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TECHNICAL ADVANCE

Protein mislocalization in plant cells using a GFP-binding chromobody

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SUMMARY

A key challenge in cell biology is to directly link protein localization to function. The green fluorescent protein (GFP)-binding protein, GBP, is a 13-kDa soluble protein derived from a llama heavy chain antibody that binds with high affinity to GFP as well as to some GFP variants such as yellow fluorescent protein (YFP). A GBP fusion to the red fluorescent protein (RFP), a molecule termed a chromobody, was previously used to trace *in vivo* the localization of various animal antigens. In this study, we extend the use of chromobody technology to plant cells and develop several applications for the *in vivo* study of GFP-tagged plant proteins. We took advantage of *Agrobacterium tumefaciens*-mediated transient expression assays (agroinfiltration) and virus expression vectors (agroinfection) to express functional GBP:RFP fusion (chromobody) in the model plant *Nicotiana benthamiana*. We showed that the chromobody is effective in binding GFP- and YFP-tagged proteins *in planta*. Most interestingly, GBP:RFP can be applied to interfere with the function of GFP fusion protein and to mislocalize (trap) GFP fusions to the plant cytoplasm in order to alter the phenotype mediated by the targeted proteins. Chromobody technology, therefore, represents a new alternative technique for protein interference that can directly link localization of plant proteins to *in vivo* function.

Keywords: ***Nicotiana benthamiana,*** **fluorescence microscopy, subcellular localization, nanotrap, llama heavy chain antibody, VHH domain.**

INTRODUCTION

Plant cells require a remarkable level of structural organization to compartmentalize a plethora of diverse cellular processes and functions. Subsequently, protein (re-)localization plays a critical role in cellular biology and has been implicated in various different processes such as organelle and membrane trafficking (Inaba and Schnell, 2008), nuclear import and export (Meier and Brkljacic, 2008), cell polarity (Geldner, 2008), cytoskeletal architecture (Guimil and Dunand, 2007), cell wall organization (Zhong and Ye, 2007), and cellular crosstalk via plasmodesmata (Maule, 2008). The current view is that dynamic changes in protein localization are critical for intra- and intercellular information exchange, which in turn enables proper cellular function and integration of extracellular signals. However, a key challenge in plant cell biology is to directly link protein localization to function.

One approach for reconciling protein localization with function is to alter protein distribution patterns and evaluate the impact on functionality. This can be achieved by removal or addition of known localization motifs such as secretion or translocation signals, nuclear localization signals (NLS) or nuclear export sequences (NES) followed by functional analysis of the mutated protein (Shen *et al.*, 2007; Schornack *et al.*, 2008). Mislocalization can also be achieved using compartment-specific antibodies that generate artificial sinks (Conrad and Manteuffel, 2001). As an example, expression of variable domains of conventional antibodies (ScFv) with specificity towards small heat shock proteins prevented their accumulation in heat shock granules and altered heat tolerance in plants (Miroshnichenko *et al.*, 2005). In general such 'mislocalization' experiments can be very informative and complement loss-of-function experi-

ments by establishing a direct link between biological function and cellular localization. However, the challenge with these approaches is to express, target and assemble the antibodies as well as to ensure sufficient specificity towards the protein targeted *in vivo*.

Antibodies of members of the Camelidae family differ from conventional mammalian antibodies by lacking light chains. Recognition of antigens by Camelidae antibodies is solely mediated by the variable domain of the heavy chain (termed the VHH or nanobody), resulting in a minimal functional antigen-binding fragment that is structurally less complex than that of conventional antibodies (Muyldermans, 2001; Conrad and Sonnewald, 2003). This particular feature facilitates heterologous expression of nanobodies in diverse cell types and enables their use in a variety of biotechnological applications. In one early approach, nanobodies directed against a plastidic starch-branching enzyme were expressed in potato, resulting in inhibition of the enzyme and reduction of starch biosynthesis (Jobling *et al.*, 2003).

In a recent major advance, Rothbauer *et al.* (2006) developed a nanobody with GFP-binding specificity (also termed a nanotrap) suitable for expression and localization *in vivo*. This GFP-binding protein (GBP) is a small 13-kDa soluble protein that binds with high affinity to GFP as well as to some of its derivatives such as yellow fluorescent protein (YFP). This nanobody has several applications for the study of GFP-tagged proteins. First, it allows efficient isolation of GFP fusion proteins and their interactors. Second, it can be used to trace GFP-tagged proteins and also to trap them to particular subcellular compartments (Rothbauer *et al.*, 2006, 2008). To facilitate this process, translational fusions of GBP to the red fluorescent protein (RFP) were made to create so-called chromobodies that enable concurrent localization studies of GBP along with the target GFP protein. The GBP:RFP chromobody was used to demonstrate that besides binding GFP *in vitro*, GBP binds and traces the localization of various GFP fusion proteins *in vivo* (Rothbauer *et al.*, 2006).

In this study, we extend the use of the nanotrap/chromobody technology to plant cells and develop a number of applications for the *in vivo* study of GFP-tagged plant proteins. We took advantage of *Agrobacterium tumefaciens*-mediated transient expression assays (agroinfiltration) and virus expression vectors (agroinfection) to express GBP:RFP fusion (chromobody) in the model plant *Nicotiana benthamiana* (Goodin *et al.*, 2008). We show that the GBP nanobody/chromobody is effective in binding GFP and YFP tagged proteins *in planta* and that this interaction is specific. Most interestingly, we observed that GBP can mislocalize (trap) GFP fusion proteins to the plant cytosol and alter the phenotype mediated by the targeted proteins. Since GBP specifically targets the GFP epitope of otherwise functional fusion proteins, this approach is ideally suited to altering the localization of functional proteins in plant cells and assess the impact on their activities. The chromobody technology therefore represents an alternative technique for linking cellular localization to function and complements current methods based on mutagenesis or fusion to heterologous localization signals.

RESULTS

GFP-binding protein binds in planta expressed GFP and YFP but not cyan fluorescent protein (CFP)

To determine the applicability of the GBP technology to plants, we first investigated the extent to which *Escherichia*

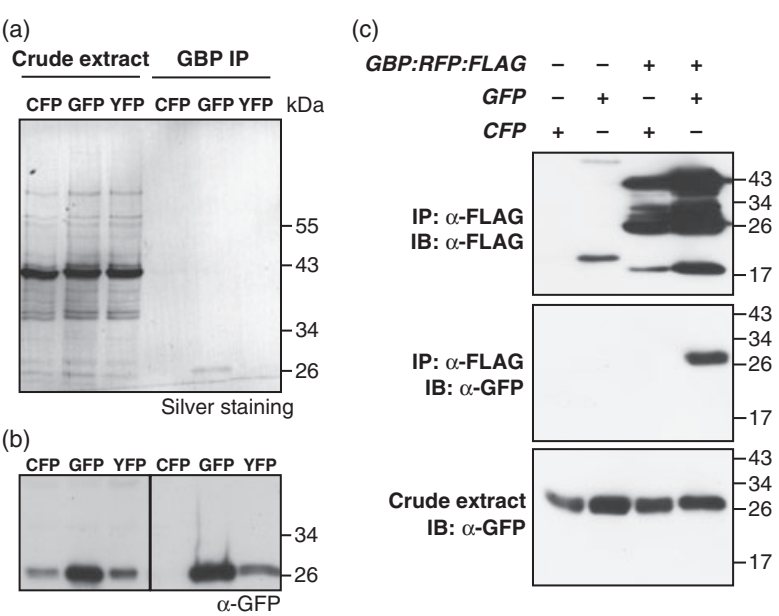

Figure 1. Green fluorescent protein-binding protein (GBP) interacts with GFP and yellow fluorescent protein (YFP) *in vitro* and *in vivo*.
(a) Silver-stained gel loaded with total protein extract (lanes 1–3) of agroinfiltrated *Nicotiana benthamiana* expressing cyan fluorescent protein (CFP), GFP or YFP and immunoprecipitated (IP) fraction after pulldown with GFP-Trap® (lanes 4–6).
(b) Immunoblot (IB) of an identically loaded gel as in (a) using anti-GFP antisera.
(c) Co-immunoprecipitation of GFP but not CFP with GBP:red fluorescent protein (RFP):FLAG *in vivo*. All constructs were agroinfiltrated into *N. benthamiana* in the combinations depicted on top. The IP obtained with sepharose coupled anti-FLAG antisera was subjected to anti-FLAG and anti-GFP immunoblot (top and middle panels, respectively). Total protein extract was also immunoblotted using anti-GFP to ensure expression of GFP and CFP.

coli purified GBP:6×His coupled to sepharose (GBP sepharose, GFP-Trap®) can be used to detect *in planta* expressed GFP and its fluorescent derivatives. We used agroinfiltration to transiently express GFP, YFP and CFP in *N. benthamiana* leaves which were then used to prepare crude soluble extracts. We incubated crude extracts with GBP sepharose

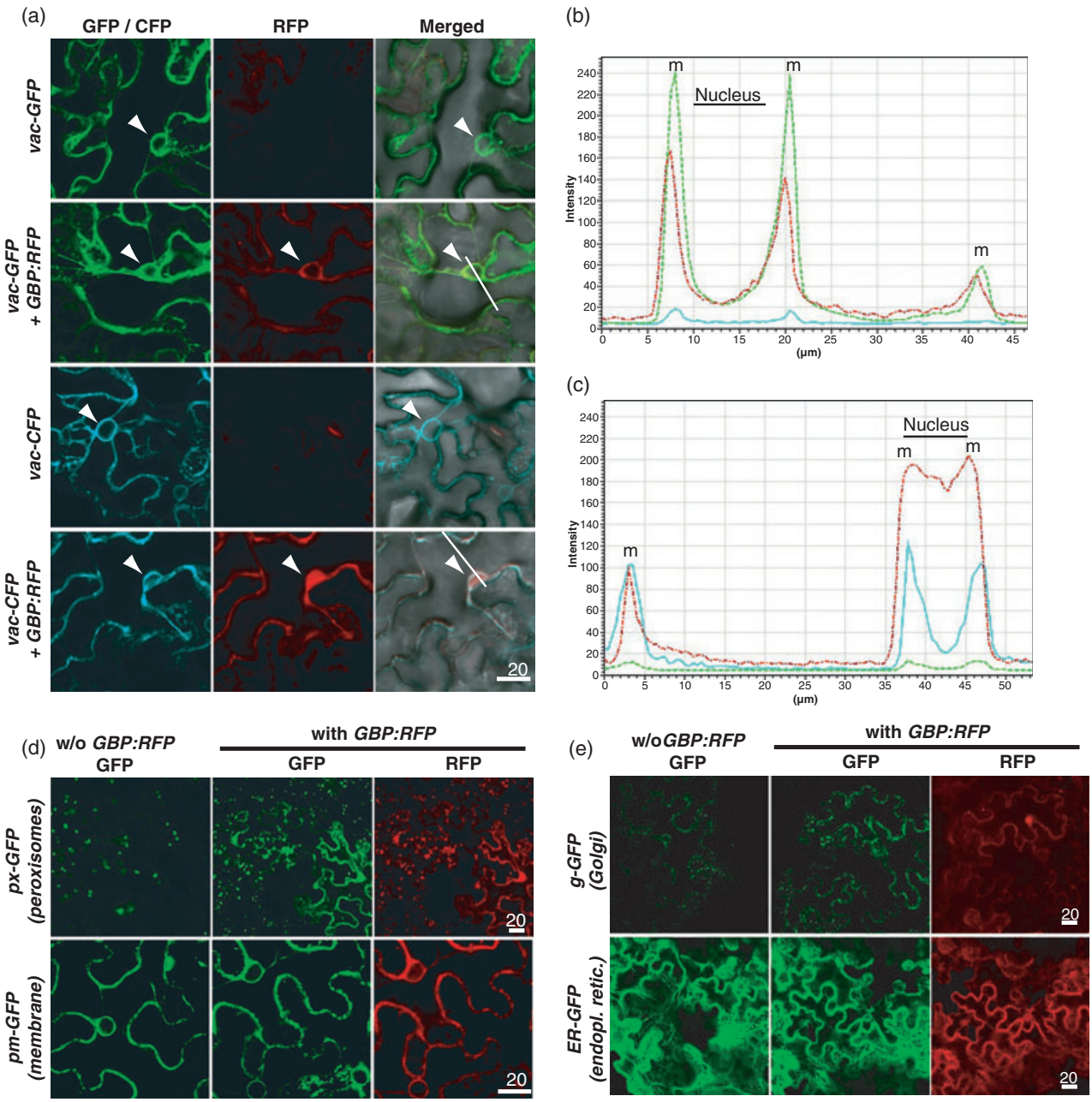

Figure 2. Membrane-anchored GFP but not cyan fluorescent protein (CFP) tethers green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) to the membrane.

(a) Confocal imaging of GBP:RFP and tonoplast anchored GFP or CFP (vac-CFP, vac-GFP) after agroinfiltration of *Nicotiana benthamiana* leaf tissue. Confocal images showing GFP (green), CFP (blue) and RFP (red). Agroinfiltrated T-DNA constructs are indicated at the left side. Arrows indicate nuclei, white bars represent transects depicted in (b) and (c). Scale bar, 20 μm. Measurements were done 2 days after agroinfiltration.

(b, c) Fluorescence intensity plot of transect through cells expressing vac-GFP (b) or vac-CFP (c) and GBP:RFP. m, non-nuclear cytosolic/tonoplast fluorescence peaks. The nuclear transect section is indicated. Colors of graphs correspond to agroinfiltrated constructs as assigned in (a).

(d, e) Confocal images of GFP and RFP 2 days post-infiltration after *Agrobacterium tumefaciens*-mediated expression of GFP fusion proteins alone (left column) or together with GBP:RFP (middle and right column). The black images representing the red channel of GFP fusions in the absence of GBP:RFP are not displayed. Scale bar, 20 μm. GBP:RFP labels peroxisomal px-GFP and plasma membrane-integral pm-GFP (d), but localization of GBP:RFP, Golgi localized G-GFP and endoplasmic reticulum localized ER-GFP remains unaffected by co-expression (e).

beads, and immunoprecipitated protein fractions were subsequently analysed on silver-stained protein gels. We detected protein bands corresponding in size to fluorescent protein in both the GFP and YFP samples but not in fractions derived from CFP preparations, suggesting specific binding to GFP and YFP only (Figure 1a,b). In addition, no other protein bands were detected in the precipitated samples (Figure 1a), indicating high specificity towards GFP and YFP and either absence or low levels of aspecific binding to endogenous proteins. Subsequent immunoblotting and detection using anti-GFP antisera showed that GBP sepharose quantitatively immunoprecipitates GFP and YFP, but not CFP (Figure 1b).

Chromobody interacts with target proteins *in planta*

The finding that GBP can bind fluorescent proteins expressed in plant cells prompted us to determine whether we could express a functional GBP *in planta*. To that end, we generated *A. tumefaciens* binary constructs containing the chromobody (*GBP* fused to monomeric *RFP* or *GBP:RFP*) driven by the constitutive CaMV 35S promoter. Two of three constructs additionally carry C-terminal FLAG or 6 × His epitopes for purification purposes. Transient *A. tumefaciens* mediated expression (agroinfiltration) of 35S-*GBP:RFP* in *N. benthamiana* resulted in red fluorescence at 2 days post-infiltration (dpi) that was readily detectable in cytoplasm and nuclei of expressing cells even at 6 dpi (Figure S1 in Supporting Information, and data not shown). A similar distribution of red fluorescence was observed when expressing the FLAG- or 6×His tagged derivatives (Figure S2). None of the constructs triggered phenotypic changes in the infiltrated areas (data not shown).

To assess the ability of the chromobody to bind GFP *in vivo*, we first infiltrated *A. tumefaciens* strains carrying the *GBP:RFP:FLAG* construct with or without the *GFP* or *CFP* constructs and then used protein extracts of infiltrated *N. benthamiana* leaves in anti-FLAG immunoprecipitation of *GBP:RFP:FLAG*. Analysis of the extracts and pull-down fractions with anti-GFP antisera revealed the presence of GFP but not CFP in addition to the GBP:RFP:FLAG (Figure 1c). These findings indicate that the chromobody is functional when expressed *in planta* and exhibits specificity by binding GFP, but not CFP.

Chromobody co-localizes with GFP but not CFP fusion proteins

In animal systems, the red fluorescent chromobody was used to follow the localization of GFP fusion proteins and amplify the fluorescence signal (Rothbauer *et al.*, 2006). To test the transferability of such experiments to plants, we co-infiltrated into *N. benthamiana* leaves *A. tumefaciens* strains harbouring a 35S-*GBP:RFP* construct together with GFP reporter constructs that localize to various subcellular structures (Mathur, 2007; Nelson *et al.*, 2007). Confocal fluorescence microscopy was then used to localize both GFP (antigen) and GBP:RFP (chromobody) fusions upon co-expression *in planta*. We first co-expressed the chromobody with vac-GFP, a GFP fusion to aquaporin that localizes to the

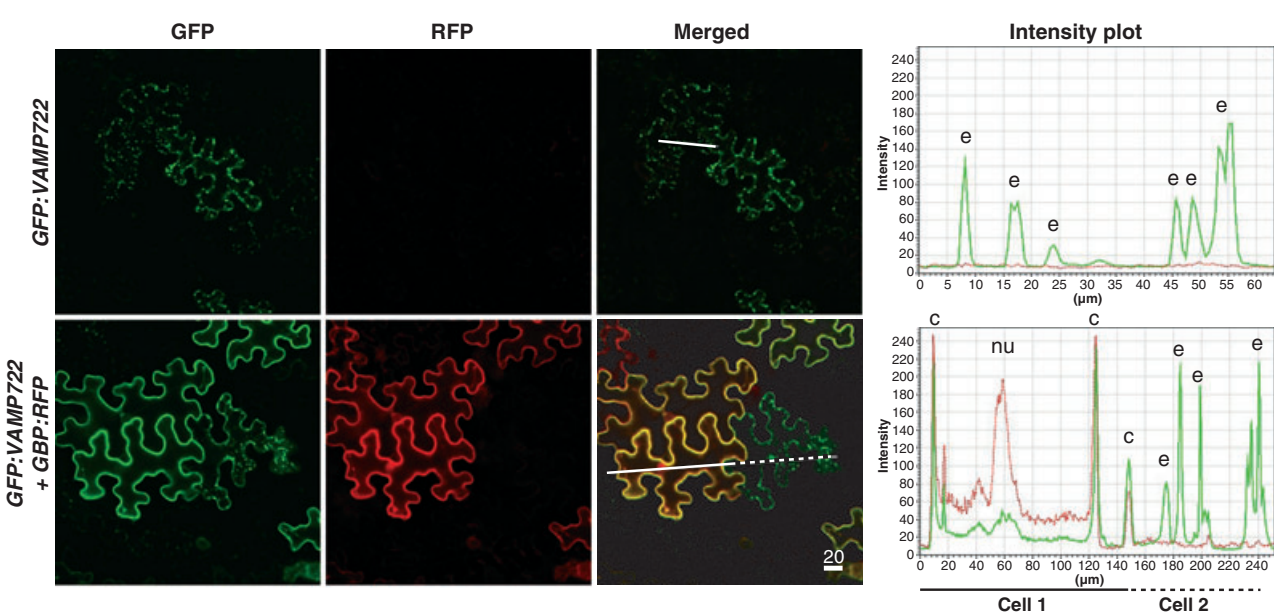

Figure 3. Chromobody alters localization of GFP:VAMP722.
Confocal imaging of green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) (red) and GFP:VAMP722 (green) 2 days after agroinfiltration of *Nicotiana benthamiana* leaf tissue. Agroinfiltrated T-DNA constructs are indicated at the left side. White solid and dashed bars represent transects through neighboring cells with their fluorescence intensities plotted on the right. Scale bar, 20 µm. e, vesicle-like endomembrane compartments, c, cytosol, nu, nucleus.

tonoplast (Nelson *et al.*, 2007) (Figure 2a). Remarkably, vac-GFP tethered the red fluorescence of the GBP:RFP to the tonoplast depleting it from the nucleus, indicating that the chromobody traced the subcellular localization of vac-GFP (Figure 2a,b).

To address the specificity of the interaction between the chromobody and its GFP antigen, we replaced GFP by CFP, resulting in a vac-CFP *A. tumefaciens* binary plasmid. In contrast to the vac-GFP experiments, co-expression of vac-CFP and GBP:RFP resulted in distinct subcellular distributions of blue and red fluorescence (Figure 2a,c). The chromobody red fluorescence was retained in the nucleoplasm and was not depleted in nuclei as observed with vac-GFP (Figure 2a,c). Thus, CFP did not sequester the chromobody, consistent with the lack of binding observed *in vivo* and *in vitro* (Figure 1).

Two other GFP constructs, a peroxisomal GFP fusion (px-GFP) and an integral membrane GFP fusion (pm-GFP), resulted in co-localization with the chromobody when co-expressed with GBP:RFP in *N. benthamiana* leaves (Figure 2d). Both GFP fusions tethered GBP:RFP to their subcellular compartments depleting it from the nucleus (Figure 2d).

In contrast to the GFP constructs described above, GFP fusions that enter the endoplasmic reticulum (ER) and Golgi pathways had no impact on chromobody distribution (Figure 2e). Co-expression of Golgi-localized GFP (G-GFP) and endoplasmic reticulum-localized GFP (ER-GFP) with GBP:RFP did not alter the subcellular localizations of either the chromobody or the antigens (Figure 2e). Considering that G-GFP and ER-GFP are thought to enter the ER lumen in a nascent state upon synthesis, it is reasonable to assume that a properly folded GFP antigen remained inaccessible to the cytosolic chromobody.

Chromobody alters the localization of several target proteins

While testing fluorescent reporters, we discovered that the localization of certain GFP fusions can be dictated by the chromobody. GFP:VAMP722 is a GFP fusion with the Arabidopsis vesicle (v)-SNARE, VAMP722, which associates with endomembrane compartments that are subject to pathogen-induced cell polarization (Kwon *et al.*, 2008). In contrast to its normal endomembrane localization, co-expression of *GFP:VAMP722* with *GBP:RFP* resulted in dispersed green fluorescence within the cytosol (Figures 3 and S2). It is noteworthy that altered localization of GFP:VAMP722 was observed only in cells co-expressing both the antigen and the chromobody, as can be clearly seen in the two adjacent cells that either show or lack chromobody expression (Figure 3).

We also determined that the chromobody triggers mislocalization of nuclear, plastid and mitochondrial YFP and GFP

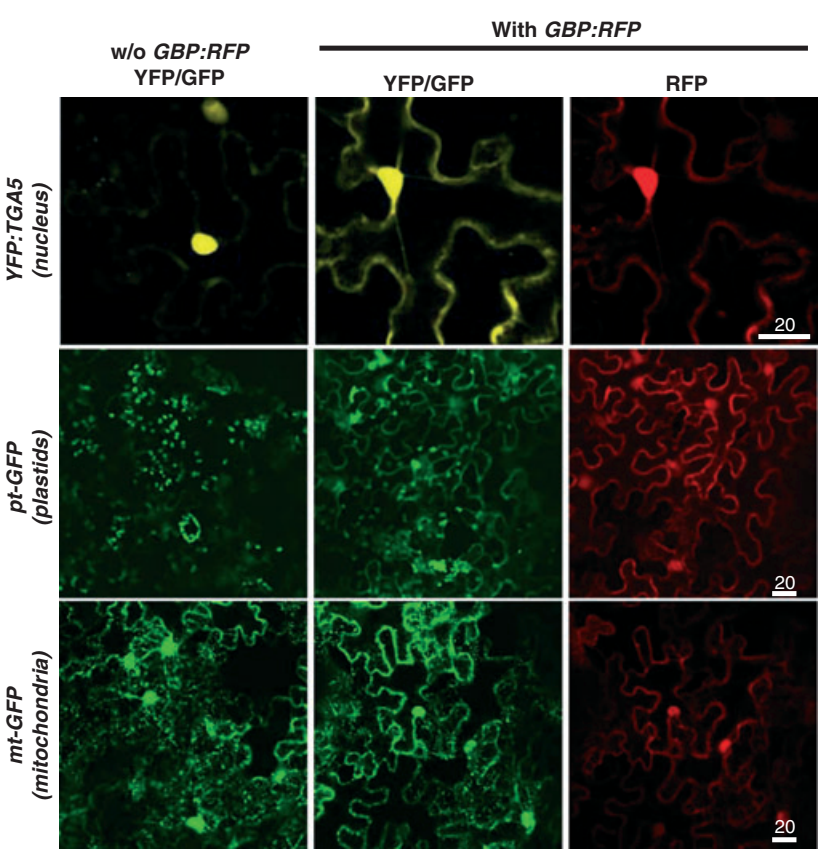

Figure 4. Expression of green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) traps nuclear, plastidic and mitochondrial GFP fusions in the cytosol.
Confocal images of GFP and RFP 2 days post-infiltration after *Agrobacterium tumefaciens*-mediated expression of GFP fusion proteins alone (left column) or together with GBP:RFP (middle and right columns). The black images representing the red channel of GFP fusions in the absence of GBP:RFP are not displayed. Scale bar, 20 μm.

fusions. YFP:TGA5 is a YFP fusion to a nuclear localized bZIP transcription factor from Arabidopsis (Kim and Delaney, 2002) that fluoresces exclusively in the nucleus when expressed in *N. benthamiana* (Figure 4). Co-expression of YFP:TGA5 with GBP:RFP resulted in the appearance of yellow fluorescence in the cytosol, indicating that the chromobody perturbed the nuclear accumulation of the YFP:TGA5 fusion (Figure 4). Similar experiments with a CFP:TGA5 fusion confirmed the specificity of the chromobody, since a similar level of nuclear accumulation of CFP:TGA5 was observed in the presence and absence of GBP:RFP (Figure S3).

In addition to the VAMP722 and TGA5 fusions, plastid localized pt-GFP and mitochondrial targeted mt-GFP also showed perturbed localization and significant increases in cytosolic GFP fluorescence in the presence of the chromobody relative to control treatments (Figure 4).

In conclusion, these results indicate that the chromobody can shift or extend the localization of several GFP fusion proteins pointing to the particularly useful application of using GBP:RFP to directly assess the link between cellular localization and function of GFP fused proteins. A summary list of all tested constructs in the chromobody co-expression experiments is shown in Table S2.

Chromobody expression using the tobacco mosaic virus vector pTRBO enables robust mislocalization of target proteins

Considering that the trapping efficiency of GBP:RFP is probably dependent on its steady-state levels in the cell, we cloned the chromobody in pTRBO, a recently developed tobacco mosaic virus (TMV) binary expression construct that enables very high expression levels and high percentage of transformed cells due to cell-to-cell spread of the virus via plasmodesmata (Lindbo, 2007a). In side-by-side expression time courses in *N. benthamiana*, we found that 35S promoter-driven expression of GBP:RFP is detectable as early as 1 dpi of the *A. tumefaciens* strain but that fluorescence levels drop after 2 dpi. In contrast, TMV-mediated expression of GBP:RFP is not detectable at 1 dpi but results in much stronger and sustainable fluorescence levels that peak at 4 dpi (Figure S4 and data not shown).

In co-expression experiments with GFP-tagged proteins, the pTRBO chromobody construct proved highly efficient in causing mislocalization, probably because the ratio of chromobody versus target GFP protein is high (data not shown). We therefore opted to use the pTRBO::*GBP:RFP* for subsequent mislocalization assays. The optimal conditions for mislocalization assays consist of an initial infiltration with the *A. tumefaciens* pTRBO::*GBP:RFP* strain, followed by infiltration with the GFP fusion strain 2–3 days later, with the microscopic and phenotypic scoring taking place in the subsequent days.

Chromobody alters the function of target proteins

Once we established that the chromobody can trap target proteins in the cytoplasm, we endeavored to test whether it can affect a set of known localization-dependent functions in plants. We selected proteins involved in innate immunity and apoptosis, such as a disease effector protein from the bacterial plant pathogen *Xanthomonas gardneri* (AvrHah1; Schornack *et al.*, 2008), *N. benthamiana* Map

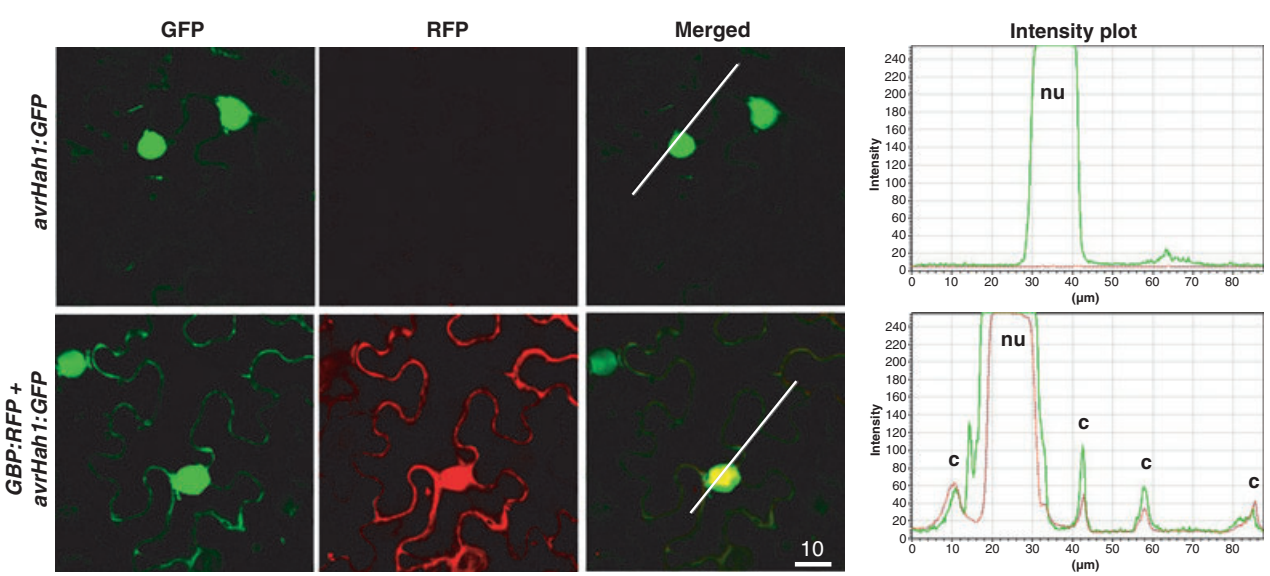

Figure 5. Chromobody alters localization of AvrHah1:GFP.
Confocal imaging of green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) (red) and AvrHah1:GFP (green) 2 days after agroinfiltration of *Nicotiana benthamiana* leaf tissue. Agroinfiltrated T-DNA constructs are indicated at the left side. White solid bars represent transects through cells with fluorescence intensities plotted on the right. Scale bar, 20 μm. c, cytosol, nu, nucleus.

Kinase Kinase 1 (NbMKK1; Yoshihiro *et al.*, 2008) and mouse Bax protein (Lacomme and Santa Cruz, 1999), all of which induce the hypersensitive response (HR), an easily visualized form of programmed cell death (Lam, 2008). The cell-death inducing activities of both AvrHah1 and NbMKK1 are dependent on nuclear localization (Schornack *et al.*, 2006; Yoshihiro *et al.*, 2008; Figure 6a,b) whereas Bax was shown to localize to mitochondria when exerting its cell death triggering activity (Lam *et al.*, 2001; Yoshinaga *et al.*, 2005). In contrast to NbMKK1 and Bax, AvrHah1 triggers cell death only in the presence of the corresponding plant resistance gene *Bs3* (Schornack *et al.*, 2008). This allows avoidance of the problematic side-effects of cell death responses on *in vivo* fluorescence microscopy by studying chromobody-dependent alteration of AvrHah1 localization in plants lacking *Bs3*, while parallel experiments with plants expressing *Bs3* could be used to assess the effect of chromobody expression on execution of cell death.

We observed that agroinfiltration of *avrHah1:GFP* in *N. benthamiana* resulted in green fluorescence confined to the nucleus, as reported before (Schornack *et al.*, 2008; Figures 5 and S5). However, co-infiltration of *A. tumefaciens* strains carrying *avrHah1:GFP* with strains with pTRBO::*GBP:RFP*, which produces red fluorescence in both the cytoplasm and nucleus, caused a significant shift of green fluorescence towards the cytoplasm (Figures 5 and S5). This indicates that a portion of AvrHah1:GFP was trapped by the chromobody in the cytoplasm. Next, we performed agroinfiltration of *avrHah1* and *Bs3* in *N. benthamiana* and observed the expected rapid cell death response due to the HR (Schornack *et al.*, 2008; and Figure 6c). Remarkably, co-expression of *avrHah1* and *Bs3* with pTRBO::*GFP:RFP* resulted in a significant reduction in cell death (Figure 6d). Cell death was also reduced following co-infiltration of the chromobody with *avrHah1:GFP* in pepper plants (*Capsicum annuum* ECW123) that carry *Bs3* as an endogene (Figure 6c). These results indicate that the par-

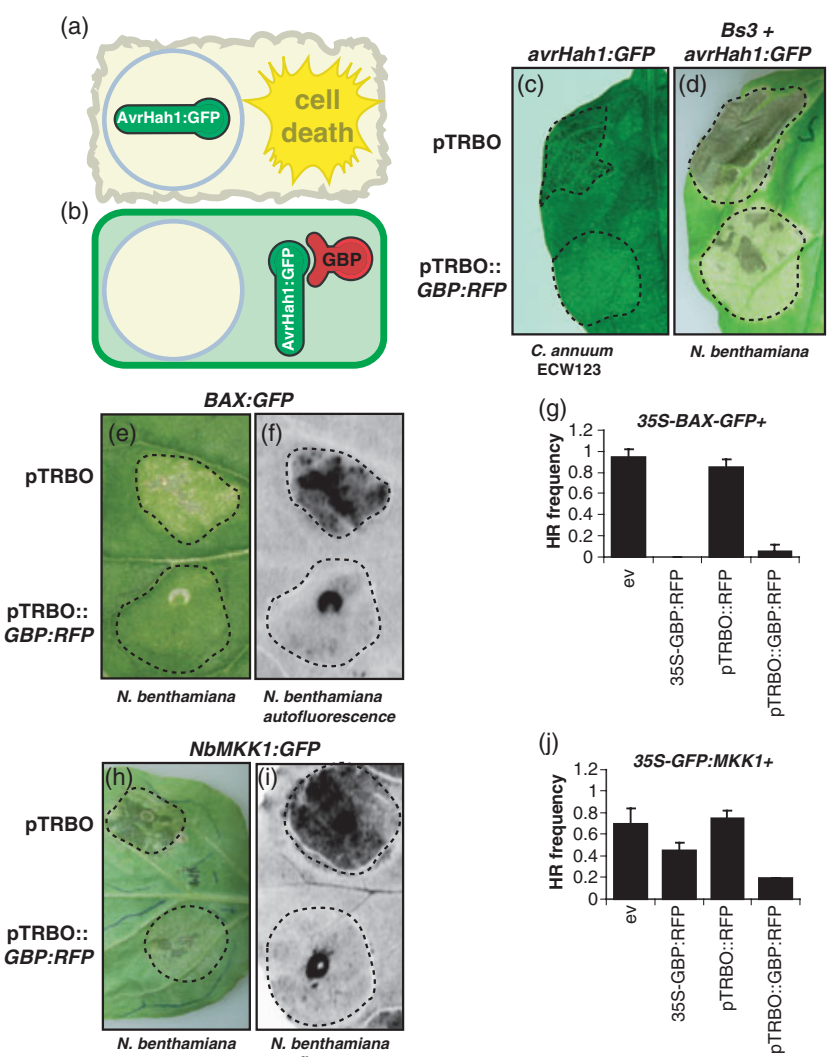

Figure 6. Chromobody affects the cell death-inducing activity of GFP fusions.
The presence of AvrHah1:GFP in the nucleus is known to trigger cell death (a) and we tested whether mislocalization of AvrHah1:GP BAX:GFP and NbMKK1:GFP to the cytoplasm results in reduced cell death activity (b).
All GFP fusions named on top were agroinfiltrated into leaves of plant species named below images. Twenty four hours prior to infiltration of the cell death-inducing construct, either empty pTRBO vector (upper infiltration spots) or pTRBO::*GBP:RFP* (lower spots) were agroinfiltrated. Pictures were taken 3 days post-infiltration (dpi; c, d, h, i) and 5 dpi (e, f). Some leaves were imaged using ultraviolet (UV) excitation to visualize autofluorescence (f, i). For Bax:GFP (g) and NbMKK1:GFP (j) activities we counted the loss-of-cell death events upon co-expression of ev (empty vector) versus 35S-*GBP:RFP* or prior expression of pTRBO::*RFP* versus pTRBO::*GBP:RFP*. Displayed are the means and standard deviation of two independent experiments.

tially altered localization of AvrHah1:GFP resulted in altered cell death-inducing activity.

We performed similar pTRBO::*GBP:RFP* or *35S-GBP:RFP* chromobody co-expression experiments with GFP fusions of BAX or NbMKK1 (Figure 6e–j). A significant reduction in cell death was also observed in the presence of the chromobody. Taken together, our experiments showed that GBP interferes with the localization-dependent activities of three GFP-tagged proteins indicating broad applicability of the method.

DISCUSSION

In this study we applied the GBP nanobody/chromobody technology recently pioneered by Rothbauer *et al.* (2006, 2008) to plant cell biology. Functional GBP can be expressed in plant cells and used for specific and high-affinity immunoprecipitation of GFP expressed in *N. benthamiana*. Most interestingly, we showed that co-expression of the chromobody (GBP:RFP fusion) with GFP-tagged proteins caused mislocalization of the GFP protein resulting in accumulation in the cytoplasm and altered functionality. Therefore, GBP nanobody/chromobody technology provides an alternative and complementary experimental approach to mislocalize plant proteins and to directly link cellular localization to function.

Depending on the nature and relative expression levels of the target protein (antigen), the chromobody might serve as a decorator and signal amplifier or trap. Co-expression of the chromobody with a GFP protein at similar levels generally results in co-localization of red and green fluorescence. Although layering an RFP signal on top of a GFP signal does not provide additional information on protein localization equal level expression provides an important control step prior to mislocalization experiments because it helps to determine whether the chromobody can mislocalize target antigens present in certain subcellular localizations. The relatively small size of the GBP:RFP chromobody (41 kDa) appears to allow passive diffusion into the nucleus but not to other subcellular compartments. Our experiments using several antigens with varying subcellular localizations indicated that the chromobody cannot enter plastids, mitochondria, Golgi or the endoplasmic reticulum (ER) to reach a given antigen (Figures 2 and 4). We conclude that accessibility of the GFP epitope to the chromobody in the plant cytosol is required for successful mislocalization. The ER- and Golgi-localized GFP fusions enter the lumen of their compartments upon synthesis and appear not to be accessible, whereas mitochondrial and plastid proteins pass through the cytosol prior to organellar import and thus can be intercepted (Table S2). Interestingly, the successful mislocalization of GFP:VAMP722 fusion is consistent with the prediction that the GFP epitope is exposed to the cytosolic side of the endomembrane compartments (Table S2). Further analysis of subcellular marker constructs will clarify which fusion proteins are best suited for mislocalization assays.

In principle, the chromobody could be used in various subcellular compartments but this requires fusion to specific targeting signals. For instance, addition of a C-terminal NLS or peroxisome targeting signal to the chromobody is expected to result in nuclear or peroxisomal traps, whereas N-terminal fusions of plastidic or mitochondrial signal sequences would generate organelle traps. Previously, Jobling *et al.* (2003) successfully targeted a VHH domain to the plastid by addition of an N-terminal plastid translocation sequence, while Rothbauer *et al.* (2008) generated fusions of GBP to a nuclear lamina-specific protein to deplete an antigen from the nuclear interior. Nevertheless, we demonstrate that a variety of GFP fusion proteins localized to endomembrane compartments, mitochondria, plastids and nuclei can be shifted in their localization by the current version of the chromobody (Figures 3–5).

Co-expression of the chromobody with GFP-fused nuclear antigens displaced them from their original location and interfered with their function (Figures 4, S3 and S5). Although mistargeting of the GFP-tagged protein is sufficient to explain the loss of function, inactivation due to chromobody binding may also be a contributing factor. For example, the chromobody blocked the cell death-inducing activity of NbMKK1 and AvrHah1, which are both known to require nuclear localization for functionality (Schornack *et al.*, 2008; Yoshihiro *et al.*, 2008). Although the chromobody caused a considerable shift in the subcellular localization of NbMKK1 and AvrHah1, levels of these proteins are still detected in the nucleus and could have been directly inactivated via binding to the GBP:RFP chromobody. Similarly, the chromobody reduced the cell death-inducing activity of BAX:GFP, which is thought to trigger cell death following association with mitochondria and subsequent cytochrome *c* leakage (Yoshinaga *et al.*, 2005). Because chromobody fluorescence was not detected in mitochondria (Figures S1 and S4), we assume that inactivation of BAX:GFP activity probably occurred in the cytoplasm, possibly through interference with BAX translocation to mitochondria. Unfortunately, we could not test this hypothesis and study BAX cellular localization in plant tissue due to the high background fluorescence and cellular disintegration caused by the cell death response.

Our experiments showed that a molecular trap created by expression of the chromobody in the cytosol can provide evidence that a given subcellular compartment, in this case the nucleus, is crucial for the function of GFP-tagged proteins. Although this method probably requires alternative experiments to support data on uncharacterized proteins, it complements the repertoire of experimental methods available to plant cell biologists to link localization to function (Reddy *et al.*, 2007) and provides an independent alternative approach to mutation or fusion of heterologous signals. Existing methods to interfere with the spatial distribution of a protein consist of irreversible modification

of the wild-type protein by mutation or deletion of critical amino acids. Deletion and replacement of secretion signal sequences or NLS motifs are routinely used in plant biology (Van den Ackerveken *et al.*, 1996; Citovsky *et al.*, 2006). However, such mutations can be intrusive and may alter the integrity and biochemical properties of the protein. A less disruptive approach consists of addition of anchor or target sequences to the protein to redirect it to a different compartment. Transmembrane stretches, nuclear exclusion signals and organelle targeting signals are among the sequences used to enrich or deplete proteins from particular subcellular compartments (Nelson *et al.*, 2007). However, this approach requires irreversible alteration of the protein under study and generation of new recombinant DNA constructs. In contrast, chromobody technology can be readily applied to any existing GFP fusion construct and thus provides the possibility of interfering with stimulus-dependent dynamic relocalization patterns of protein pools, and should therefore prove immediately useful to functionally and biochemically characterize GFP-tagged proteins.

For proof of concept, we applied chromobody-mediated mislocalization to proteins involved in plant immunity. It is well established that pathogenic invasion of plants induces substantial subcellular reorganization and that microbial pathogens can deliver disease effector proteins to subcellular compartments of host plant cells (Deslandes *et al.*, 2003; Burch-Smith *et al.*, 2007; Wiermer *et al.*, 2007; Hoefle and Hückelhoven, 2008; Lipka *et al.*, 2008). Bacterial plant pathogens of the genus *Xanthomonas* deliver effector proteins to host plant nuclei. These effectors, known as transcription activator-like (TAL) or AvrBs3-family effectors, alter plant gene expression, resulting in virulence-like effects or induction of host immunity depending on the plant genotype (Schornack *et al.*, 2006; Kay *et al.*, 2007). One such effector protein, the *Xanthomonas gardneri* AvrHah1, activates the pepper *Bs3* resistance gene resulting in hypersensitive cell death (Schornack *et al.*, 2008). Here we demonstrate that trapping of AvrHah1:GFP in the cytosol significantly attenuates the induction of cell death in *Bs3* pepper plants, suggesting that the avirulence activity occurs in the host nucleus (Figure 6). Our data provide independent confirmation of prior experiments based on NES fusion to AvrHah1 that also indicated that activation of *Bs3*-dependent cell death requires localization in the host nucleus (Schornack *et al.*, 2008). To conclude, we focused on plant immunity to demonstrate that chromobody technology does work in plants, but that this method should be applicable to other areas of plant cell biology whenever a functional GFP-tagged protein is available. It should be noted, however, that only partial depletion of GFP-fused proteins might be observed in some cases. This requires a quantitative read-out to study the effect on a given phenotype. In our case we observed significant reduction in cell death (Figure 6g,j).

We confirmed the findings of Rothbauer *et al.* (2008) that the GBP:RFP chromobody has selective affinity towards GFP and YFP *in vitro* and *in vivo* but does not effectively bind CFP. Why is that a useful attribute of GBP? A CFP-tagged protein can be used as a negative control in chromobody mislocalization experiments for any given GFP/YFP-tagged construct (Figure S3). Also, because CFP and YFP fluorescence spectra have little overlap and relative fluorescence ratios can be measured, constructs with the two fluorescent tags can be co-expressed in the presence of the chromobody resulting in an intracellular negative control. Finally, any chromobody-mediated phenotype can be complemented with a CFP fusion. This should enable generation of inducible mutant phenotypes by interfering with otherwise fully functional proteins along with the possibility of complementing and monitoring *in planta* localization.

Another consequence of the high specificity of GBP is to use it as an antibody to label fluorescent proteins in western blots and enable immunoaffinity purification of GFP/YFP-tagged proteins (Rothbauer *et al.*, 2008). Partially purified plant extracts containing GBP could be used in immunological experiments in a similar way to mammalian antisera. It should be pointed out that although GFP is the most widely used epitope in biology it is rarely used as an immunotag, probably due to the lack of reasonably effective anti-GFP sera (SS and SK, unpublished results).

We foresee many applications of chromobody technology to several areas of plant biology. In addition to GBP-mediated mislocalization, GBP can also be used to enable purification of protein complexes containing GFP-fused bait proteins and enrichment of cellular compartments by membrane-anchored GFP fusions. For instance, we are currently using GBP to affinity purify GFP-tagged endomembrane compartments (RF and VL, unpublished results). GBP can also be exploited in stable transgenic lines. Expression of the chromobody under inducible stage- or tissue-specific promoters would enable interference with otherwise fully functional GFP fusions at particular developmental stages or in tissue-specific processes. In conclusion, nanobody/chromobody technology provides an alternative to existing plant cell biology methods and should contribute to addressing the challenge of linking cellular localization to function.

EXPERIMENTAL PROCEDURES

Plants, bacterial strains and plasmids

Nicotiana benthamiana and pepper (*Capsicum annuum* ECW123, obtained from T. Lahaye, Halle, Germany) were grown at 25°C, 60% humidity and under 16-h light/8-h dark cycles. *Escherichia coli* DH5α, BL21 and *Agrobacterium tumefaciens* GV3101 were routinely grown in LB medium (Bertani, 1951) at 37 and 28°C, respectively.

A plant codon-optimized GBP:RFP open reading frame (ORF) was synthesized by Genscript Corp. (http://www.genscript.com/) based on amino acid sequences of GBP (Rothbauer *et al.*, 2006) and RFP

(GenBank AAM54544), and was cloned into the TMV vector pTRBO (Lindbo, 2007a) resulting in pTRBO::*GBP:RFP*. Gateway technology (Invitrogen, http://www.invitrogen.com/) was used for subsequent cloning. An entry clone harboring GBP:RFP was generated by PCR amplification of the *GBP:RFP* ORF without its stop codon. Entry clones harboring the coding sequence of Arabidopsis TGA5 (GenBank Q39163) and *N. benthamiana* MKK1 (GenBank AB243987 (Takahashi *et al.*, 2007) were generated by amplification from cDNA using primers that bypass the stop codon. Mouse BAX was amplified from a cDNA clone harboring the coding sequence. All amplicons were cloned into the entry vector pENTR/D-TOPO (Invitrogen). The GFP fusions were generated by Gateway LR recombination (Invitrogen) of entry clones with pK7FWG2 (Karimi *et al.*, 2002) resulting in 35S-*NbMKK1:GFP* and 35S-*BAX:GFP*, respectively. Gateway LR recombination was also used to generate other fusions (see Table S1 for details) using the destination vectors pGWB11 (C-terminal FLAG epitope), pGWB8 (C-terminal 6×His epitope), pGWB42 (N-terminal YFP) and pGWB45 (N-terminal CFP; Nakagawa *et al.*, 2007).

35S-*GFP:VAMP722* was constructed by amplifying VAMP722 ORF from cDNA and subsequent cloning via primer-introduced *Nco*I and *Xma*I sites into pGJ2185 (Kwon *et al.*, 2008).

35S-*AvrHah1:GFP* (Schornack *et al.*, 2008) and an *A. tumefaciens* binary construct expressing *Bs3* under the control of its native promoter (Bs3p-*Bs3*, (Roemer *et al.*, 2007) were obtained from T. Lahaye (Halle, Germany). Subcellular fluorescent marker constructs (Nelson *et al.*, 2007) were obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org/).

Transient *in planta* expression

In planta transient expression by agroinfiltration (35S promoter-based *A. tumefaciens* T-DNA binary constructs) or agroinfection (TMV-based binary constructs) was performed according to methods described elsewhere (Huitema *et al.*, 2004; Lindbo, 2007b; Schornack *et al.*, 2008). *Agrobacterium tumefaciens* GV3101 (Van Larebeke *et al.*, 1974) was used to deliver T-DNA constructs into 3-week-old *N. benthamiana* or pepper plants. Overnight *A. tumefaciens* cultures were harvested by centrifugation at 10 000 ***g***, and resuspended in infiltration medium [10 mM $MgCl_2$, 5 mM 2-(*N*-morpholine)-ethanesulfonic acid (MES), pH 5.3, and 150 μM acetosyringone) to an OD_{600} = 1.0 prior to syringe infiltration into leaf panels. To enhance expression, bacterial suspensions were infiltrated together with a construct expressing the gene-silencing inhibitor *p19* expressing construct (Voinnet *et al.*, 2003).

Protein extraction and immunoprecipitation

GFP-Trap® was provided by ChromoTek GmbH (http://www.chromotek.com/). Protein extraction and immunoprecipitation was essentially carried out as described (Moffett *et al.*, 2002) using 0.15% NP-40, but omitting IgG pre-clearing. For FLAG IP, bound proteins were eluted with 3 × FLAG peptides. The GFP and derivatives were detected using anti-GFP (rabbit IgG fraction, SKU# A-11122, Invitrogen) at a dilution of 1:5000.

Confocal microscopy

Cut leaf patches were mounted in water and analyzed on a Leica DM6000B/TCS SP5 confocal microscope (Leica Microsystems, http://www.leica-microsystems.com/) with the following excitation wavelengths: CFP, 458 nm; GFP, 488 nm; YFP, 514 nm; RFP, 561 nm. Scanning was performed in sequential mode to prevent signal bleed-through. Identical microscope power settings were applied for all individual images of a figure to allow comparison of fluorescence intensities between samples. Fluorescence intensity transects were generated using the Leica analysis software LAS AF1.8.2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Subcellular distribution of green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) *in vivo.*

Figure S2. C-terminal epitopes do not affect trapping function and subcellular distribution of the antibody.

Figure S3. Green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) alters localization of yellow fluorescent protein (YFP):TGA5 but not cyan fluorescent protein (CFP):TGA5.

Figure S4. Levels of transiently expressed green fluorescent protein-binding protein:red fluorescent protein (GBP:RFP) are affected by the expression vector used.

Figure S5. Chromobody alters localization of AvrHah1:GFP.

Table S1. Constructs used in this study.

Table S2. Localization/orientation of GFP/yellow fluorescent protein (YFP) fusions.

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