1987). By partaking in nuclear import and export of actin, the nuclear ADF/cofilin and profilin proteins undoubtedly help in maintaining critical levels of wild type actin in the nucleus.

In conclusion, we show that vegetative class actin, profilin, and ADF/cofilin variants are present in fixed and isolated *Arabidopsis* nuclei that are relatively free from cytoplasmic G-actin contamination. We illustrate that a wider variety of monoclonal antibodies, apart from the previously characterized nuclear actin-specific 2G2, detect actin present in the *Arabidopsis* plant nucleus. One of these, MAbGEa, is known to react with actin isolated from all eukaryotes, and should provide a second universal reagent for nuclear actin studies. Using vegetative actin subclass 1- or subclass 2-specific monoclonal antibodies and *Arabidopsis* mutants lacking in the expression of one or the other actin subclass, we have further confirmed the specificity of nuclear actin identification and demonstrated that ACT7 has different pattern of nuclear distribution from ACT2 and ACT8. The different molecular activities of the various subclasses of actin and ABPs in the nucleus, and the proteins and protein complexes that may be co-localized in ACT7 speckles remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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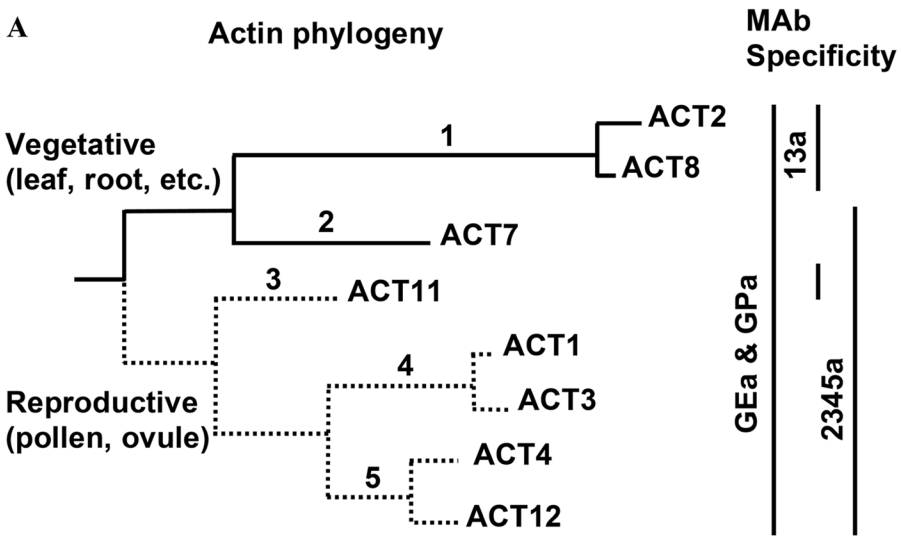

B

	NES1	NES2
Rr aACTIN	ALPHAIMRLDLA --	DIKEKLCYVALD
At ACT7	ALPHAI**L**RLDLA --	DIKEKL**A**YVALD
At ACT2	**S**LPHAI**L**RLDLA --	DIKEKL**SF**VA**V**D
At ACT8	**S**LPHAI**L**RLDLA --	DIKEKL**SF**VA**V**D

C

	C Term ACT7
ACT7	...IVHRKCF
	C Term ACT7-Spacer-NLS
ACT7-NLS	...IVHRKCF- GSGS -SVVHPKKKRKV

Fig. 1.

Actin family of *Arabidopsis*. (**A**) Actin protein sequence tree depicting the two major classes (Vegetative and Reproductive) and five (1–5) subclasses of actin variants. The specificities of different anti-actin monoclonal antibodies to the various protein variants are shown to the right. (**B**) Comparison of the bi-partite nuclear-export signal sequence among diverse actins. Rat α-actin is shown first followed by the three *Arabidopsis* vegetative class actins. The first and last amino acids of the ACT7, ACT2 and ACT8 bipartite sequences are A172 or S172 and D224, respectively. Amino acid differences from the rat sequence are shown in bold. (**C**) Sequence of the nuclear localization signal (NLS) and the spacer appended to the C-terminus of the ACT7 protein.

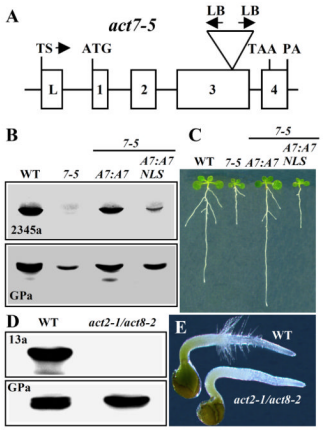

Fig. 2.

Mutants deficient in the expression of one of the two subclasses of vegetative actins. (**A**) A map of *act7-5* allele. The T-DNA insertion in exon 3 causes a 10 pb deletion in *ACT7* gene sequence and the mutant allele encodes a truncated protein missing 87 amino acid at the 3′ end, but includes 22 amino acid T-DNA sequence before the stop codon TAA (exons are boxes, introns and flanking sequence are lines, TS transcription start site, ATG initiation codon, PA polyadenylation site, LB left boarder of T-DNA). (**B and C**) Morphological and molecular phenotypes of ACT7-deficient line (*7–5*) and the mutant line complemented with an *ACT7* cDNA under the control of *ACT7* regulatory sequences (*7-5/A7:A7*) or with the *ACT7* transgene appended at the C-terminus with a signal sequence encoding a putative NLS (*7-5/A7:A7 NLS*). (B) Western blots probed either with MAb2345a for ACT7 protein (top panel) or with MAbGPa for total actin. Because amino acid Threonine 43 is the epitope recognized by MAb2345a, it could detect the C-terminal truncated protein encoded by the mutant allele, if present. (C) Eight-day-old seedlings. Note the transgene encoding ACT7 but not ACT7-NLS complemented the retarded root growth mutant phenotype. (**D**) Actin expression in wild-type (WT) and *act2-1/act8-2* double mutant shoot samples. Top panel was probed with MAb13a for ACT2 and ACT8 and the bottom panel was probed with MAbGPa for total actin. (**E**) 36-h-old seedlings. Note the complete lack of root hairs in the double mutant.

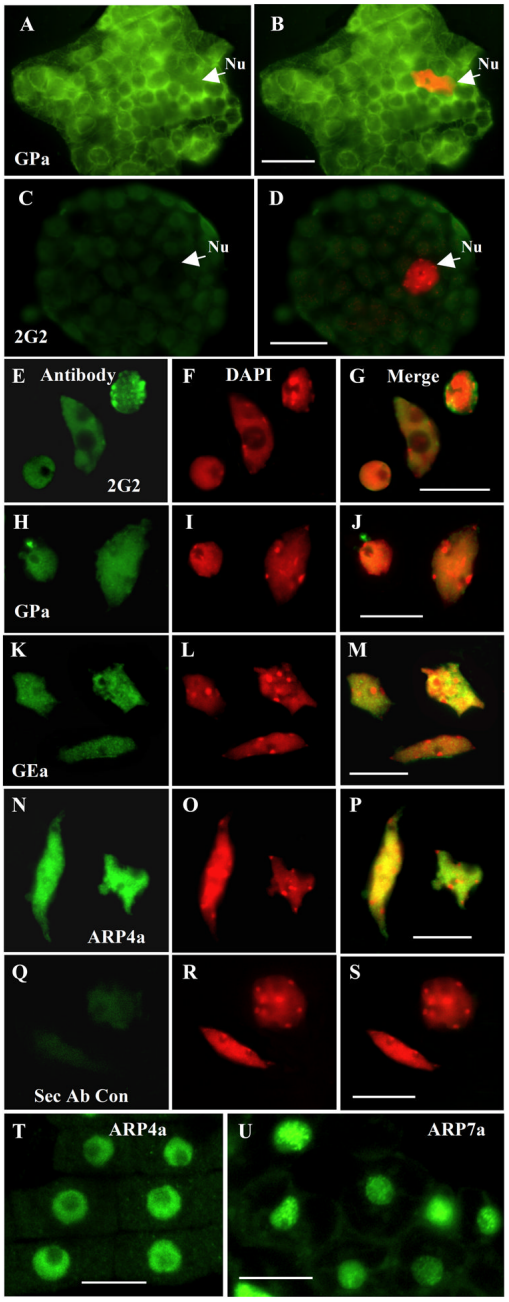

Fig. 3.

Localization of actin in isolated nuclei with diverse anti-actin antibodies. (**A-D**) Paraformaldehyde fixed whole leaf mesophyll cells labeled with the general plant actin monoclonal antibody MAbGPa (A and B) or the conformation specific nuclear actin antibody 2G2 (C and D). (A, C) Actin antibody staining. (B, D) Actin with DAPI co-staining for DNA. Arrows point to the nucleus (Nu). (**E-P**) Isolated leaf nuclei labeled with actin or ARP antibodies. (E-G) Nuclear actin-specific antibody 2G2. (H-J) General plant actin antibody MAbGPa. (K-M) General eukaryotic actin antibody MAbGEa. (N-P) Nuclear ARP4-specific antibody MAbARP4a. (**Q-S**) Control nuclei labeled only with FITC-conjugated secondary antibody. Column 1: antibody stained images (green); Column 2: DAPI images (red); Column 3: merged images (orange to yellowish-green suggests co-

localization of both fluorophores). (**T**) Root cells labeled with ARP4-specific antibody MAbARP4a. (**U**) Young leaf cells labeled with nuclear ARP7-specific antibody MAbARP7a. Scale bars −25 μm in A-D, 10 μm in E-U.

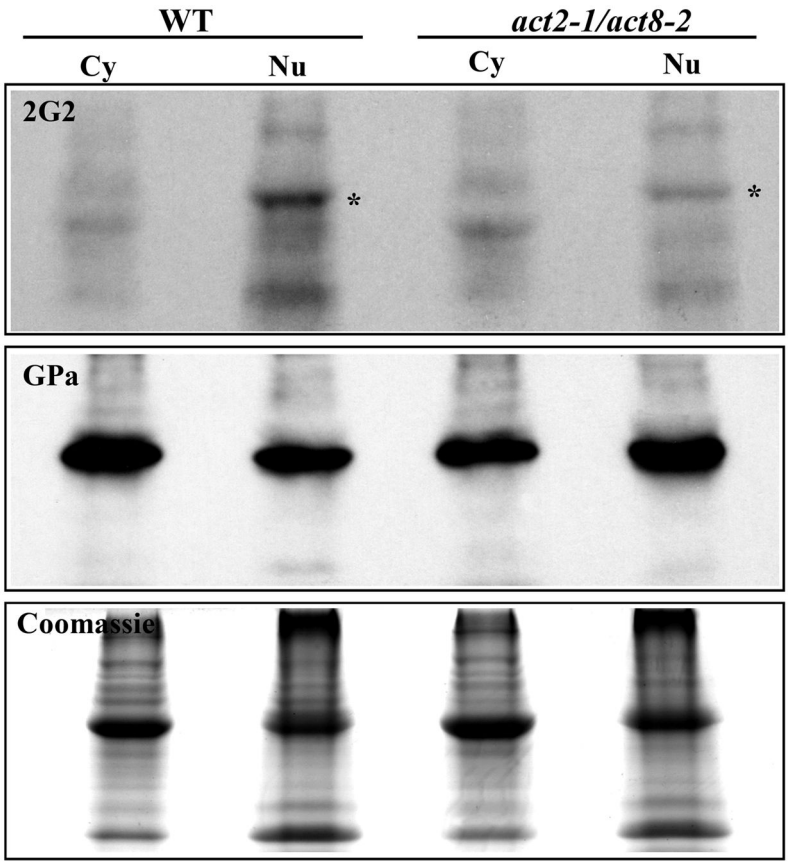

Fig. 4.

Western blot analysis of actin in the cytoplasmic and nuclear enriched fractions of wild type (WT) and *act2-1/act8-2* double mutant seedlings. Top panel was probed with 2G2 antibody and the middle panel probed with MAbGPa. Coomassie Brilliant Blue image of an identical gel is shown in the bottom panel. MAb2G2 detected about 45 kD actin band (*) in the nuclear (Nu) but not cytoplasmic (Cy) fraction, whereas MAbGPa detected similar sized bands in both fractions.

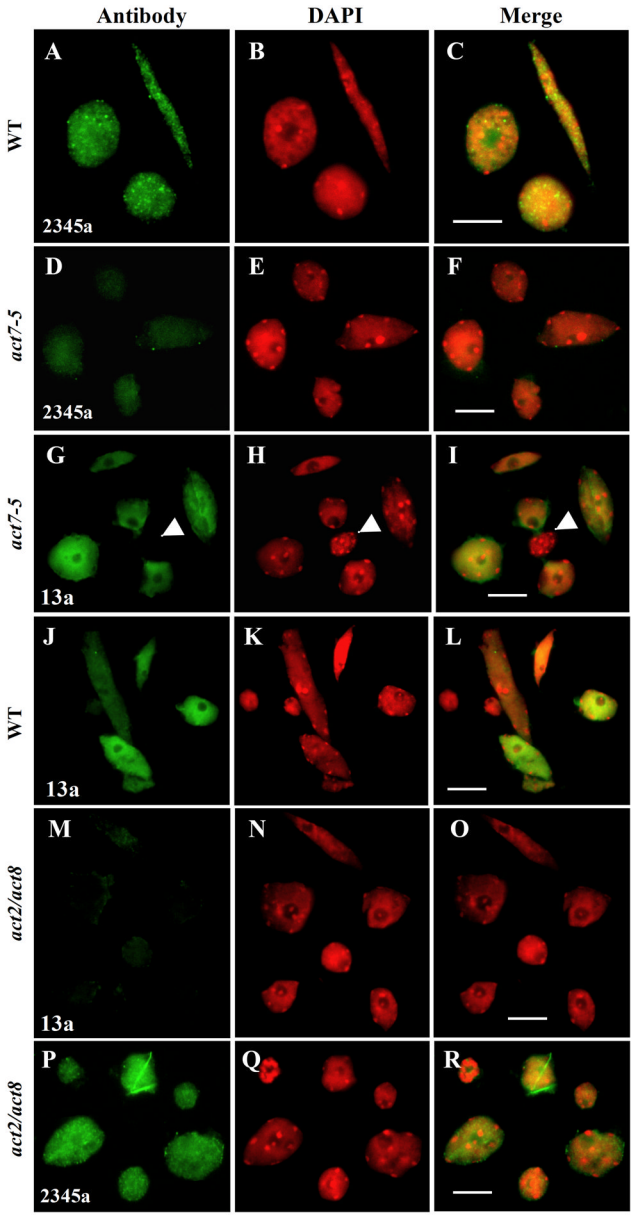

Fig. 5.

Localization of the subclass 1 and subclass 2 vegetative actin variants in isolated nuclei. (**A-C**) Wild-type nuclei labeled with MAb2345a for ACT7 (subclass 2) variant. (**D-F**) *act7-5* mutant nuclei labeled with MAb2345a for ACT7 variant. (**G-I**) *act7-5* mutant nuclei labeled with MAb13a for ACT2 and ACT8 (subclass 1) variants. Arrowheads point to a mitotic nucleus with condensed chromosomes. (**J-L**) Wild-type nuclei labeled with MAb13a for ACT2 and ACT8 variants. (**M-O**) *act2/act8* double mutant nuclei labeled with MAb13a for ACT2 and ACT8 variants. (**P-R**) *act2/act8* double mutant nuclei labeled with MAb2345a for ACT7 variant. Column 1: antibody stained images; Column 2: DAPI images; Column 3: merged images. Scale bars −10 μm.

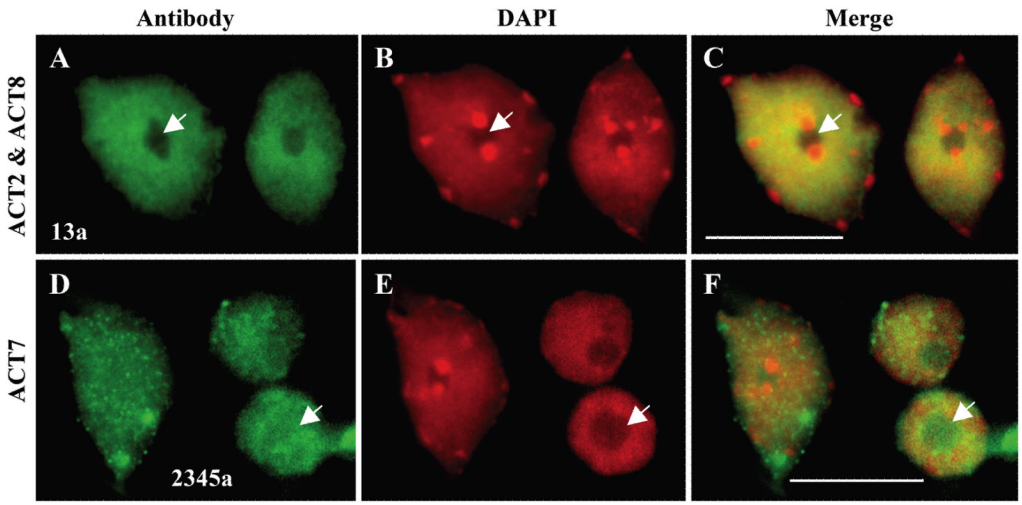

Fig. 6.

Differential sub-nuclear distribution of subclass 1 and subclass 2 variants.

(**A-F**) Leaf nuclei. (A-C) MAb13a labeling of ACT2 and ACT8. (D-F) MAb2345a labeling of ACT7. Column 1: antibody stained images; Column 2: DAPI images; Column 3: merged images. Arrows point to nucleolus. Scale bars −10 μm.

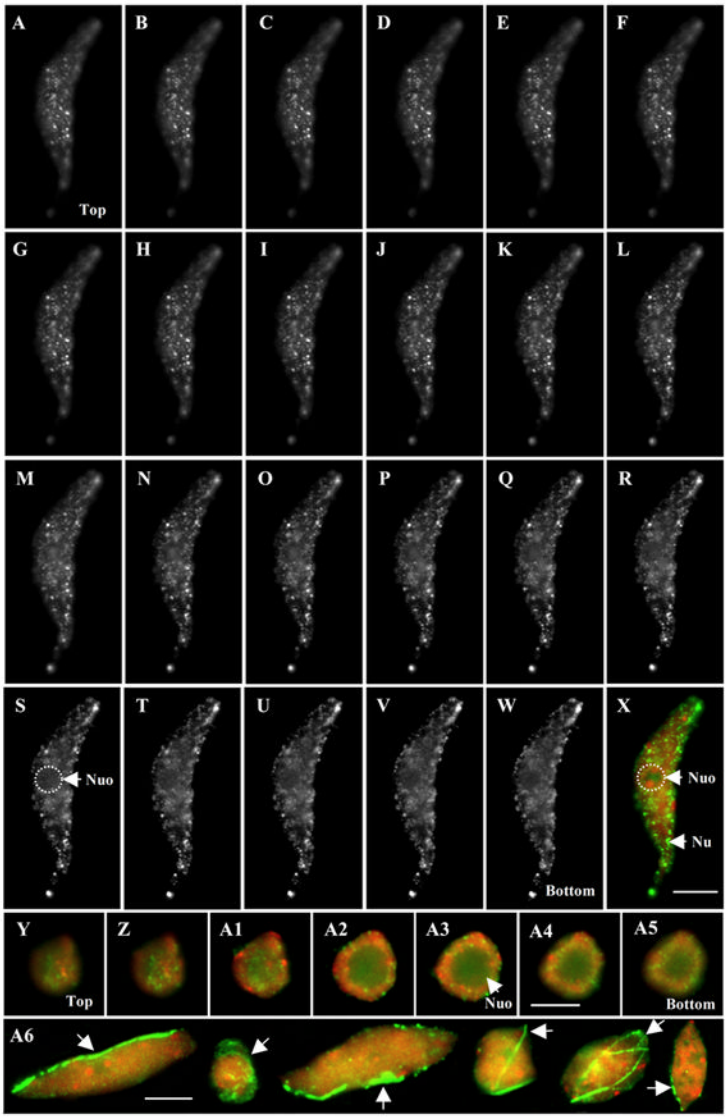

Fig. 7.

ACT7-rich nuclear speckles are present throughout nucleoplasm. (**A-W**) 0.2 μm optical sections through different regions of an isolated, sickle-shaped leaf nuclei labeled with MAb2345a for ACT7. (**X**) A merged optical image of nuclear ACT7 (green) and DNA (DAPI, red) staining of the same nucleus shown in A-W. (**Y-Z**, **A1-A5**) Merged optical images (ACT7 in green and DNA in red) at different regions of a round-shaped root nucleus. (**A6**) Merged images (actin and DNA) of nuclei showing part of nuclear actin basket of microfilaments still attached to the nuclear surface (arrows), as found in a subset of fixed isolated nuclei. Nu: nucleus; Nuo: nucleolus. Scale bars −5 μm.

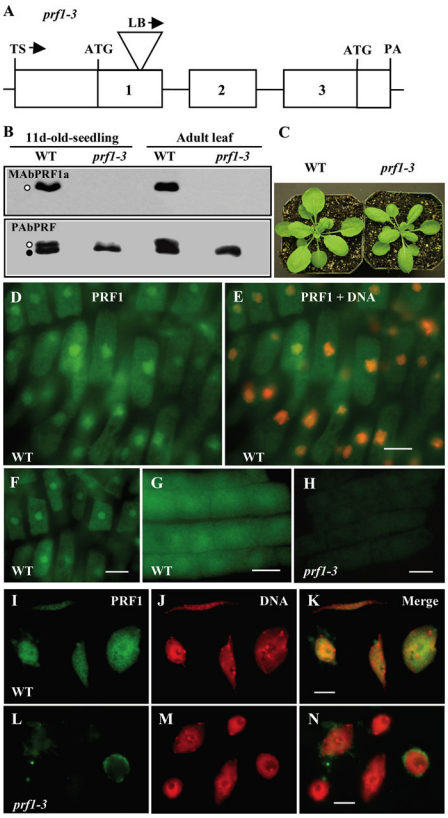

Fig. 8.

Expression and subcellular distribution of the PRF1 variant. (**A**) A map of *prf1-3* mutant allele with a T-DNA insertion after codon 20 in exon 1 (see legend to Fig. 2 for annotation). (**B**) Western blot analysis of PRF1 protein levels in the wild-type (WT) and *prf1-3* mutant line. Top panel is probed with the monoclonal antibody MAbPRF1a recognizing specifically PRF1 and the bottom panel is probed with the polyclonal antibody PAbPRF recognizing all five *Arabidopsis* profilins. In wild-type vegetative tissue, this polyclonal antibody reacts with all three constitutive profilins, PRF1, PRF2 and PRF3. (**C**) 4-week-old wild-type and *prf1-3* mutant plants exhibiting almost identical morphology. (**D-H**) Immunolabeling of wild-type (D-G) and *prf1-3* mutant (H) root cells with MAbPRF1a. (D-F) Root cap cells. (G and H) Files of root apical cells. (**I-N**) Immunolabeling of wild-type (I-K) and *prf1-3* mutant (L-N) isolated seedling nuclei labeled with MAbPRF1a. Antibody staining is shown in green and the DNA staining with DAPI is shown in red. E, K and N represent merged images of both staining. Scale bars −10 μm in D-H and −5 μm in I-N.

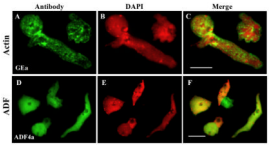

Fig. 9.

Localization of ADF/cofilins in isolated leaf nuclei. (**A-C**) Actin labeled with MAbGEa for positive control. (**D-F**) Nuclei labeled with MAbADF4a for the four subclass I ADF variants (ADF1, ADF2, ADF3 and ADF4). Column 1: antibody staining; Column 2: DAPI staining; Column 3: merged images. Scale bars −10 μm.

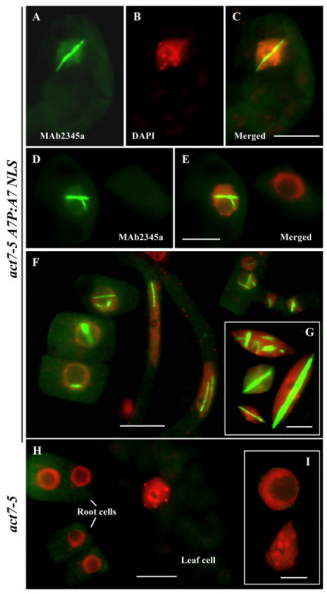

Fig. 10.

Targeting of ACT7 appended with a putative NLS to the nucleus induces formation of intranuclear actin rods. (**A-G)** Immunolabeling of leaf (A-E) or root cells (F) and dissociated seedlings nuclei (G) from *act7-5* mutant plants transformed with *A7P:A7 NLS.* All samples were labeled with MAb2345a for ACT7 protein. C and F-G are merged images of actin (green) and DNA (red) staining. (**H** and **I**) Immunolabeling of root and leaf cells (H), and the nuclei (I) of untransformed *act7-5* mutant plants for ACT7 protein. Note the absence of any actin staining in the nucleus. Scale bars −10 μm in A-F and H, and −5 μm in G and I.