In Vivo Imaging of Diacylglycerol at the Cytoplasmic Leaflet of Plant Membranes

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Diacylglycerol (DAG) is an important intermediate in lipid biosynthesis and plays key roles in cell signaling, either as a second messenger itself or as a precursor of phosphatidic acid. Methods to identify distinct DAG pools have proven difficult because biochemical fractionation affects the pools, and concentrations are limiting. Here, we validate the use of a genetically encoded DAG biosensor in living plant cells. The sensor is composed of a fusion between yellow fluorescent protein and the C1a domain of protein kinase C (YFP–C1aPKC) that specifically binds DAG, and was stably expressed in suspension-cultured tobacco BY-2 cells and whole Arabidopsis thaliana plants. Confocal imaging revealed that the majority of the YFP–C1aPKC fluorescence did not locate to membranes but was present in the cytosol and nucleus. Treatment with short-chain DAG or PMA (phorbol-12-myristate-13-acetate), a phorbol ester that binds the C1a domain of PKC, caused the recruitment of the biosensor to the plasma membrane. These results indicate that the biosensor works and that the basal DAG concentration in the cytoplasmic leaflet of membranes (i.e. accessible to the biosensor) is in general too low, and confirms that the known pools in plastids, the endoplasmic reticulum, mitochondria and glycolipids at plastids (Bruce 1998, Dörmann and Benning 2002, Benning 2009, Shimojima and Ohta 2011, Muthan et al. 2013). In signaling, DAG is typically generated through activation of the phospholipase C (PLC) pathway, which causes the minor lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) to be hydrolyzed into the second messengers, DAG and inositol 1,4,5-trisphosphate (IP3). While the latter diffuses into the cytosol where it triggers the release of Ca2+ from an internal store through activation of a ligand-gated calcium channel, DAG remains in the plasma membrane where it recruits and activates members of the TRPC (transient receptor potential, canonical) family of ion channels (Albert 2011).

While DAG and IP3 classically act downstream of the canonical ‘PLC signaling cascade’ in animals, in plants this pathway is still obscure, basically because they lack homologs for PKC, TRPC and IP3 receptors in their genomes (Munnik 2014). Nonetheless, evidence is accumulating that plants use the phosphorylated forms of DAG and IP3 as second messengers, i.e. phosphatidic acid (PA) and inositolpolyphosphates (IPPs), such as IP4, IP5 and IP6 (Arisz et al. 2009, Munnik and Vermeer).
active PLC continuously hydrolyzing PI(4,5)P$_2$ into DAG and 1998a, Munnik et al. 1998b, Meijer and Munnik 2003, Munnik and Testerink 2009, Munnik 2014), and the so-called ‘non-specific PLC’ (abbreviated as NPC), which hydrolyzes structural phospholipids such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as substrate (Munnik et al. 1994, Munnik et al. 1998a). In mammalian cells the concentration of these minor lipids is <0.1% of all glycerolipids (Munnik et al. 1994, Munnik et al. 1998a). In mammalian cells for examples PI(4,5)P$_2$, PI(3,4,5)P$_3$, PI(3,5)P$_2$, and PI(3,4)P$_2$ are localized (Munnik et al. 1998a, Mueller-Roeber and Pical 2002, Heilmann 2008a, Heilmann 2008b, Ischebeck et al. 2010, Munnik and Nielsen 2011, Pokotylo et al. 2014, Heilmann 2016), but basically this has never been addressed (Arisz et al. 2000, Heilmann 2008a, Xue et al. 2009, Ischebeck et al. 2010, Boss and Im 2012, Dong et al. 2012). One of the exciting developments in cell biology has been the ability to visualize certain lipids in vivo using so-called ‘lipid biosensors’, i.e. fusions of a fluorescent protein to a lipid-bind- ing domain specific for a particular lipid (Vermeer and Munnik 2013). When the concentration of this lipid is below the detection level, the probe will be in the cytosol (and often in the nucleus too due to its free diffusion). However, when the concentration of the lipid is high enough, or increases in response to stimulation, then the biosensor will bind the lipid and locate the membrane where the lipid is formed. Obviously, this method only detects lipids at the cytoplasmic leaflet of membranes, but the advantage is that it can reveal its subcellular localization in vivo, in real time, and disclose dynamics that otherwise would have remained hidden. In the mammalian field, such lipid biosensors have been a great success (Hammond and Balla 2015). Similarly, for the plant field, four distinct lipid biosensors have successfully been characterized, i.e. for PI(4,5)P$_2$ (Vincent et al. 2005, van Leeuwen et al. 2007, Simon et al. 2014), PI4P (Thole et al. 2008, Vermeer et al. 2009, Simon et al. 2014), PI3P (Voigt et al. 2005, Vermeer et al. 2006, Simon et al. 2014) and phosphatidylserine (PS) (Yamaoka et al. 2011). Here, we describe and validate a biosensor that monitors DAG. It consists of a fusion between the yellow fluorescent protein (YFP) and the cysteine-rich 1a (C1a) domain of human PKC$_\gamma$ that specifically binds DAG and has been extensively characterized in mammalian cells (Oancea et al. 1998, Kim et al. 2011). The probe has been used in tobacco pollen tubes previously, but in that study only transient expression using a pollen-specific promoter after particle bombardment was reported, and DAG binding was never validated (Helling et al. 2006). Here we used transient expression of the DAG biosensor in cowpea proplastids and also stably expressed it in two model systems, i.e. tobacco BY-2 cells and Arabidopsis thaliana plants. DAG binding was validated using a short-chain analog and PMA (phorbol-12-myristate-13-acetate), a phorbol ester that mimics DAG binding to the C1a domain and in animal cells activates PKC (Oancea et al. 1998). We found that the biosensor was mostly localized in the cytosol, indicating that the concentration of DAG in the cytoplasmic leaflet of membranes is normally too low to be detected by YFP–C1aPKC. Detailed further analysis, however, revealed four novel DAG pools: one at the cytoplasmic leaflet of Golgi membranes and three very local and temporal pools at the plasma membrane, i.e. in root epidermal cells of the transition zone, in dividing cells at the growing cell plate and during polarized tip growth in root hairs. The results provide new insights into the spatiotemporal dynamics of plant DAG and offers a new tool to monitor this in vivo.
Results

YFP–C1aPKC localization in tobacco BY-2 cells

In mammalian cells, YFP–C1aPKC has been shown to function as a robust DAG biosensor (Oancea and Meyer 1998, Oancea et al. 1998). To investigate its use in plant cells, stable transgenic tobacco BY-2 cells were generated that expressed YFP–C1aPKC under the control of the constitutive 35S promoter. As shown in Fig. 1, most of the YFP–C1aPKC fluorescence was localized in the cytosol and nucleus, like YFP alone (Fig. 1). Nonetheless, some signal was present as motile, punctate structures (arrowheads in Fig. 1), but no clear plasma membrane signal was visible. To test the functionality of the DAG biosensor, the phorbol ester PMA was tested. PMA mimics the binding of DAG to the C1a domain and is therefore a potent activator of PKC activity in vivo and causes a rapid recruitment of YFP–C1aPKC to the plasma membrane of animal cells (Oancea and Meyer 1998, Oancea et al. 1998). Treatment of our tobacco YFP–C1aPKC cells with 1 μM PMA also resulted in a strong relocalization of the biosensor to the plasma membrane (Supplementary Fig. S1). As a second control, we tested a short-chain analog of DAG, i.e. 1,2-dioctanoyl sn-glycerol (DiC8), which has the advantage of being water soluble compared with normal DAG. While DiC8 failed to induce a relocalization of the biosensor in BY-2 cells (data not shown), it did trigger the relocation of YFP–C1aPKC to the plasma membrane of cowpea protoplasts in a fashion similar to that of PMA (Supplementary Fig. S2). Most probably, the DiC8 gets stuck in the cell wall. Together these results indicate that YFP–C1aPKC monitors DAG in the cytoplasmic leaflet of plant membranes in vivo, but that the basal concentrations of such pools are relatively low.

DAG dynamics during cytokinesis in BY-2 cells

During cytokinesis, distinct labeling patterns and dynamics for PI3P, PI4P and PI(4,5)P2 biosensors were found in tobacco BY-2 cells. For example, PI3P was always present on punctate structures that surrounded the cell plate like a donut structure but was never part of the cell plate (Vermeer et al. 2006). In contrast, the PI4P biosensor labeled the cell plate right from the start of its formation (Vermeer et al. 2009), while the PI(4,5)P2 biosensor only labeled the leading edges of the growing cell plate (van Leeuwen et al. 2007). Hence, we decided to follow the dynamics of the DAG biosensor during cytokinesis.

Following cell divisions in the YFP–C1aPKC BY-2 cell line, we found that the biosensor labeled the newly formed cell plate right up until fusion with the parental plasma membrane occurred (Fig. 2; Supplementary Movie S1). Co-labeling with the endosomal tracer FM4-64 suggested that the YFP–C1aPKC appeared only slightly later (Fig. 2; Supplementary Movie S2).
which is reminiscent of what we observed for PI4P (Vermeer et al. 2009). These results indicate that during cell division, relatively high amounts of DAG are formed.

Localization of YFP–C1aPKC in Arabidopsis seedlings

In order to image DAG dynamics in whole plants, we generated transgenic Arabidopsis lines that expressed 35S-EYFP–C1aPKC. To increase the avidity of the biosensor, we also generated lines that expressed two C1a domains in tandem (EYFP–2x/C2C1aPKC). Such an approach has had success before to monitor PI3P and PI(4,5)P2, whose concentrations are also very low (Vermeer et al. 2006, Simon et al. 2014). To avoid potential problems due to the strong activity of the 35S promoter, we also generated lines expressing the DAG biosensor under the control of the UBQ10 promoter. This drives stable and lower levels of expression, which are still sufficient for confocal microscopy (Geldner et al. 2009). At least 6–10 independent lines were analyzed and all showed similar localization patterns. Importantly, all lines appeared and grew like wild-type plants (Supplementary Fig. 3).

In Fig. 3, a typical fluorescence pattern of UBQ10:EYFP, 35S:EYFP–C1aPKC, UBQ10:EYFP–C1aPKC and UBQ10:EYFP–2x/C1aPKC in the epidermis of roots of 5-day-old Arabidopsis seedlings is shown. Unlike free YFP that is located in the cytosol and nucleus, YFP–C1aPKC fluorescence was found in punctate structures that were also observed in the tobacco BY-2 cells expressing the DAG biosensor (Fig. 3A–L). Interestingly, in the epidermis of the transition zone of the primary root, we could now also detect some YFP–C1aPKC labeling at the plasma membrane (Fig. 3). Although the UBQ10:EYFP–C1aPKC lines showed much weaker fluorescence compared with the 35S line, the observed labeling pattern was very similar (Fig. 3B, C). The UBQ10:EYFP–2x/C1aPKC lines displayed a similar localization pattern, but with a much stronger labeling of both punctate structures and the plasma membrane (Fig. 3D, J). This is likely to be due to the higher avidity of this biosensor. Interestingly, the plasma membrane localization of the biosensor gradually disappeared as the epidermal cells started to elongate (Fig. 3D–F, J–L). Western blot analysis of the lines expressing the different biosensors indicated that most of the fusion protein was intact and had the predicted size (Supplementary Fig. S4).

Differentiated root cell types and leaf pavement cells revealed similar fluorescence patterns of YFP–C1aPKC and YFP–2x/C2C1aPKC, although we did not observe labeling of the plasma membrane (Supplementary Fig. S5).

To validate DAG binding, we also generated plants expressing a fluorescent protein fused to the C1aPKC domain carrying a P46G mutation, which is essential for DAG binding (Oancea et al. 1998). As shown in Supplementary Fig. S6, mCherry–C1aPKC-P46G was localized in the cytosol of root epidermal cells and root hairs. Upon PMA treatment, it no longer translocated to the plasma membrane while the intact EYFP–C1aPKC did. These data support that the observed localization pattern of EYFP–C1aPKC is dependent on its lipid-binding capacity.

YFP–C1aPKC reveals a pool of DAG at the trans-Golgi network

In mammalian cells, small amounts of DAG have been reported to reside at the trans-Golgi network (TGN) where it is involved...
in membrane fission to form transport carriers (Yeaman et al. 2004, Bossard et al. 2007). To investigate the identity of the punctate structures that we observed, plants expressing YFP–C1aPKC were crossed with UBQ10::mCherry–RabA1g and with the PI4P biosensor 35S::mRFP–PH FAPP1. RabA1g accumulates at the TGN, while mRFP–PH FAPP1 also labels, in addition to the plasma membrane, Golgi membranes (Geldner et al. 2009, Vermeer et al. 2009, Simon et al. 2014). As is clear from Fig. 4, a partial co-localization between YFP–C1aPKC and both Golgi markers was observed.

The fungal toxin brefeldin A (BFA) has been shown to inhibit Golgi trafficking and to induce the appearance of large, so-called BFA compartments (Geldner et al. 2003). BFA treatment (50 μM, 45 min) resulted in a strong accumulation of both YFP–C1aPKC and RabA1g in BFA compartments (Fig. 4), again suggesting that YFP–C1aPKC detects DAG at the TGN. As was reported previously, mRFP–PH FAPP1 labeled structures that were much more resistant to BFA treatment and only showed a weak accumulation in BFA compartments (Fig. 4). BFA treatment of UBQ10::EYFP–C1aPKC and UBQ10::EYFP–2 × C1aPKC seedlings co-incubated with FM4-64 (a fluorescent lipophilic membrane dye) also revealed a clear accumulation of both YFP and FM4-64 signal in BFA compartments (Supplementary Fig. S7).

**YFP–C1aPKC accumulates at the tip of growing root hairs**

When analyzing growing root hairs, a polar localization of the YFP–C1aPKC signal was observed, which changed during development (Fig. 5). In young root hairs establishing tip growth, YFP–C1aPKC was localized to the apex of the root hair, whereas in older root hairs that had fully established tip growth, YFP–C1aPKC labeled the shanks of the root hair, just below the apex, but also faintly a compartment just below the tip of the growing root hair (Fig. 5). This localization pattern resembled that of the TGN-localized small GTPase, AtRABA4b (Preuss et al. 2006, Kang et al. 2011) and may indicate that DAG is involved in membrane trafficking required for the polar growth of root hairs. Interestingly, EYFP–2 × C1aPKC showed a much stronger plasma membrane localization, which could already be detected during bulge formation (Fig. 5). During establishment of tip growth, EYFP–2 × C1aPKC shifted its plasma membrane localization from the apex to the shanks (Fig. 5; Supplementary Movie S3).

**Discussion**

**Using YFP–C1aPKC as a biosensor to visualize DAG in living plant cells**

In mammalian cells, YFP–C1aPKC is a well-established biosensor for imaging the localization and dynamics of DAG (Oancea and Meyer 1998, Oancea et al. 1998, Kim et al. 2011). In this study, its potential to image DAG in two well-established plant systems is described, i.e. tobacco BY-2 cells and Arabidopsis seedlings.

Confocal imaging revealed that most of the YFP–C1aPKC fluorescence was located in the cytosol and nucleus; a pattern that is similar to cells expressing YFP alone (Figs. 1, 3). However, PMA, a phorbol ester that mimics DAG binding, and a short-chain analog of DAG (DiC8) were both able to relocalize the biosensor to the plasma membrane (Supplementary Figs. 1200
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Fig. 4 YFP–C1aPKC detects DAG at the TGN. Confocal fluorescence images of Arabidopsis seedlings co-expressing 35S::EYFP–C1aPKC and UBQ10::mCherry–RabA1g (A–C and E–G) or 35S::EYFP–C1aPKC and 35SmRFP–PHFAPP1 (H–J and L–N). EYFP–C1aPKC shows partial co-localization with both the TGN marker mCherry–RabA1g (D) and the PI4P biosensor mRFP–PHFAPP1 (K). (E–G and L–M) The effect of BFA treatment (50 μM 45 min) on the subcellular localization of the marker used. Whereas both EYFP–C1aPKC and mCherry–RabA1g show strong accumulation in BFA compartments, mRFP–PHFAPP1 only shows weak accumulation. (D and K) Quantification of co-localization based on the images including mCherry is shown in magenta. Scale bars = 10 μm.

S1, S2). Mutating the amino acid residue critical for lipid binding rendered the fusion protein cytosolic and insensitive to PMA treatment in Arabidopsis seedlings (Supplementary Fig. S6). Together these data confirm that the biosensor is bona fide and detects DAG. The predominant cytosolic localization of the fusion protein indicates that the DAG level in the cytoplasmic leaflets of most plant membranes is relatively low, which is actually similar to animal cells. Some DAG could be in the nucleus (Albi et al. 2008, Dieck et al. 2012a, Dieck et al. 2012b), although we did not observe any clear relocalization of YFP–C1aPKC into the nucleus, while PMA treatment diminished this nuclear fluorescence, suggesting that the sensor can freely diffuse between the cytosol and nucleus (Supplementary Fig. S1).

We did not observe labeling of ER, mitochondria or plastids, which are organelles known to contain DAG (Dörmann and Benning 2002, Dong et al. 2012, Muthan et al. 2013). Although this appears to be contradictory at first, one should realize that YFP–C1aPKC is localized in the cytosol so can only detect DAG facing the cytoplasmic leaflet of membranes. Since the biosensor is not detecting any ER-, mitochondrial- or plastid-resident DAG, our results suggest that these DAG pools are located at the luminal side of these organelles. The latter has recently been confirmed for plastids (Muthan et al. 2013). Alternatively, DAG could be bound to proteins with a higher affinity than the biosensor, or, due to its flip–flop behavior, may not be available long enough for the sensor to bind it. Nonetheless, YFP–C1aPKC did detect some transient accumulation of DAG at the plasma membrane of epidermal cells in the transition zone of Arabidopsis roots, and by employing a biosensor with increased avidity, EYFP–2 × C1aPKC, this became more evident. The fact that the latter biosensor also did not reveal any signal at the ER, mitochondrion or plastid confirms that these pools are inaccessible to the biosensor. A recent study in mammalian cells, using a similar approach, did not detect DAG at the ER either, but found a novel motile organelle (Kim et al. 2015).

The observed transient accumulation of YFP–2 × C1aPKC in the root epidermis might reflect a specific role for the phosphoinositide signaling circuit during the transition of epidermal cells into differentiation. This is supported by the recently observed bipolar localization of PI4P, PI(4,5)P2 and the PIP5-kinases, AtPIP5K1 and AtPIP5K2, which was shown to be required for polarity and patterning (Ischebeck et al. 2013, Tejos et al. 2014). The fact that YFP–C1aPKC also detected DAG at the TGN, cell plate and growing root hairs points to differential behavior of DAG in different membranes, which may reflect distinct functions (see below).

An important feature of YFP–C1aPKC and YFP–2 × C1aPKC is that they apparently do not outcompete endogenous DAG binding/metabolism, permitting their use to monitor DAG in living plant cells. Targeting of the biosensor to specific organelles may even be used to investigate intraorganelle DAG pools, although the resolution of the confocal microscope may be too limited to discriminate between soluble and membrane-bound YFP–C1aPKC. Nonetheless, immunogold labeling and electron microscopy could extend the use of this DAG sensor.

The presence of DAG at TGN membranes has also been reported for mammalian cells, where DAG locally activates protein kinase D (PKD) to mediate protein release from the TGN via its C1 domain (Maeda et al. 2001, Baron and Malhotra 2002, Yeaman et al. 2004). Whether similar mechanisms exist in plants is unknown. At least this will not involve PKC or PKD since plant genomes lack homologs of these kinases (Testerink
They do, however, encode proteins containing a C1 domain (or C1-like), but it is unknown whether these domains can actually bind DAG. While direct evidence for a signaling role for DAG is lacking in plants, there is substantial evidence that PLC-generated DAG is rapidly phosphorylated to PA (Munnik et al. 2000, Arisz et al. 2009), and that it is the latter that functions as a lipid second messenger, recruiting and activating signaling proteins, including protein kinases.

**Fig. 5** Polar localization of YFP–C1aPKC in growing root hairs. Confocal fluorescence images of root hairs of 5-day-old Arabidopsis seedlings expressing 35S::YFP–C1aPKC (A–D) or UBQ10::EYFP–2 × C1aPKC (E–J). (A, B) Young bulging root hair, (C, D) root hair undergoing tip growth, (E, F) two bulging root hairs and emerging root hair, (G, H) Plasma membrane labeling of EYFP–2 × C1aPKC at the apex as well as labeling of punctated structures, (I, J) Root hair undergoing tip growth showing accumulation of YFP–2 × C1aPKC fluorescence in the shanks of the root hair apex (see also Supplementary Movie S3). (K–N) Growing root hairs of plants expressing UBQ10::mCherry–C1aPKCmut showing cytosolic localization and absence of a tip-focused gradient. YFP and mCherry are shown in gray (A, C, E, G, I, K, M) or in a false color gradient to enhance signal intensity ranging from 0 to 255 gray values. Scale bar = 10 μm.

The current data may also have implications for the PLC signaling pathway in plants. As mentioned earlier, higher plants exhibit extremely low PI(4,5)P2 levels, and one of the explanations for this could be a high intrinsic PLC activity at the plasma membrane, constantly hydrolyzing PI(4,5)P2 (van Leeuwen et al. 2007). If so, one would expect then to find a steady-state localization of the DAG at the plasma membrane, and this is not what we typically observed (Fig. 1; Supplementary Fig. 4). If, however, this DAG would be instantly converted into PA by DGK, then this could explain the lack of correlation. While PLC and DGK activity are indeed enriched at the plasma membrane of various plant species (Munnik et al. 1998a), it remains unknown whether this occurs in a complex.

Ultimately, the development of a bona fide PA biosensor will provide further insight into the connection between DAG and PA, in localization and kinetics. For pollen, a PA biosensor has recently been reported. It consists of a fluorescent protein fusion with the PA-binding domain of yeast protein, Spo20, which was transiently expressed in pollen tubes (Potocky et al. 2014). We have stably expressed the same biosensor in tobacco BY-2 cells and Arabidopsis plants, but, in contrast to pollen, no typical membrane localization was found, even when cells or seedlings were challenged with cold, heat or salt stress (R. van Wijk, J.E.M. Vermeer and T. Munnik, unpublished), which are known to boost PA levels within minutes (Munnik et al. 2000, Bargmann et al. 2009a, Bargmann et al. 2009b, Mishkind et al. 2009, Arisz et al. 2013). Moreover, a recent lipid binding study on the Spo20 domain suggests that it interacts non-specifically with anionic lipids, including PA, PS and PI(4,5)P2 (Horchani et al. 2014). Hence, future research and clarity will be required to image PA conclusively in plant cells.

Role for DAG during plant cytokinesis

In both plant systems, we found that YFP–C1aPKC and YFP–2×C1aPKC accumulated at forming cell plates (Figs. 2, 3F; Supplementary Movie S1). Co-labeling with the endocytic tracer FM4-64 revealed that this dye labeled the cell plate just slightly before (Fig. 2; Supplementary Movie S1), resembling a pattern that we observed earlier for the PI4P biosensor, YFP–PH4APP1 (Vermeer et al. 2009). The latter clearly labels the Golgi membranes and TGN compartment positioned under the tip, and weak labeling of the plasma membrane at the shanks just below the tip (Fig. 5). The latter localization at the shanks of the root hair tip became very clear in growing root hairs expressing YFP–2×C1aPKC (Fig. 5). The localization of YFP–C1aPKC and YFP–2×C1aPKC is in agreement with the observed accumulation of NtPLC3 in growing pollen tubes, although Helling et al. (2006) did not observe clear labeling of a

TGN compartment using the same C1α-based sensor (Helling et al. 2006). Besides the difference in experimental design (stable vs. transient overexpression), and although tip growth of pollen tubes and root hairs are very much alike, there may also be some differences.

To dissect the possible role(s) of DAG in polar growth, a further in-depth characterization of Arabidopsis PLC and DGK enzymes will be required. Combined with forward (chemical) genetic screens, and based on the relocalization of single and double DAG biosensors, this is likely to provide new insights into plant lipid signaling and metabolism.

**Materials and Methods**

### Constructs

All constructs were produced using standard molecular biological procedures. To generate pEYFP–C1αPKC, the C1α domain (amino acids 26–86) from rat PKCγ was amplified from plasmid pClpcMC–GFP using the primers BglII-C1α-fw gagagacttaggca-gaaggggctgcac and EcoRI-C1α-rv ecgagttcgactttgcagctgc, and ligated to pEYFP-C1. Next, the EYFP–C1αPKC fragment was cloned into pMOND35S (Vermeer et al. 2004) using XbaI and EcoRI. For plant transformation, the 35S-EYFP–C1αPKC–Tnos or the 35S-mRFP–PHAP1–Tnos fragments were transferred to pGreen179 using NotI. To generate pUBQ10:EYFP-C1αPKC and pUBQ10:EYFP-2×C1αPKC, we first cloned C1αPKC and 2×C1αPKC in pUN151, pUN151-C1αPKC and pUN151-2×C1αPKC were recombined into pNIGEL07 using Cre/Lox recombination as described (Geldner et al. 2009). To generate pGreen179-UBQ10:mCherry-C1αPKCPEG, the C1αPKC domain was PCR amplified and cloned in-frame with mCherry using the primers C1α-PanEGF gcggacttaggca-gaaggggctgcac and C1α_XbaIrv gcagtgactgcagaaggtTCCctgcttgaagaaacg. The P46G point mutation was introduced with site-directed mutagenesis using the primers C1α_P > Gw gcgtcttcacagGGAaaccttgcgatctgtactgcactg and C1α_P > Grv cattgaccaagttCTCgcttgagaaagcg. pGreen179-35S-mRFP-PHAP1 has been described before (Vermeer et al. 2009).

### Plant material

Cowpea protoplasts were isolated and transfected as described before (Vermeer et al. 2006). Tobacco BY-2 cells were transformed and subcultured as described previously (Vermeer et al. 2006). Arabidopsis plants were transformed using floral dip transformation (Clough and Bent 1998). Homozygous T3 plants were used for further analysis. Seeds were germinated on 0.5× Murashige and Skoog (MS) containing 1% sucrose and 1% agar. Arabidopsis plants expressing free EYFP or mCherry–Rabar1Ag were from the Wave Line collection (Geldner et al. 2009). F1 seedlings were used for co-localization experiments.

### Confocal microscopy and image analysis

BY-2 cells (4–5 d old) or protoplasts were mounted in eight-chambered cover slides (Nalge Nunc International; www.nunc-brand.com). Five- to seven-day-old seedlings were mounted on cover slides or in Nunc chambers and covered by a cover glass or a block of agar, respectively. Fluorescence microscopy was performed using a Zeiss LSM 510, 710, Leica SPS or SP8 confocal laser scanning microscope implemented on an inverted microscope. Excitation was provided at 488 nm or 561 nm for GFP/ FM4-64 and mCherry, respectively. For YFP fluorescence, an excitation/emission combination of 488 nm and 505–550 nm detection was used. For YFP/FM4-64 fluorescence, an excitation/emission combination of 488 nm/505–550 nm for YFP was combined with long pass 650 nm for FM4-64. For YFP/mRFP/ mCherry dual scanning, we used the excitation/emission combinations of 488 nm/505–550 nm for YFP and 561 nm/600–650 nm for red fluorescent protein (RFP)/mCherry. Cross-talk-free images were acquired by operating the microscope in the sequential-acquisition mode. A Zeiss × 40 water-immersion objective, Zeiss × 40 oil objective, Zeiss × 63 water objective or a Leica × 63 water objective were used. Images were captured and analyzed using ZEISS LSM510 software (version 3.2 SP3), Zeiss ZEN 2012 and LAS-X and processed using FIJI with the macbiophotonics bundle (www.macbiophotonics.ca/imagej/installing_imagej.htm). Intensity correlation analysis delivers a coefficient that ranges between 0.5 and zero for complete co-localization or non-co-localization, respectively. We found some advantages of intensity correlation analysis over other methods, in that it takes into account the relative intensities of co-localizing pixels, and can be performed without thresholding. For the analysis of YFP–C1αPKC-positive structures, regions of interest (ROIs) of entire cells, excluding the plasma membrane, were examined. At least 20 different ROIs were analyzed. Error bars indicate the SD of the mean.

### Western blot analysis

Roots of 11-day old seedlings were ground in liquid nitrogen and proteins were extracted using 2× (w/v) protein extraction buffer (0.5% (v/v) NP-40, 75 mM NaCl, 100 mM Tris-HCl pH 8.0, 1% (w/v) polyvinylpolypyrrolidone (PV/PP), 100 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA (pH 8.0) and complete, EDTA-free Protease Inhibitor Cocktail (Roche)). The total protein concentration in the supernatant (30 min, 18,000 × g, 4°C) was determined by nanodrop (ThermoFisher). A 60 μg aliquot of total protein per sample was separated on a 12% SDS–polyacrylamide gel and blotted on polyvinylidene fluoride (PVDF; Immobilon P, Millipore). The Western blot analysis was performed using a polyclonal anti-GFP rabbit serum (A-6455, ThermoFisher) and a second antibody, goat anti-rabbit peroxidase (131460, Pierce).

### Supplementary data

**Supplementary data** are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

References


