Isoform-specific Subcellular Localization among 14-3-3 Proteins in *Arabidopsis* Seems to be Driven by Client Interactions[□]

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In most higher eukaryotes, the predominantly phosphoprotein-binding 14-3-3 proteins are the products of a multigene family, with many organisms having 10 or more family members. However, current models for 14-3-3/phosphopeptide interactions suggest that there is little specificity among 14-3-3s for diverse phosphopeptide clients. Therefore, the existence of sequence diversity among 14-3-3s within a single organism begs questions regarding the *in vivo* specificities of the interactions between the various 14-3-3s and their clients. Chief among those questions is, Do the different 14-3-3 isoforms interact with different clients within the same cell? Although the members of the *Arabidopsis* 14-3-3 family of proteins typically contain highly conserved regions of sequence, they also display distinctive variability with deep evolutionary roots. In the current study, a survey of several *Arabidopsis* 14-3-3/GFP fusions revealed that 14-3-3s demonstrate distinct and differential patterns of subcellular distribution, by using trichomes and stomate guard cells as *in vivo* experimental cellular contexts. The effects of client interaction on 14-3-3 localization were further analyzed by disrupting the partnering with peptide and chemical agents. Results indicate that 14-3-3 localization is both isoform specific and highly dependent upon interaction with cellular clients.

INTRODUCTION

The 14-3-3s are a family of acidic, soluble proteins with a native dimeric size of ~60 kDa. They bind phosphoproteins (as well as some nonphosphorylated proteins) and effect changes in the client proteins. Those changes can vary from inactivation to activation of the enzymatic activity of the client, the degradation or protection from degradation of the client, and the movement of the client from one cellular location to another (Bachmann *et al.*, 1996; Muslin and Xing, 2000; Dougherty and Morrison, 2004). In most eukaryotes, the 14-3-3s are represented by a fairly large family of gene products. *Arabidopsis* has 12 expressed 14-3-3 genes that produce characterized protein (Ferl, 1996; Wu *et al.*, 1997b; Rosenquist *et al.*, 2000; DeLille *et al.*, 2001). Humans have seven (Yaffe, 2002). *Drosophila* has only two (Chang and Rubin, 1997; Kockel *et al.*, 1997), but alternative splicing of the gene that encodes the ζ isoform (*leo*) contributes to additional 14-3-3 protein diversity (Philip *et al.*, 2001). Within the families of individual species and even among diverse members of the eukaryota, the 14-3-3 proteins are highly conserved in many areas of the molecule, but variation does exist, especially within the termini and certain

smaller internal regions (Ferl, 1996; Fu *et al.*, 2000; Sehnke *et al.*, 2002).

One possible reason for isoform diversity is simply to ensure fundamental 14-3-3 presence in all cell types where 14-3-3 function is required. Under the extreme form of this model, the diversity of 14-3-3 sequences would be a result of genetic drift within the coding regions while various promoter elements were captured and altered to ensure expression of 14-3-3 gene products in certain cell types. The core 14-3-3 function would not be altered in this scenario, and those 14-3-3s, although diverse in sequence, would be homogenous in function. In seeming support of this notion, there is clear evidence for cell- and tissue-specific expression of 14-3-3s in both animals and plants (Daugherty *et al.*, 1996; Testerink *et al.*, 1999; Qiu *et al.*, 2000; Han *et al.*, 2001; Philip *et al.*, 2001; Subramanian *et al.*, 2001; Aitken, 2002; Fulgosi *et al.*, 2002; Maraschin *et al.*, 2003; Alsterfjord *et al.*, 2004; van Hemert *et al.*, 2004; Qi *et al.*, 2005; Wijngaard *et al.*, 2005).

Another possible reason for isoform diversity is that different isoforms interact with different protein clients, and isoform diversity is a reflection of the coevolution of the sequences within the phosphoprotein client and the specific 14-3-3s with which it interacts. Under the extreme form of this model, the diversity of 14-3-3 sequences would be a result of selection for specific interactions with specific clients. The core 14-3-3 function, the binding of phosphoproteins, would be altered in that the various 14-3-3s within an organism would be differentiated by the specific clients or client sets with which they associate.

These two possibilities set the stage for a simple test of *in vivo* 14-3-3 client specificity based on the subcellular localization characteristics of 14-3-3/green fluorescent protein (GFP) fusions. If all 14-3-3s interact with the same clients, then various 14-3-3/GFP fusions will all similarly localize

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Abbreviations used: GFP, green fluorescent protein.

within a given cell type. If each 14-3-3 isoform has a specific and unique client set, then the subcellular localization for each isoform/GFP fusion will be different.

MATERIALS AND METHODS

Plant Material

Arabidopsis plants (*Arabidopsis thaliana* 'Wassilewskija') were transformed through vacuum infiltration (Bechtold and Pelletier, 1998) with fusion proteins composed of the coding region of various 14-3-3 isoforms coupled to GFP (S65T) and driven by the CaMV35s promoter (Sehnke *et al.*, 2002). The isoforms investigated include κ , λ , ω , and ϕ . Between four and six plant lines of each transformant were screened by visual inspection before selecting a single line for each isoform for subsequent analyses. Although there are some differences in the intensity of expression among lines, the patterns of subcellular distribution of 14-3-3 isoform/GFP expression are consistent among lines. In addition, a positive control consisting of CaMV35s/GFP alone was used for comparison. Plants were grown horizontally in plates on nutrient agar (Paul *et al.*, 2001) and were typically 2 weeks old when they were used for analyses.

Microscopy and Photography

Plants were examined with an Olympus BX51 fluorescent microscope coupled to an Evolution MP cooled charge-coupled device camera with Q-capture 2.60 software (Quantitative Imaging, Burnaby, British Columbia, Canada). For trichomes, photos were taken through the 20 $\times$ objective and captured with no binning. The specific region of interest was subsequently cropped from the resulting large (14.2-MB) digital file. Images of nuclei and guard cells were similarly completed using the 40 $\times$ objective. Slide samples were prepared in water or phosphate-buffered saline-Tween (PBS-T) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ plus 0.1% Tween), depending on the application. For Figures 2 and 3, leaves were cut from the living plants just before mounting on the slide in water. For Figures 5–7, the slides were prepared for the in situ incubation of leaf sections on the slide with biochemical reagents or were prepared with excised leaves that had been incubated in a given reagent for 1–4 h in a separate Microfuge tube (below).

Quantification of Relative GFP Expression in Trichomes

Digital images (TIF format) of single trichomes were opened in Bio-Rad's Quantity One program, and the Volume Tools software was used to establish relative amounts of GFP signal within sections of the trichome image. The pixel volume integration of the nuclear region alone was divided by total pixel volume integration of an area within the body of the trichome to find the proportion of the signal that resides in the nucleus. Because portions of the trichome exit the focal plane, this calculation does not represent percentage of nuclear 14-3-3/GFP in any strict sense (van Hemert *et al.*, 2004), but it does represent a comparison of relative nuclear localization within the body of the trichome. Five trichomes from 14-3-3- κ /GFP plants and five from 14-3-3- ϕ /GFP plants were used in the analysis. The values for each isoform are presented in Figure 3e as averages with standard deviations indicated as error bars.

Biochemical Treatments

In all cases, the plant material was prepared fresh (as described above) and either treated in situ on a microscope slide or incubated in the given solution in a 1.5-ml microcentrifuge tube.

5-Aminoimidazole-4-carboxamide Ribonucleoside Monophosphate (AICAR). Leaves were excised from the plant, the midvein was removed with a scalpel, and the leaf sections were placed on slides flooded with 10 mM AICAR in water. Trichomes were photographed at ~1-h intervals (Man and Kaiser, 2001).

Peptide Competition. Leaves were excised from the plant, the midvein was removed with a scalpel, and the leaf sections were incubated in Microfuge tubes containing either the peptide R18 (R18 peptide, PHCVPRDLWLDEANMCLP) (Wang *et al.*, 1999) or a peptide composed of the carboxyl-terminal region of 14-3-3 ν (UpC peptide, CPKEVQKVDEQAQPPPSQ) (Sehnke *et al.*, 2001). Both peptides used at a concentration of 4 μ g/ml in PBS-T. As with the AICAR treatments, trichomes were photographed at ~1-h intervals. Leaves incubated in PBS-T alone were indistinguishable with those incubated in water (our unpublished data).

Native Pore Gradient Gel Analyses

Leaves from 2-wk-old transgenic *Arabidopsis* plants expressing the 14-3-3/GFP fusions were extracted in 1 $\times$ native sample buffer (50 mM Tris, pH 6.8) in ice at a ratio of 100 μ l of buffer for every 200 mg of leaf tissue. A motor-driven homogenizer was used to grind the tissue into a slurry in a Microfuge tube, and then the slurry was centrifuged at 12,000 $\times$ g at 4°C to

Figure 1. Resolution of 14-3-3/GFP fusion proteins on native, pore-gradient gel electrophoresis provides evidence of both homo- and heterodimers. Each numbered lane shows the resolution of a different isomeric form of the fluorescing 14-3-3/GFP fusion proteins. The highest molecular weight band in each lane represents the 14-3-3/GFP homodimer. The set of lower molecular weight bands represent heterodimers composed of one 14-3-3/GFP molecule and one native 14-3-3 molecule. The band size within that lower set is dependent on the specific native isoform associated with that heterodimer. Lane 1, 14-3-3- κ /GFP; lane 2, 14-3-3- λ /GFP; lane 3, 14-3-3- ω /GFP; lane 4, 14-3-3- ϕ /GFP; and lane 5, GFP. Fluorescently labeled molecular weight markers composed of ferridoxin (440-kDa), catalase (232-kDa), bovine serum albumin (BSA) dimer (132 kDa), and BSA (66 kDa) flank the 14-3-3/GFP lanes.

remove debris. Extracts were resolved with nondenaturing acrylamide electrophoresis (Wu *et al.*, 1997a) with the modification that the gel was prepared with a pore gradient (5–15% acrylamide). The resultant gel was scanned on a Bio-Rad Molecular Imager FX at 488 nm and rendered into a digital image with the Bio-Rad FX software.

RESULTS

Biochemical Status of 14-3-3/GFP Fusion Proteins in Plant Cells

Native gel electrophoresis was used to survey the expression, size, and dimerization status of the 14-3-3/GFP fusions within plants transformed with the fusions. Native protein extracts from the leaves of transgenic *Arabidopsis* expressing the 14-3-3/GFP fusions were subjected to native pore gradient gel electrophoresis. The green fluorescence image of the resulting gel is shown in Figure 1. Comparison of the GFP fusion leaf extracts (lanes 1–4) with flanking molecular weight markers indicates that the all four 14-3-3/GFP fusions produced singlet major bands in the range of 130 kDa and a small family of bands in the general range of 90–100 kDa. Extracts from plants expressing GFP alone produce a band of ~50 kDa. These data indicate that the GFP signal present in the transgenic 14-3-3/GFP fusion plants is a sole and direct result of GFP that is fused to 14-3-3 with little or no cleavage of the GFP signal away from the fusion protein.

In all 14-3-3 fusion extracts, the GFP signal is in protein complexes that are in molecular weight multiples that are consistent with 14-3-3 homodimers and heterodimers, but not with 14-3-3/GFP monomers. The bands near 150 kDa in the 14-3-3/GFP fusion lanes are consistent with predicted molecular weights of 14-3-3/GFP homodimers, whereas the bands in the 90- to 100-kDa range are consistent with the predicted sizes of 14-3-3/GFP fusions heterodimerized with endogenous 14-3-3 protein. Monomer fusions of 14-3-3s to GFP should be in the range of 50–60 kDa, and no bands

Figure 2. Subcellular distribution of 14-3-3/GFP fusions shows isoform specific patterns of localization. Green indicates fluorescence of the fusion proteins, whereas any red or yellow is a contribution of the natural fluorescence of chlorophyll at 488 nm. 14-3-3- κ /GFP is largely nuclear in trichomes and guard cells (a, f, and k). 14-3-3- λ is largely nuclear in trichomes (b and g) but is absent from guard cell nuclei, being present instead along the edge of the cell adjacent to the stomatal pore (l). 14-3-3- ω (c, h, and m) and 14-3-3- ϕ (d, i, and o) are widely distributed throughout trichomes and guard cells. GFP alone (e, j, and p) is also widely distributed throughout the trichomes and guard cells. Bar, 50 μ m (2a), bar in 10 μ m (2f), and 100 μ m (2k). The micrographs throughout the manuscript are similarly scaled with respect to cell type displayed.

were observed at this molecular weight in any of the lanes containing extracts from the 14-3-3/GFP fusion. The slight variations in apparent size among the 14-3-3/GFP fusions are consistent with the different predicted masses of the various isoforms. λ is the smallest of the isoforms, with a predicted molecular weight of 27.9 kDa, followed by κ at 28.0, ω at 29.1, and ϕ at 30.2 kDa. These data indicate that the GFP signal observed in the leaves of the 14-3-3/GFP fusion plants is, in general, derived from 14-3-3/GFP fusion proteins that are present in a state that is consistent with native properly folded 14-3-3 proteins, in both homodimeric and heterodimeric states.

The expression levels of the transgenic lines are similar when the overall signal of bands on the gel are used as an estimate of the amount of fusion protein produced. In addition, Western analysis of the extracts with representative isoforms indicates that the 14-3-3/GFP fusions are produced at levels that approximate typical 14-3-3 expression (our unpublished data).

The Subcellular Distribution of 14-3-3/GFP Isoforms

The 14-3-3/GFP isoforms demonstrated differential subcellular localization in trichomes. Trichomes proved to be excellent cell types for examination of subcellular localization of the 14-3-3/GFP isoforms, providing clear distinction among nuclear and other locations within a prominent cellular morphology and without potentially competing chlorophyll fluorescence. The 14-3-3- λ /GFP and 14-3-3- κ /GFP fusions demonstrated a predominantly nuclear localization (Figure 2, a and b), whereas the 14-3-3- ω /GFP and 14-3-3- ϕ /GFP fusions were much more widely distributed within both cytoplasmic and nuclear locations within the trichomes (Figure 2, c and d). In both 14-3-3- ω /GFP and 14-3-3- ϕ /GFP,

the fusion protein was seen associated with components of the trichome cytoskeleton (Figure 2, c and d) as well as the nucleus. GFP expressed without fusion to 14-3-3 showed a generalized subcellular distribution in both nuclear and cytoplasmic compartments (Figure 2e).

In addition to the differences in gross subcellular localization demonstrated by 14-3-3- λ /GFP and 14-3-3- κ /GFP versus 14-3-3- ω /GFP and 14-3-3- ϕ /GFP, each isoform also displayed individual differences in localization within the nucleus. 14-3-3- κ /GFP was fairly concentrated within the nucleolus (Figure 2f), whereas 14-3-3- λ /GFP was less prominent in the nucleolus than in the surrounding nuclear material (Figure 2g). The 14-3-3- ω /GFP and 14-3-3- ϕ /GFP fusions were more uniformly distributed within the nucleus, showing no apparent affinity for or exclusion from the nucleolus (Figure 2, h and i). Differences in the subcytoplasmic localization between 14-3-3- ϕ /GFP and 14-3-3- ω /GFP were subtle but apparent. 14-3-3- ω /GFP seemed to be consistently more widely expressed throughout the cytoplasm and cell membrane than 14-3-3- ϕ /GFP, which demonstrated a more limited and punctate distribution.

Subcellular localization of 14-3-3/GFP fusions was dependent upon cell type. Guard cells demonstrated distinctive subcellular localization among the 14-3-3/GFP fusions, but those localizations were not necessarily the same as those observed in trichomes. 14-3-3- κ /GFP was again decidedly nuclear in localization within guard cells (Figure 2k), whereas 14-3-3- λ /GFP localization seemed to be absent from the nucleus and limited to the peripheral guard cell edges in contact with the stomatal pore (Figure 2l). The distributions of 14-3-3- ω /GFP and 14-3-3- ϕ /GFP were similar to each other, although distinctly different from the distributions of either 14-3-3- κ /GFP or 14-3-3- λ /GFP.

Figure 3. Patterns of 14-3-3/GFP localization in trichomes are consistently specific. The panels on the left (a and c) show two views of a typical 14-3-3-κ/GFP plant, and the panels on the right (b and d) show views of a typical 14-3-3-φ/GFP plant to demonstrate the number of trichomes available for analyses and the consistency of localization within those trichomes. The graph on the far right (e) shows the relative proportion of nuclear 14-3-3/GFP localization compared with total 14-3-3/GFP presence in the trichomes averaged for five trichomes each of 14-3-3-κ/GFP and 14-3-3-φ/GFP. The error bars represent the SD.

The subcellular distribution of 14-3-3/GFP in trichomes is consistent among trichomes and leaves. Figure 3, a and b, provides a field view of 14-3-3-κ/GFP and 14-3-3-φ/GFP plants to show the number of trichome cells available for analysis on typical leaves. Figure 3, c and d, present a closer view of leaves showing the subcellular distribution in eight to 10 trichomes scattered across the leaf surface. Figure 3e provides a relative quantification of the distribution of the GFP signal in 14-3-3-κ/GFP and 14-3-3-φ/GFP. On the average, 14-3-3-κ/GFP signal in the nucleus comprises ~35% of the total signal in the trichome body. In 14-3-3-φ/GFP trichomes, nuclear signal comprises ~18% of the total signal in the trichome body.

In all cases, the distribution of the 14-3-3/GFP fusion was the result of dynamic processes and proteins in differential motion within the cells. Figure 4 displays a subset of still images from a movie that can be found online as supplemental material (Supplemental Figure S1). These frames illustrate the dynamic movement of φ/GFP along the cytoskeletal network in trichomes.

Subcellular Localization Dependency on 14-3-3 Client Interactions: AICAR

AICAR is a 5' AMP analog that is known to disrupt the biochemical and biological influence of 14-3-3s upon their

target clients (Toroser *et al.*, 1998). AICAR is useful in an *in vivo* experimental environment because it can penetrate plant cell walls and membranes while retaining its activity as a 5' AMP mimetic.

AICAR treatment of leaves resulted in a dramatic alteration in the subcellular localization of 14-3-3-κ/GFP and 14-3-3-λ/GFP fusions within trichomes. Figure 5 displays trichomes from the AICAR treatments of leaves. The top panel shows trichomes photographed before treatment with AICAR (Figure 5, a–e), and the bottom panel shows trichomes after 2-h incubation in AICAR. Where possible, the same trichome was photographed before and after treatment. (The reapplication of the slide after treatment made it difficult to use the same cell for before and after photography, but occasionally the same cell was identified, as in Figure 5, c, d, h, and i.).

Instead of the characteristic nuclear localization of 14-3-3-κ/GFP and 14-3-3-λ/GFP, AICAR treatment resulted in GFP signal widely and diffusely spread throughout the distal portions of the trichome, with no indication of nuclear localization (Figure 5, f and g). AICAR treatment of leaves also disrupted the 14-3-3-φ/GFP localization within trichomes, resulting in distinctively globular regions of fluorescence (Figure 5i) that were nonnuclear but still mobile within the cell (our unpublished data). Similar AICAR treat-

Figure 4. Distribution of 14-3-3/GFP fusion protein can be dynamic. A series of stills showing the redistribution of 14-3-3-φ/GFP associated with cytoskeleton over time. Each panel represents a 20-s interval (a movie composed of a series of 5-s intervals can be found on line as Supplemental Figure S1: 14-3-3-φ/GFP movie).

Figure 5. AICAR disrupts 14-3-3/GFP localization in some isoforms. Top, example of trichomes from untreated leaves. In each cell, the nuclear and cytoplasmic components are clearly visible as distinct features. Bottom, effects of treating excised leaves with 10 mM AICAR for 2–3 h. 14-3-3- κ /GFP, 14-3-3- λ /GFP, and 14-3-3- ϕ /GFP (f, g, and i) all show a disrupted pattern of 14-3-3/GFP distribution. 14-3-3- ω /GFP (h) seems less affected by AICAR, and GFP (j) is unaffected.

ment had no apparent effect on nonfusion GFP (Figure 5, e and j).

Treatment with AICAR had little overt effect on the distribution of 14-3-3- ω /GFP (Figure 5, c and h); however, a close inspection of the cells after treatment revealed that the dynamic motion of the fusion proteins characteristic of these cells is greatly diminished in the presence of AICAR (our unpublished data).

Subcellular Localization Dependency on 14-3-3 Client Interactions: R18

The R18 peptide is a very high-affinity 14-3-3 target, having a higher affinity for 14-3-3 binding than any known target (Petosa *et al.*, 1998; Wang *et al.*, 1999). Thus, it can act as an antagonist of client binding and is expected to outcompete any native protein to disrupt the partnering of the 14-3-3s with their targets. Figure 6 illustrates the effects of incubating excised leaf sections with R18. The top panel of Figure 6 (Figure 6, a–e) shows trichomes photographed before treatment, and the middle panel (Figure 6, f–j) shows trichomes photographed after 3–3.5 h of incubation with R18. The bottom panel (Figure 6, k–p) demonstrates the effect on GFP signal distribution by using an unrelated peptide that does not have an affinity for 14-3-3 binding. Treatment with R18 peptide had a dramatic effect on the distributions of all 14-3-3/GFP fusions. The 14-3-3- κ /GFP and 14-3-3- λ /GFP distributions changed from their characteristic nuclear predominance (Figures 5b and 6a) to a generalized and diffuse distribution (Figures 5g and 6f) similar to that seen with AICAR. The 14-3-3- ϕ /GFP fusion similarly lost its native localization, becoming distributed in globules similar but not identical to those seen with AICAR treatment of 14-3-3- ϕ /GFP. In contrast to treatment with AICAR, the 14-3-3- ω /GFP distribution was clearly affected by treatment with R18 peptide, losing its characteristic cellular specific localization (Figure 6c) and becoming very diffusely distributed throughout the trichome (Figure 6h).

Similar experimental treatment of leaves with R18 peptide had no influence on the localization of GFP alone (Figure 6, e and j). In addition, similar experimental incubation with a nonrelated peptide had no influence on the distribution of

GFP signal in any of the 14-3-3/GFP fusions or on GFP itself (Figure 6, k–p). Figure 7 displays an extended time course of the treatment of 14-3-3- ϕ /GFP with R18 (also see Figure 6, d and i). These sequential images of the same trichome illustrate the progressive nature of the effect that incubation with R18 has on the distribution of the 14-3-3- ϕ /GFP fusions in 30-min intervals.

DISCUSSION

It is well established that members of the 14-3-3 family seem to be ubiquitous in eukaryotes and play a diversity of roles in metabolism (Aitken, 1996; Ferl, 1996; Babakov *et al.*, 2000; Fu *et al.*, 2000; Muslin and Xing, 2000; Rosenquist *et al.*, 2000; Sehnke *et al.*, 2000, 2002; Aitken *et al.*, 2002; Dougherty and Morrison, 2004; Wijngaard *et al.*, 2005). 14-3-3s have been associated with many subcellular processes and various methodologies have placed them in and within the influence of specific organelles. Evidence of a direct association of 14-3-3s with nuclear material has been demonstrated with localization studies (Bihn *et al.*, 1997) and implied with the discovery of 14-3-3s as components of promoter complexes (de Vetten and Ferl, 1994; Lu *et al.*, 1996; Schultz *et al.*, 1998; Pan *et al.*, 1999), and a variety of studies focused on the roles of 14-3-3s in nuclear export (Bodendorf *et al.*, 1999; Kanai *et al.*, 2000; Brunet *et al.*, 2002; Eilers *et al.*, 2002). The binding of 14-3-3s also has been shown to regulate the subcellular redistribution of proteins between the nucleus and the cytoplasm (Pietromonaco *et al.*, 1996; Cutler *et al.*, 2000; Seimiya *et al.*, 2000; Merla *et al.*, 2004; Uchida *et al.*, 2004; van Hemert *et al.*, 2004). In addition, 14-3-3s have been localized to or shown to have a role in localization to the endoplasmic reticulum (Subramanian *et al.*, 2004), the chloroplast (Sehnke *et al.*, 2000; Man and Kaiser, 2001), and mitochondria (Bunney *et al.*, 2001; Datta *et al.*, 2002).

Although there have been many studies that indicate a particular subcellular role for 14-3-3s, subcellular localization per se has had limited application as a diagnostic tool for developing an understanding of 14-3-3 diversity within an organism (van Hemert *et al.*, 2004). Many apparent conclusions of 14-3-3 function and distribution within particular

Figure 6. 14-3-3/GFP localization is disrupted by the R18 peptide. Top, example of trichomes from untreated leaves. In each cell, the nuclear and cytoplasmic components are clearly visible as distinct features. Middle, effects of treating excised leaves with R18 peptide for 3–4 h. In all 14-3-3/GFP isoforms, R18 causes a disruption of subcellular distribution. Bottom, effects of treating excised leaves with a nonspecific peptide for the same amount of time, which has little or no effect on 14-3-3/GFP distribution.

cell types are based on observations of a single isoform, and comparative data among isoforms are rare. The experiments presented here are expressly designed to be comparisons among isoforms of *Arabidopsis*, within easily observed specific cell types, to answer fundamental questions of 14-3-3 biology and diversity.

The four isoforms chosen for this study come from evolutionarily divergent groups within the *Arabidopsis* 14-3-3 family (Feri *et al.*, 2002; Sehnke *et al.*, 2002). ϕ and ω are members of a small 14-3-3 group that is clearly separated on the *Arabidopsis* 14-3-3 phylogenetic tree. κ and λ constitute a completely separate grouping with a very deep branch from the main 14-3-3 line. Thus, the experiments presented here sample the characteristics of two distinct gene family subgroups.

Given the dimeric nature of 14-3-3s, conclusions regarding *in vivo* isoform specificity must be developed with caution. Indeed, data from this study indicate that any localization data for a particular isoform must be interpreted as representing a population of dimeric molecules that will not be homogeneous. Each of the 14-3-3/GFP fusions used in this study produces a population that is largely represented by homodimers but also encompasses a range of heterodimeric 14-3-3 pairs.

Subcellular Localization of *Arabidopsis* 14-3-3s

In trichomes, the subcellular distribution patterns were distinct among the expressed 14-3-3/GFP fusions. 14-3-3- κ /GFP and 14-3-3- λ /GFP were largely nuclear in localization, whereas the ω and ϕ fusions were more generally distrib-

Figure 7. Effects of peptide R18 develop slowly over time. The panel shows a time course following the progression of the disruption of 14-3-3- ϕ /GFP localization. The pictures were taken 30 min apart, beginning at the 2-h time point. The series demonstrates the progression of the effect R18 binding has on subcellular distribution of 14-3-3- ϕ /GFP.

uted throughout the cell. This rather simple observation leads to a fairly direct conclusion that the λ and κ isoforms participate in functions that differ from those involving ω and ϕ isoforms.

The observation of isoform-specific localization is perhaps surprising given that a portion of any isoform is apparently associated in any one of a number of heterodimer pairings. Heterodimerization among differentially localizing or differentially functioning 14-3-3 monomers would result in a blended pattern of localization or function at the very least, tending to spread the 14-3-3/GFP fusion signal among the family of localization patterns available to the entire family of 14-3-3s present in the cell. This also leads to the possibility that, for example, the nuclear localized GFP signal present in the 14-3-3- κ /GFP plants is due to homodimers of 14-3-3- κ , whereas the cytoplasmic signal is due to the heterodimers. In any case, the presence of heterodimer pairs within the 14-3-3 population in the cells expressing the 14-3-3/GFP fusions stringently tests the concepts of isoform-specific localization and strengthens the conclusion that different isoforms can have different functions within a cell.

The localizations of the 14-3-3/GFP isoforms seem to reflect the evolutionary relationships among the isoforms (DeLille *et al.*, 2001; Ferl *et al.*, 2002), with κ /GFP and λ /GFP both predominating in the nucleus, whereas 14-3-3- ϕ /GFP and 14-3-3- ω /GFP exhibit a more uniform distribution throughout the trichome cell. The localization of other isoforms should be investigated before making the case for confining a particular subcellular local and, by extension, function to any particular branches in the *Arabidopsis* 14-3-3 tree. Also, the observation of a differential presence of 14-3-3- κ /GFP and 14-3-3- λ /GFP within the nucleolus indicates that additional layers of specific localization, and perhaps function, exists even within these closely related 14-3-3s.

It is important to note, however, that the localization of a particular isoform may be dependent upon the cell type and that generalizations about the localization of a 14-3-3 isoform should be limited to those cell types where the localization was directly observed. The 14-3-3- λ /GFP fusion demonstrated a dramatic affinity for the nucleus of trichome cells, but in guard cells was conspicuously absent from the nuclei while present in a specialized band near the stomata. This observation indicates that λ is not solely nor entirely a "nuclear" isoform. Although such dramatic cell type-specific localization was not observed for the other isoforms in this study, it is clear that cell type can have a role in determining the localization of 14-3-3 isoforms.

From this and other studies, it seems that 14-3-3s are in motion within cells and cellular compartments and that the distribution of any 14-3-3 at any one time is but a snapshot of that dynamic process. Real-time observations of 14-3-3- ϕ /GFP illustrate how active the movement of these fusion proteins can be within the cell. Although best viewed in the supplemental movie (Supplemental Figure S1), Figure 4 provides an indication of the cytoplasmic movement of ϕ /GFP along the cytoskeleton, intersecting with nuclear and plasma membranes over time. The subcellular distribution of the 14-3-3- ω /GFP also shows an active intersection with the nucleus along the cytoskeletal network (Figure 2 and in real-time observations) and is consistent with observations that associate the ω isoform with nuclear shuttling (Cutler *et al.*, 2000). Cytoplasmic distribution of 14-3-3s is well documented in the literature, but again, the emphasis is on the dynamic nature of these localizations. 14-3-3s also have been implicated in mediating the release of protein complexes from the ER (Yuan *et al.*, 2003), shown to partner with a microtubule-localized Rho exchange factor (Zenke *et al.*,

2004), and seem to play a role in organelle targeting through associations with the actin cytoskeleton (Roth *et al.*, 1999; Jin *et al.*, 2004).

The 14-3-3-mediated exchange between subcellular localizations has been extensively explored in systems where 14-3-3s are involved in the shuttling, or exclusion, of proteins to and from the nucleus. For example, in mammals histone deacetylases (HDACs) are sequestered in the cytoplasm through 14-3-3 binding, and 14-3-3s thereby act as negative mediators of HDAC activity (Grozinger and Schreiber, 2000; Wang *et al.*, 2000). 14-3-3s also have been shown to interact with many members of the Cdc25 family of phosphatases by either facilitating export or in the active exclusion of those proteins from the nucleus (Zeng and Pwnica-Worms, 1999; Lee *et al.*, 2001; Giles *et al.*, 2003). The interaction of chromatin HMGN proteins with 14-3-3s also retards their entry into the nucleus (Prymakowska-Bosak *et al.*, 2002). The active movement of the fusion proteins within the cell, and intersecting with the nucleus, could reflect a real-time demonstration of the dynamic nature of these proteins.

Client Interactions as Drivers of 14-3-3 Localization

Because the major established role for 14-3-3 proteins is the interaction with client proteins, the subcellular localization of 14-3-3 isoforms within a cell could be driven by specific client associations rather than (or in addition to) the intrinsic properties of the 14-3-3s themselves. If client associations are a major driver of localization, then isoform-specific localization would reflect the specificity of the client interaction with specific isoforms. Although many reports have indicated that various 14-3-3 isoforms have different affinities for peptide and protein interactions (Rosenquist *et al.*, 2000; Huber *et al.*, 2002; Sehnke *et al.*, 2002), there are no reports indicating that a given isoform has higher affinity for one client over another, whereas another isoform demonstrates a different affinity hierarchy with the same set of clients. Indeed, there are several reports indicating that evolutionarily diverse 14-3-3s can perform clearly redundant roles, most notably that plant and animal isoforms can complement yeast 14-3-3 knockouts (van Heusden *et al.*, 1996; Kuromori and Yamamoto, 2000; Callejo *et al.*, 2002; Vasara *et al.*, 2002). Therefore, there is little biochemical support for the notion that the various *Arabidopsis* isoforms would have specific and selective pools of client proteins.

The use of agents recognized to interfere with 14-3-3/client interactions in this study does, however, indicate that client interactions are major determinants of 14-3-3 subcellular localization. 14-3-3s contain a 5' AMP binding site that plays a role in client binding (Athwal *et al.*, 1998; Camoni *et al.*, 2001). The AMP site also can be occupied by the analog compound AICAR and the monophosphate derivative ZMP. AICAR will activate plant enzymes such as NADH-nitrate reductase, sucrose phosphate synthase, and glutamine synthase that rely on a 14-3-3 partner for regulation (Huber and Kaiser, 1996; Toroser *et al.*, 1998; Weiner and Kaiser, 1999; Man and Kaiser, 2001). In each case, AICAR disrupts 14-3-3 binding to its designated target, and the capacity for 14-3-3s to mediate downstream activity is lost in the presence of AICAR. The premise for AICAR treatments in the experiments presented here is that if the subcellular distribution of the 14-3-3/GFP fusion is reliant on partner binding, then a disruption of that partnering also will disrupt the subcellular distribution of the fusion protein. Three of the four isoforms examined displayed such a response when exposed to AICAR. The distribution of κ , λ , and ϕ fusions was rendered diffuse or randomly globular after treatment with AICAR for 2 h, although distribution of 14-3-3- ω /GFP

seemed to be only minimally affected by AICAR (Figure 5, c and h). The subcellular distribution of the control construct (GFP alone, Figure 5, e and j) was unaffected by AICAR, reinforcing the indication that 14-3-3/GFP distribution is driven by the partner to which it is bound.

Another approach to disrupting the partner association was to block binding with a competing peptide. The peptide R18 has a higher affinity for 14-3-3 binding than any known target, making it an extremely effective antagonist to 14-3-3/client interactions. The R18 peptide was specifically selected from a random peptide library designed to be screened for high-affinity 14-3-3 binding (Petosa *et al.*, 1998; Wang *et al.*, 1999). The subcellular distribution of 14-3-3/GFPs in cells from excised leaves incubated with R18 display similar responses to that seen by AICAR treatment. All four isoforms examined showed a disruption of subcellular distribution of their localization patterns after 3–4 h of incubation with R18 (Figure 6). The parallel treatments with buffer alone or buffer with a nonspecific peptide did not affect the distribution of the 14-3-3 fusion proteins, and R18 had no effect on the distribution of GFP alone. The efficacy of R18 as an in vivo antagonist for 14-3-3 partner binding also has been demonstrated with transient expression assays (Jin *et al.*, 2004).

The distinct in vivo subcellular localization patterns of 14-3-3/GFP fusions within specific cell types, together with the scope of the effect interaction antagonists on the localization of the fusions, provides compelling evidence that the subcellular distributions of native 14-3-3 isoforms can be driven by client interactions and that those interactions are isoform specific in nature. In the absence of client interactions, 14-3-3 seems to be diffusely spread and without subcellular character. In the presence of client specific interactions, 14-3-3s take on isoform-specific localizations that, although dynamic, are individually distinct. This leads to a model where specific 14-3-3 isoforms interact with specific subsets of client proteins. As such, this model endows each 14-3-3 isoform with a specific client set and thereby a specific function. Heterodimeric 14-3-3 pairs could potentially interact with two or more client types, offering a layer of complexity to the model. However, the observation of any differential subcellular specificity among isoforms argues that the number of actual clients in any one cell must be limited, because multiple clients could dictate multiple localizations.

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