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# Wall-associated kinase 1 (WAK1) is crosslinked in endomembranes, and transport to the cell surface requires correct cell-wall synthesis

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## Summary

**The *Arabidopsis thaliana* wall-associated kinases (WAKs) bind to pectin with an extracellular domain and also contain a cytoplasmic protein kinase domain. WAKs are required for cell elongation and modulate sugar metabolism. This work shows that in leaf protoplasts a WAK1-GFP fusion protein accumulates in a cytoplasmic compartment that contains pectin. The WAK compartment contains markers for the Golgi, the site of pectin synthesis. The migration of WAK1-GFP to the cell surface is far slower than that of a cell surface receptor not associated with the cell wall, is influenced by the presence of fucose side chains on one or more unidentified molecules that might include pectin, and is dependent upon cellulose synthesis on the plasma membrane. WAK is crosslinked into a detergent-insoluble complex within the cytoplasmic compartment before it appears on the cell surface, and this is independent of fucose modification or cellulose synthesis. Thus, the assembly and crosslinking of WAKs may begin at an early stage within a cytoplasmic compartment rather than in the cell wall itself, and is coordinated with synthesis of surface cellulose.**

Key words: Cell wall, Receptor kinase, Endomembranes

## Introduction

The extracellular matrix or cell wall of plant cells is composed of cellulose fibers embedded in a gel of pectin and includes a variety of glycans and proteins (Carpita and McCann, 2000; Cosgrove, 2001). The pectin is thought to be the more flexible part of the matrix, and can be modified to alter the physical properties, extension and osmolarity of the external environment of the cell (O'Neill et al., 2004; Ridley et al., 2001; Willats et al., 2001). Cellulose is synthesized by a rosette of cellulose synthases on the plasma membrane, such that cytoplasmic sucrose synthase supplies UDP-glucose monomers that are fed to cellulose synthase to become polymerized into cellulose fibers in the cell wall (Kohorn, 2000). Pectin and hemicellulose, and a number of other carbohydrate polymers, are instead synthesized in the Golgi, and then transported to the cell surface to complex with cellulose and other wall components (Willats et al., 2001). Numerous proteins are also secreted via the Golgi and are thought to be complexed with the carbohydrate component of the wall at the cell surface. However, the site of assembly of these protein-carbohydrate polymer complexes is not known. Only a few proteins have been shown to bind pectin in angiosperms, including arabinogalactan proteins (AGPs) (Nothnagel, 1997) and stigma/stylar cysteine-rich adhesins that help to guide pollen tubes (Mollet et al., 2000; Park et al., 2000).

The wall-associated kinases (WAKs) also bind to pectin, because WAKs can be detected on denaturing polyacrylamide gels to harbor an epitope recognized by Jim5 that binds homogalacturonan, the pectin backbone (Wagner and Kohorn, 2001; Willats et al., 2000). The extracellular domain of WAK1 also binds to $Ca^{2+}$-induced polymers, but not monomers, of pectin in vitro (Decreux and Messiaen, 2005). However, it is not known where in the cell WAKs, AGPs or any cell-wall proteins first bind to the cell-wall-carbohydrate polymers. WAKs are required for cell elongation and vacuolar invertase activity (Kohorn et al., 2006) and as they contain an extracellular domain within the cell wall and a cytoplasmic serine/threonine protein kinase domain (Anderson et al., 2001), they are positioned to be able to effect communication between the cell wall and cytoplasmic events. They also provide the potential to serve as a physical link between the cell wall and plasma membrane, similar to a role suggested for the formins (Deeks et al., 2002), AGPs (Nothenagel, 1997), COBRA (Schindelman et al., 2001) and PERKS (Nakhamchik et al., 2004).

To understand the biogenesis of WAKs, WAK1 fused to green fluorescent protein (WAK1-GFP) was expressed in cells that were regenerating their cell wall. WAK1-GFP accumulates in a cytoplasmic compartment whose detection depends upon WAK1-GFP expression in protoplasts. This compartment contains pectin and its migration to the cell surface is far slower than that of a cell surface receptor not associated with the cell wall. The biogenesis of WAK1-GFP is also influenced by the presence of fucose side chains on one or more unidentified molecules, which might include pectin, and is affected by the inhibition of cellulose synthesis. WAK1 is crosslinked into a detergent-insoluble complex within the cytoplasmic

compartment before it accumulates at the cell surface. Thus, the assembly and crosslinking of WAK1 may begin at an early stage within a cytoplasmic compartment, and is coordinated with the synthesis of surface cellulose.

## Results

### WAK1-GFP expression in protoplasts

To follow the biogenesis of WAKs, a WAK1-GFP fusion protein driven by the 35S CaMV promoter (in pCambia 1302) was transiently expressed in mesophyll protoplasts isolated from wild-type *Arabidopsis* leaves. The 35S promoter was used because the WAK1 promoter did not provide sufficient expression to allow detection of the GFP fusion protein. GFP was visualized by confocal laser scanning microscopy. Considerable variation in the size and position of the vacuole made it necessary to look at multiple cross sections of each and of many cells to determine whether GFP was on cytoplasmic compartments or on the cell surface. Some variation in the amount of expression of the WAK1-GFP fusion protein was seen between cells, but the patterns of localization varied only with time. Representative cells are shown in Fig. 1A and quantitation of the localization (from multiple experiments) in Fig. 1B. Within 24 hours, WAK1-GFP was detected in a compartment within the protoplasts but no WAK1-GFP was seen on the cell surface (Fig. 1A, 1 day WAK1-GFP). The size of the compartments varied considerably and only in larger ones could it be seen easily that WAK was on their surface (Fig. 1A, 1 day WAK1-GFP). By contrast, a GFP fusion protein of BAK1 (BAK-GFP), a receptor involved in brassinosteroid signaling and one not associated with the cell wall (Li et al., 2002), was detected on the surface within 12 hours of synthesis when driven by the same 35S CaMV promoter (Fig. 1A, 1 day BAK-GFP). Expression levels of WAK1-GFP and BAK-GFP fusion constructs appeared to be similar based on the level of GFP fluorescence. If the transformed protoplasts were incubated for 3 days, WAK-GFP was detected on the surface but also in internal compartments (Fig. 1, 3 days). By day 5, most but not all WAK1-GFP was on the cell periphery and surface (Fig. 1A). A WAK-GFP fusion protein that contained the protein kinase domain, transmembrane domains and signal sequence but lacked all of the extracellular domain (WAKFIN) appeared on the surface of protoplasts within 1 day. This is similar to BAK-GFP and indicates that the delay of WAK1-GFP depends upon the WAK extracellular domain. To quantify the location of WAK-GFP in protoplasts, an entirely cytoplasmic localization of the GFP signal scored '1', a localization at the surface only scored '5', and cells showing a mix of either localization were given a value between 1 and 5. The results shown in Fig. 1B indicate that WAK1-GFP first accumulates in a cytoplasmic compartment and then appears on the surface, far slower than BAK-GFP and far slower than expected.

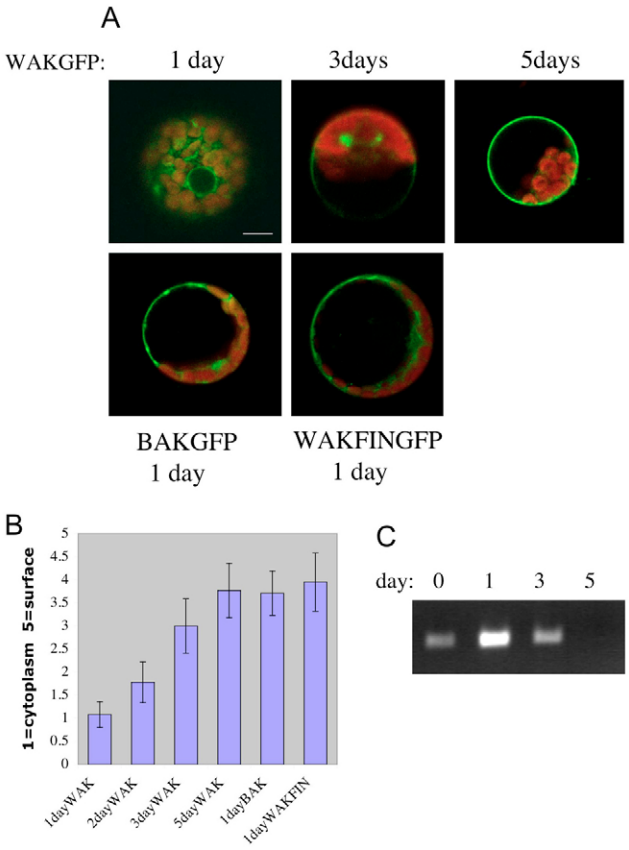


**Fig. 1.** (A) Confocal images of WAK1-GFP localization in *Arabidopsis* protoplasts. 35S CaMV WAK1-GFP expressed from pCambia 1302 was transformed into leaf protoplasts, and cells were incubated in W5 for 1 day and then transferred to regeneration medium for additional times as indicated. GFP was detected in the green channel and chlorophyll in the red. Shown are single optical sections through representative cells. BAK-GFP and WAKFIN-GFP (lacking extracellular domain) were also expressed from pCambia 1302. Bar, 10 μm. (B) Quantification of cells on indicated day post transformation of WAK1-GFP, BAK-GFP or WAKFIN-GFP location; 1, cytoplasmic localization; 5, surface localization. (C) Ethidium-bromide staining of an agarose gel of RT-PCR from WAK-GFP mRNA with equal amounts of RNA isolated from WAK-GFP transformed protoplasts incubated for the indicated time.

Reverse transcriptase (RT)-PCR was used to determine when the WAK1-GFP fusion gene was expressed (see Fig. 1C). WAK1-GFP mRNA was detected within 2 hours after transformation (day 0). Expression increases at day 1 and is then greatly reduced by day 3. By day 5 no RNA is detected (day 5). Thus, the WAK-GFP signal detected by microscopy at day 5 must be the same protein that was detected at day 3 in internal compartments, but had by day 5 moved from the interior to the surface.

### WAK and pectin colocalize

WAK is associated with pectin in the cell wall and also in plant extracts (Wagner and Kohorn, 2001), and in vitro its binding to pectin is enhanced by $Ca^{2+}$ (Decreux and Messiaen, 2005). To determine when WAK might associate with pectin, protoplasts transformed with WAK-GFP were fixed and probed with anti-pectin serum Jim5 that recognizes homogalacturonan, the most abundant pectic glycan (Willats et al., 2000). Fig. 2 shows an optical section of a protoplast not transformed with WAK1-GFP but stained with Jim5 alone. Pectin is seen in multiple small cytoplasmic compartments [Jim5 (No WAKGFP)]. Cells transformed with WAK1-GFP for 36 hours and stained with Jim5 are shown in Fig. 2, where images of the GFP (WAKGFP), Jim5 [Jim5 (WAKGFP)] or merged (Jim5 and WAKGFP) fluorescence signals are presented. This single cross section of the double labeled cell

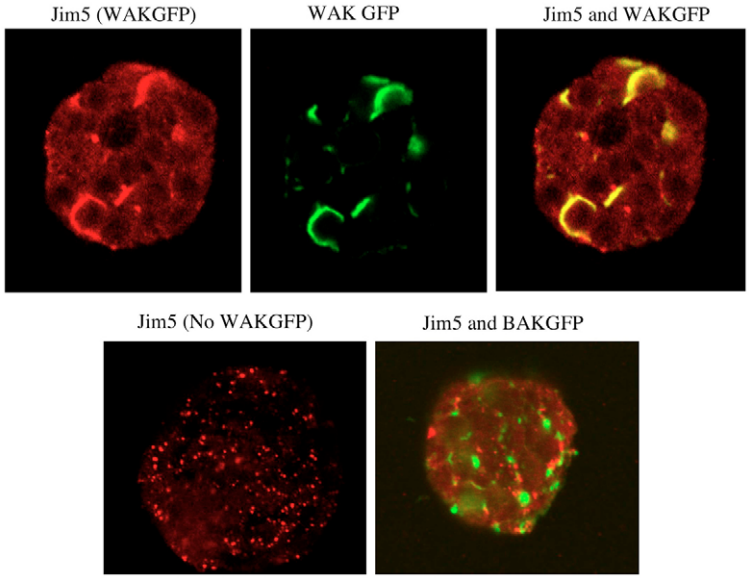


**Fig. 2.** Localization of WAK-GFP and pectin. Pectin was detected using Jim5 and cy3 anti-rat IgG serum (red) and WAKGFP by GFP fluorescence (green), and visualized by confocal microscopy. Jim5 (No WAKGFP): single optical section of protoplast not expressing WAK-GFP but stained with Jim5. Cells transformed with WAK1-GFP were fixed after 36 hours incubation, and stained with anti-pectin Jim5 antibody, and visualized by confocal microscopy. Jim5 (WAKGFP), pectin signal alone; WAKGFP, GFP signal alone; Jim5 and WAKGFP, merged signals; Jim5 and BAKGFP, cells transformed with BAK-GFP were fixed after 5 hours incubation, and stained with anti-pectin Jim5 antibody. Magnification, ×450.

shows that WAK1-GFP is on the surface of compartments that contain pectin, and these compartments have a varied surface contour. BAK-GFP-transformed cells were also stained with Jim5 at 5 hours post transformation, such that expressed protein would be found within the endomembrane system, similar to WAK1-GFP. Fig. 2 shows that the vesicles containing BAK-GFP are mostly distinct from those staining heavily with Jim5 [image (Jim5 and BAKGFP)]. Thus, WAK1-GFP accumulates at the same sites as pectin, but not all areas that have pectin contain WAK1-GFP. These results also indicate that the expression of WAK1-GFP is inducing the formation of the WAK1 compartments.

### WAK1-GFP is localized with Golgi markers

Pectin is thought to be synthesized in the Golgi (Willats et al., 2000). It was of interest to determine in what type of compartment WAK accumulates before being placed on the surface. To identify this compartment, protoplasts were transformed with WAK1-GFP and with a red fluorescent protein (RFP) fusion to a protein whose cellular location is known. Confocal microscopy of RFP and GFP was used to localize the signals, and the results are shown in Fig. 3. The membrane protein AtSRC2 has been shown to enter the ER and then to accumulate in the protein storage vacuole (PSV) (Oufattole et al., 2005; Jiang and Rogers, 1998; Muriyasu et al., 2003). Thirty-six hours post transformation, the RFP-AtSRC2 fusion protein shows distinctly different localization compared with that of WAK1-GFP (Fig. 3, see SRC/WAK1). In a few cells some overlap was seen (data not shown), but this probably reflects the passage of both proteins through the ER. A mutant of α tonoplast intrinsic protein (ΔαTIP) that lacks the PSV targeting sequence is thought to pass through the ER and then directly to the lytic and central vacuole (Oufattole et al., 2005; Jiang and Rogers, 1998). An RFP fusion of this was co-transformed with WAK1-GFP, and the results are shown in Fig. 3 (ΔTIP/WAK1). The RFP and GFP signals appear in distinct locations, suggesting that WAK1-GFP does not accumulate in the lytic and central vacuole. There is a small amount of overlap in these signals that might represent ER colocalization or indeed a small amount of WAK1-GFP that is sent to the lytic vacuole. ARA7 passes from the ER to a variety of endosomes (Ueda et al., 2004) and there is some but mostly no overlap between RFP-ARA7 and WAK1-GFP (Fig. 3, see ARA7/WAK1). Colocalization (yellow) of WAK1-GFP is seen with two Golgi markers SYP31 and SYP41 (Ueda et al., 2004) (Fig. 3, see SYP31/WAK1 and SYP41/WAK1). Single-channel emissions used to create the merged images are shown to the left of the merged images (Fig. 3, top two rows). These results suggest that WAK1-GFP is associated with the Golgi and not the vacuolar sorting pathway.

While WAK is not in the endosome, we wished to determine whether the WAK1 compartment contained membranes that had recently undergone endocytosis from the cell surface. Therefore, cells were incubated with FM4-64 which rapidly labels the surface of a variety of cells, and is subsequently incorporated by endocytosis into internal organelles (Vida and Emr, 1995; Ueda et al., 2001). No colocalization of FM4-64 and WAK-GFP was detected (Fig. 3, see FM4-64/WAK1). Thus, it is unlikely that WAK-GFP accumulates in the endomembrane system by retrieval from the cell surface.

### Transport of WAK requires cellulose synthesis

Protoplasts are created by digestion of the cell wall with multiple enzymes and, hence, many of the cell-wall components are fragmented or lost. However, protoplasts do synthesize a new cell wall, and this was observed by calcofluor staining of cellulose and by conventional fluorescence microscopy (Fischer and Cyr, 1998). For cell walls to be regenerated within a few days, cells must be placed in regeneration medium (RM) (Fischer and Cyr, 1998; Schirawski et al., 2000). At 1 hour post transformation, protoplasts have little to no calcofluor staining (Fig. 4A) and no detectable WAK1-GFP. Blue calcofluor staining increased dramatically over a 3-day period (Fig. 4A, 3 days), and was inhibited by the cellulose-synthase-specific inhibitor isoxaben (Fig. 4B, 0 vs +isox) (Heim et al., 1990; Corio-Costet et al., 1991). Thus, the appearance of WAK1 on the surface of cells correlated with the concurrent regeneration of the cell wall.

To determine whether the movement of WAK1 to the surface requires concurrent cellulose synthesis, protoplasts transformed with WAK1-GFP were treated with isoxaben. Representative transformed cells are shown in Fig. 5A and quantitation of the population in Fig. 5B. Inhibition of cellulose synthesis delays movement of WAK1-GFP to the surface even after five days, and protoplasts accumulate WAK1-GFP within the endo-membrane system. Isoxaben does not affect the rapid movement of BAK-GFP to the surface, indicating that the observed drug effect is specific to a cell-wall-associated receptor.

## WAK is crosslinked in a cytoplasmic compartment

WAKs in plant tissue have only been detected in the cell wall (Kohorn, 2000), and appear to be crosslinked in a Triton-X-100-insoluble complex containing pectin (He at al, 1996). To determine whether the WAK1 expressed in protoplasts was becoming crosslinked – and hence detergent-insoluble – the protoplasts were lysed and separated into a Triton-X-100-soluble and -insoluble fraction. The fractions were analyzed by denaturing gel electrophoresis and western blotting with anti-GFP serum. The fusion protein was detected in the Triton-soluble fraction at day 1, but accumulated into the Triton-insoluble fraction by day 3. However, the serum, and hence the signal, was weak and therefore a WAK1-TAP fusion construct (tandem-affinity-purification tag also driven by the 35S CaMV promoter) was transformed into protoplasts; the peroxidase-antiperoxidase (PAP)-soluble complex was used to follow the TAP-tag and fusion protein (Rivas et al., 2002). WAK1-GFP was also transformed at the same time and used to visually follow the location of WAK1 in the cell. At day 1 (post transformation) WAK1-TAP was detected in the Triton-soluble fraction (Fig. 6, S), consistent with its location within the endo-membrane system indicated by microscopy. Relatively low signal intensity was detected in the insoluble pellet (1 day P). After 2 and 3 days post transformation, increasingly less WAK1-TAP was detected in the soluble fraction (Fig. 6, S), and little was detected to enter the gel in the insoluble fraction (Fig. 6, P). To determine whether the WAK1-TAP was still present in the cell (not degraded), the protoplast extracts were slot-blotted rather than run on a denaturing gel, and WAK1-TAP was indeed detected (Fig. 6, slot-blots S and P). Thus, WAK1 protein was still present, but not in a detergent-soluble form. Cells expressing only GFP did not contain a protein that reacted with PAP (Fig. 6, pSmGFP). Since microscopy indicated at day 3 that there was still a significant amount of WAK within the endo-membrane system, WAK1 must have moved into the insoluble material within the cell. These results indicate that WAK1 forms a complex within the endo-membrane system before it is exported to the cell surface.

Since the transport of WAK1 to the cell surface may require concurrent cellulose synthesis, it was of interest to know if the inhibition of cellulose synthesis would affect the solubility of WAK1. Fig. 7 shows the triton soluble fraction of cells 1 and 3 days post transformation run in an SDS-PAGE and probed for WAK1-TAP (Fig. 7A, soluble). In a separate experiment the Triton X-100 supernatant and pellet were slot-blotted (Fig. 7B, total) and probed for WAK1-TAP. The results indicate that in isoxaben-treated protoplasts, WAK1-TAP still moves into the insoluble fraction at 3 days post transformation. Thus, the complex WAK1 forms within the cell does not require concurrent cellulose synthesis.

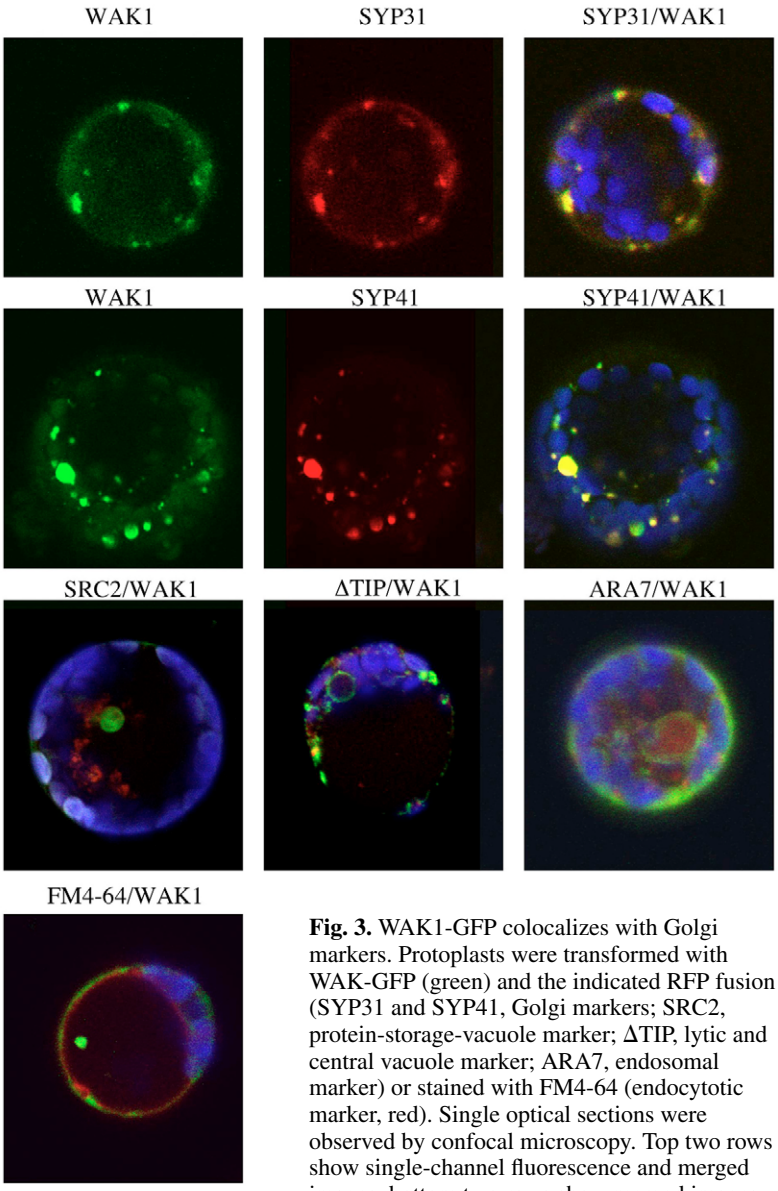


**Fig. 3.** WAK1-GFP colocalizes with Golgi markers. Protoplasts were transformed with WAK-GFP (green) and the indicated RFP fusion (SYP31 and SYP41, Golgi markers; SRC2, protein-storage-vacuole marker; ΔTIP, lytic and central vacuole marker; ARA7, endosomal marker) or stained with FM4-64 (endocytotic marker, red). Single optical sections were observed by confocal microscopy. Top two rows show single-channel fluorescence and merged images, bottom two rows show merged images only. Chlorophyll is false-colored blue. Magnification, ×450.

## *mur1-1* speeds up WAK1 transport

The *mur1-1* mutation of *Arabidopsis* inhibits the addition of fucose residues to numerous proteins and carbohydrate polymers, including pectic rhamnogalacturonan II (RG-II); this decreases pectin crosslinking (O'Neill et al., 2001). High

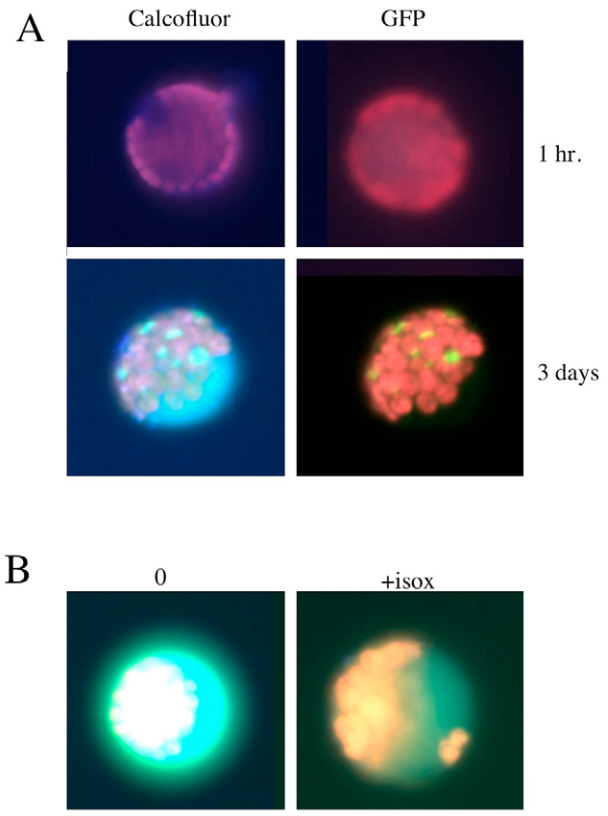


**Fig. 4.** Calcofluor staining of cellulose in transformed protoplasts. (A) single protoplasts transformed with WAK1-GFP were stained with 1 μg/ml calcofluor and the cellulose (calcofluor) or the GFP fluorescence was captured on a standard fluorescence microscope 1 hour or 3 days after transformation. Cells were incubated in RM. (B) Protoplasts were incubated in RM (0) or RM plus 2.5 μM isoxaben (+isox) for 3 days, and then stained with calcofluor and visualized by standard fluorescence microscopy for calcofluor. Red, chlorophyll; green, GFP; blue, calcofluor. Magnification, ×300.

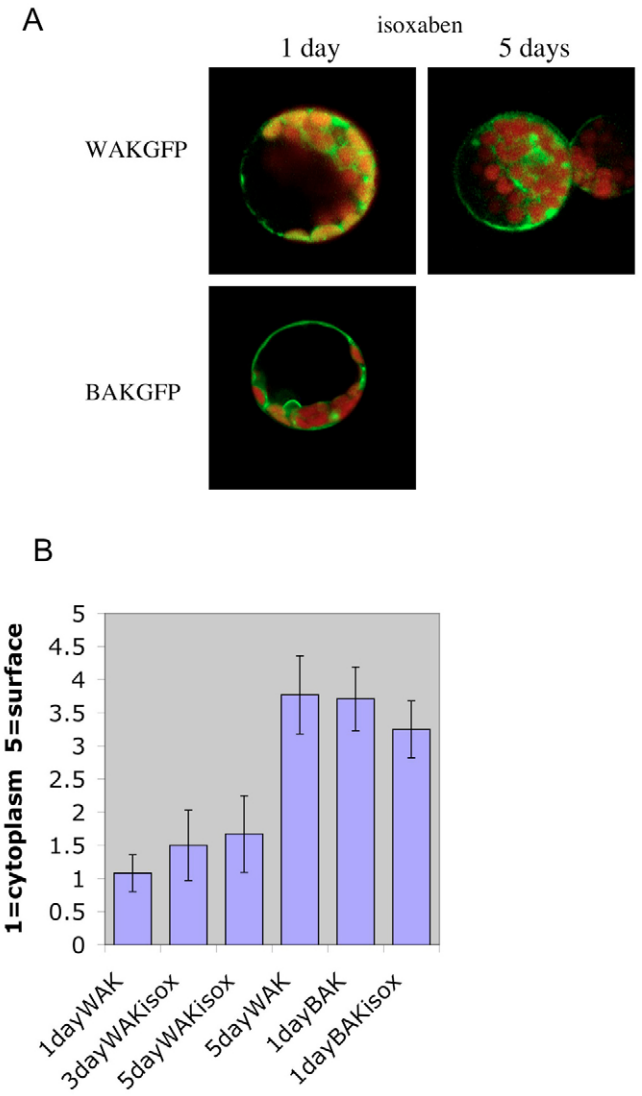


**Fig. 5.** WAK1-GFP movement to the surface is slowed down by isoxaben. (A) Protoplasts were transformed with WAK1-GFP or BAK-GFP and incubated for the indicated times in 2.5 μM isoxaben. Shown are representative confocal optical sections. Green, GFP; red, chlorophyll. See Fig. 1 for comparison. (B) Quantification of WAK1-GFP or BAK-GFP location on indicated day post transformation. 1, cytoplasmic localization; 5, surface localization. Magnification, ×300.

concentrations of boron in the cell wall can crosslink pectin monomers and, to a lesser extent, promote fucose-deficient RG-II crosslinking (O'Neill et al., 2001). Indeed, plants homozygous for the *mur1-1* allele are dwarf in boron-deficient soil, but are rescued by exogenous addition of boron. Since 99% of the cellular boron is in the cell wall and far less is concentrated within the cell (Matoh et al., 1992), it is likely that RG-II in *mur1-1* cytoplasmic compartments is not crosslinked by boron, whereas in wild-type cells RG-II crosslinking requires less boron, and thus may be crosslinked in cytoplasmic compartments. However, whereas the enzymes responsible for pectin synthesis reside in the Golgi, the location within the cell of the pectin crosslinking and binding to other wall components is not known (Willats et al., 2000; Ridley et al., 2001). To investigate the relationship between RG-II and WAK biogenesis, protoplasts from plants homozygous for *mur1-1* were transformed with WAK1-GFP, and visualized by confocal microscopy. Surprisingly, WAK1-GFP reached the surface of *mur1-1* protoplasts within 1 day post transformation (Fig. 8, mur1) whereas WAK1-GFP in wild-type protoplasts remained within cytoplasmic compartments (Fig. 8, +). These results suggest that WAK1-GFP movement to the cell surface is delayed in the endomembrane system of wild-type cells owing to a binding event that requires one or more fucosyl residues in a required glycan or glycoconjugate. Although it is tempting to speculate that the retention is due to a fucose-dependent crosslinked RGII, numerous other molecules, such as AGPs, xyloglucan and *N*-linked glycans, are also not fucosylated in this mutant (Bonin et al., 2003; Rayon et al., 1999; van Hengel and Roberts, 2002). Thus it remains possible that WAK is influenced by these changes rather than by the change in RG-II.

The *mur1-1* mutation does not, however, change the movement of WAK1 into the Triton-X-100-insoluble fraction, as compared with wild-type cells, because 3 days after transformation little WAK1-TAP is detected in the Triton-X-

100-soluble fraction (Fig. 7A, day 1 vs day 3; Fig. 7B soluble), but far more can be found in the Triton X-100-insoluble pellet in slot-blots (Fig. 7B). Thus, fucosylation of an as-yet-unidentified molecule that influences the rate at which WAK reaches the cell surface, is not required for the formation of the Triton-insoluble fraction. Furthermore, if *mur1-1* protoplasts that express WAK1-GFP are treated with isoxaben, the export of WAK1-GFP is delayed relative to non-treated cells (Fig. 8A,B, mur1 isoxaben), and this is similar to wild-type protoplasts. Despite this transport delay, isoxaben does not alter the time at which WAK1-TAP becomes insoluble in *mur1-1* or in wild-type (col+) cells (Fig. 7). This indicates that the requirement of cellulose synthesis for WAK1 transport does not depend upon fucose addition to any molecules and thus, perhaps, pectin crosslinking within the endomembrane system.

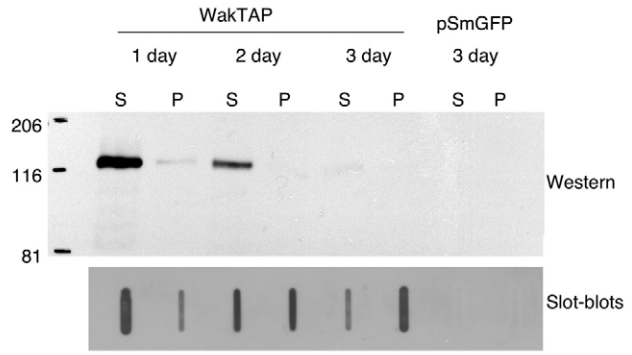


**Fig. 6.** Detection of Triton-X-100-soluble and -insoluble WAK1-TAP in protoplasts. Equal amounts of protoplasts transformed with WAK-TAP after 1, 2 or 3 days (as indicated) were extracted with 1% Triton X-100, centrifuged at 10,000 ***g*** for 5 minutes, and supernatant (S) and pellet (P) were boiled in loading buffer and either run on an SDS-PAGE (western blot) or (in a separate experiment) were slot-blotted onto nitrocellulose (slot blot). Then, both were probed with anti-TAP serum. pSmGFP, cells transformed with plasmid expressing only GFP.

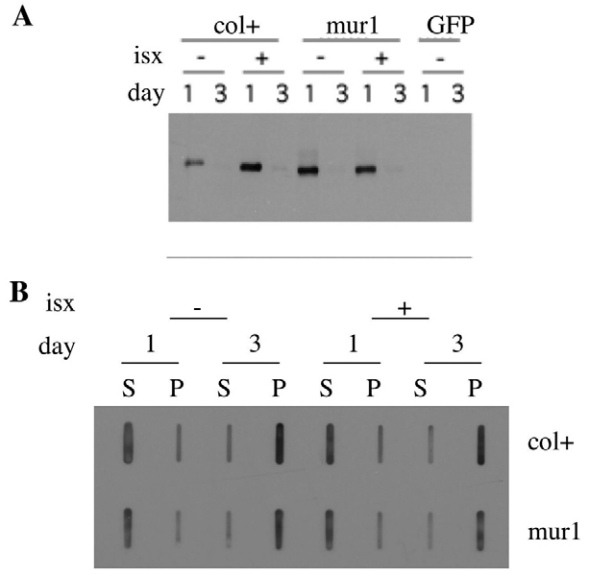


**Fig. 7.** *mur1-1* does not influence WAK1-TAP insolubility. Equal amounts of wild-type (col+) and *mur1-1* cells transformed with WAK1-TAP after 1 or 3 days (as indicated) were extracted with 1% Triton X-100 and centrifuged at 10,000 ***g*** for 5 minutes. The supernatant was run in an SDS-PAGE (A), or (in a separate experiment) the supernatant and the pellet were boiled in gel loading buffer and slot-blotted onto nitrocellulose (B). Pellet and supernatant were then both probed with anti-TAP serum. isx; 2.5 μM isoxaben added for 1 or 3 days. Wild-type cells were also transformed with GFP alone (GFP).

## WAK1-GFP in plant cells

WAK1-GFP accumulates in cytoplasmic compartments in protoplasts, but it was not clear whether this is representative of events that occur in intact plants. The presence of these WAK compartments depends on WAK1-GFP expression in protoplasts, but overexpression of other receptor-GFP fusion constructs does not induce the formation of these bodies (Fig. 1). Antibodies against WAK only detect protein at the surface of plant cells (He et al., 1996), indicating, not surprisingly, that in plants insufficient material is synthesized to accumulate or to be detected in the endomembrane system. *Arabidopsis* was therefore transformed with p35S:WAK1-GFP and the F1 and

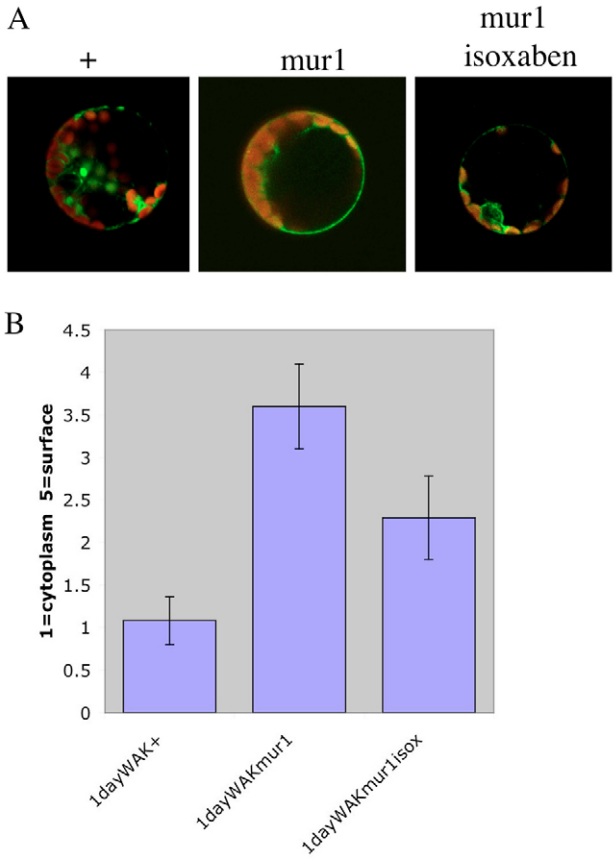


**Fig. 8.** (A) surface transport of WAK1-GFP is speeded up by *mur1-1*. Protoplasts from wild-type (+) and *mur1-1* plants were transformed with WAK-GFP and after 1 day were visualized by confocal microscopy. *mur1-1* cells were also treated with 2.5 μm isoxaben 1 hour post transformation. Red, chlorophyll; green, GFP.
(B) Quantification of WAK1-GFP location in cells on indicated day post transformation. 1, cytoplasmic localization; 5, surface localization. Magnification, ×300.

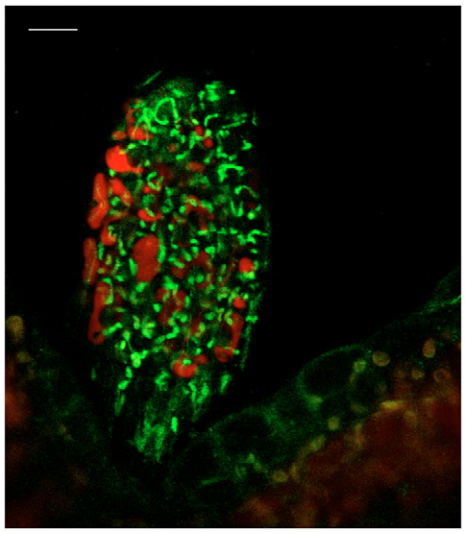

**Fig. 9.** WAK-GFP accumulates in cytoplasmic compartments of the leaf bud. Single optical section of an emerging leaf bud of an *Arabidopsis* plant transformed with WAK-GFP (green). Chlorophyll was detected in the red channel. Bar, 10 μm.

F2 generations were selected for co-transformation with the hygromycin marker. Hygromycin-resistant seedlings were screened for GFP expression. Most transformants did not express detectable levels of GFP, indicating that the gene is silenced. Indeed, no WAK1-GFP mRNA is detected in mature leaves (data not shown). In several plants out of hundreds, however, GFP was detected in rapidly growing and expanding leaf buds. Fig. 9 shows a confocal section though a leaf bud where WAK1-GFP is seen in multiple large bodies within cells. These compartments are irregular in shape and size. In expanded leaves of the same plant, GFP expression can still be detected in the periphery of each cell (Fig. 9, lower right) but not in older tissue. These results suggest that WAK1-GFP does indeed accumulate in endomembranes in plants, and that this protein becomes part of the cell wall. Since the cytoplasmic compartments are so transient and the signal is lost in older cells, the true nature of the association of WAK1-GFP with the cell wall cannot be verified at present.

## Discussion

Expression of a WAK1-GFP fusion protein in *Arabidopsis* protoplasts leads to the accumulation of WAK1 in a cytoplasmic compartment that also contains pectin, and the detection of this compartment depends upon WAK1-GFP expression. WAK1-GFP colocalizes with markers for the Golgi, indeed the site of pectin synthesis, but does not colocalize with markers of the endosome or vacuoles. The movement of WAK to the cell surface is inhibited by a cellulose synthase inhibitor, and is also influenced by one or more fucosylated metabolites whose identity has not yet been determined. WAK1-TAP, and presumably WAK1-GFP, is found in a detergent-insoluble fraction even within the cell, and thus possibly forms some form of complex before it enters the cell wall. Whether the insoluble complex represents part of the cell wall when on the cell surface remains to be determined. Since little is known of how proteins and carbohydrates of the cell wall are bound and crosslinked during their biogenesis, these results indicate that the biogenesis begins within the cell and not just at the site of cellulose synthesis.

Although it is not known whether the WAK1-GFP is functional in the cell, the WAK1 within the compartment does reach the cell surface; WAK1-GFP expression ceases after day 3, yet protein still continues to accumulate at the cell surface at day 5. The WAK compartments are not present or are not as large when WAK1-GFP is not expressed. It remains to be determined whether cells expressing normal levels of WAK1 do indeed have smaller and less abundant WAK organelles, but to date only low levels of native WAK are found on the surface of most vegetative cell types (He et al., 1996). Numerous attempts to express WAK1-GFP fusions with less strong promoters or with the WAK1 promoter failed to lead to the detection of GFP. The results are consistent with an exaggeration of this compartment by overexpression in protoplasts, thus permitting the detection of this interesting compartment that might serve as a model for the analysis of cell-wall biogenesis. Indeed, in a few plants transformed with WAK1-GFP, cytoplasmic compartments with WAK1-GFP are detected in young expanding leaves. As cells mature, the cell surface signal is also lost and, thus, cannot be analyzed at present.

That WAK1-GFP does not colocalize with markers of the endosome, lytic or storage vacuole, indicates that most WAK1-GFP is not targeted for digestion or storage in a vacuole. The small overlap of WAK1-GFP with the RFP markers probably reflects their common passage through the ER. The fact that WAK does not appear in the endosome also suggests that it is not being recycled from the plasma membrane at a fast rate. The lack of colocalization with the endocytotic marker FM4-64 is consistent with this.

Overexpression of the receptor kinase BAK-GFP to similar levels does not lead to the accumulation of this receptor in the cytoplasm, again supporting the idea that overexpression alone cannot account for the presence of slowly maturing WAK vesicles. Also consistent with this view is the observation that the *mur1-1* mutant speeds up the migration of WAK1 even though the levels of WAK1-GFP expression are still high.

Early in its biogenesis, WAK becomes crosslinked into a complex within the endomembrane system. At day 1 after transformation, most WAK1 is Triton-X-100-soluble, and colocalizes with pectin, which is first synthesized in the Golgi. Whether the pectin plays a role in forming this insoluble complex cannot yet be determined. Since WAK1 binds pectin polymers but not monomers in vitro (Decreux and Messiaen, 2005) and WAKs are covalently bound to a pectin fragment in the cell wall of plants (Wagner and Kohorn, 2001), it is possible that WAKs initiate complex formation with pectins within this cytoplasmic compartment. However, this has yet to be determined. The absence of a fucose on a number of molecules including pectin appears to release WAK1 from the WAK compartment so as to speed up its migration to the cell surface. Since this WAK1 ‘released’ by *mur1-1* is still insoluble, pectin cannot play a solitary role in the formation of a complex. It remains to be seen which proteins are associated with WAK1 within the compartments, and indeed, glycine-rich proteins can be modified with fucose and have been shown to bind WAK1 both in vitro and in vivo (Cosgrove, 1998; Park et al., 2001).

The WAK1 complex migrates to the cell surface in a cellulose-dependent manner. Previous studies have shown that cellulose and pectin synthesis are coordinated by a possible feedback mechanism (His et al., 2001), and the results here indicate that this path also includes an assembly step within the cell. Whereas isoxaben indeed cellulose synthesis, its effect on WAK biogenesis could nevertheless be indirect, perhaps through an effect on pectin synthesis or gene expression. Future studies will help to understand how the correct assembly of WAK and pectin within the endomembrane system is necessary for either a structural or a signaling function of WAK in the cell wall, such that these receptors can play a role in cell expansion (Wagner and Kohorn, 2001) and sugar metabolism (Kohorn et al., 2006).

## Materials and Methods

### Transient expression with *Arabidopsis* protoplasts

Plants were grown under alternating light conditions, 14 hours in the light (under 150 μE light) and 10 hours in the dark, at 60% humidity. 100 μl of $5\times10^5$/ml protoplasts were transformed with 30 μg of the appropriate plasmids as described by Sheen et al. 2002 (Sheen et al. 2002, see also http://genetics.mgh.harvard.edu/sheenweb/). Protoplasts were incubated in W5 medium containing 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl and 2 mM MES-KOH (pH 5.7) for 1 day, then transferred to cell-wall regeneration medium (RM) containing 1 M glucose, 0.25 M mannitol, 3.2 g $l^{-1}$ Gamborg's basal salts with vitamins, 1 μM indolacetic acid and adjusted to pH 5.8 (Schirawski et al., 2000) to promote growth of the cell wall.

### Cloning

The WAK1 coding region was amplified with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) from *Arabidopsis thaliana* (ecotype Columbia) genomic DNA, and cloned into the *Nco*I-*Spe*I site of pCambia1302 (CAMBIA, Canberra, Australia) in-frame with the C-terminal GFP. For the WAK1-TAP expression construct, a 3.2-kb *Sac*I-*Spe*I fragment of pCambia1302-WAK1-GFP, containing the CaMV35S promoter and the WAK1 coding sequence, was ligated with a 1.0-kb *Spe*I-*Hin*dIII fragment of CTAPi vector (Rohila et al., 2004), containing the TAP-tag sequence and the CaMV35S terminator, and cloned into *Sac*I-*Hin*dIII site of pBluescript SK+. RFP gene fusions were created by inserting the *Bam*HI-*Xho*I fragment containing RFP from RFP fusion JR888 (AtSRC2) into the appropriate sites of the GFP fusion clones JR 908 (ΔαTIP) and JR909 (αTIP) (Jiang and Rogers, 1998). WAKFIN-GFP; PCR was used to amplify the N-terminal 51 amino acids of WAK1 (inserting a 5′ *Nco*I- and 3′ *Xba*I-site) and the 20 aa-long transmembrane domain, inserting a 5′ *Xba*I- and 3′ *Spe*I-site. These were ligated and used to replace the *Nco*I-*Spe*I fragment of pCambiaWAK1-GFP, thereby creating a WAK-GFP that contained a signal peptide but lacked the extracellular domain.

### RT-PCR

A WAK-specific forward primer 5′-GCGAAACCAGTAGCAGCATTGGCTATG-3′ and a GFP-specific reverse primer 5′-GATGGTCCTCTCCTGCACGTATCCCTC-3′ were used to amplify RNA from transformed cells with a reverse transcriptase (RT)-PCR kit from Promega. RNA was isolated according to Wagner and Kohorn (Wagner and Kohorn, 2001).

### Immunodetection

Transformed protoplasts ($4\times10^4$) were pelleted by centrifugation at 100 ***g*** for 2 minutes and resuspended in 10 μl of W5 (1 day post transformation) or RM (3 or 5 days post transformation). A 3.5-μl aliquot was withdrawn and saved as a sample for total protein. The remaining 6.5 μl were mixed with 19 μl of buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 150 mM NaCl, and 1.25% (v/v) Triton X-100. The mixture was incubated on ice for 5 minutes, centrifuged at 10,000 ***g*** for 5 minutes at 4°C and the supernatant was taken as a Triton-soluble fraction. The pellet was resuspended in the same volume as the supernatant and the samples were mixed with an equal volume of 2× sample loading buffer (100 mM Tris-HCl, pH 6.8, 100 mM DTT, 8% SDS, 0.05% Bromphenol Blue, 20% glycerol) and boiled for 3 minutes. Aliquots (10 μl) of total protein samples were slot-blotted onto nitrocellulose membrane. Aliquots of the samples (30 μl) for the Triton-soluble fraction were separated by SDS-PAGE using 8% gel and transferred onto nitrocellulose membrane. The blots were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline supplemented with 3% (v/v) Tween 20, probed with peroxidase-antiperoxidase (PAP)-soluble complex (Sigma) (Rivas et al., 2002) at 1:5000 dilution for 2 hours (western blot) or overnight (dot-blot), and the signal was detected by chemiluminescence.

Immunodetection in protoplasts was done as described in Fisher and Cyr (Fisher and Cyr, 1998). Rat Jim5 and anti-rat Cy3 (Santa Cruz Biologicals, CA) were used at 1:100.

### Confocal microscopy

Live cells were visualized using a 40× objective with a Zeiss 510 Meta confocal laser scanning microscope, and optical sections were analyzed with Zeiss software. Multi tracking, where each scan was performed with individual laser excitation and emission, was used for colocalization studies. Location of GFP in the cell was determined in a blind assay where individual pictures were assigned cytoplasmic-to-surface identity on a scale of 1-5, and the average and standard deviation was calculated for each cell type.

### Conventional fluorescent microscopy

Cells were imaged with a BX51 Olympus fluorescent microscope under a 40× objective. Calcofluor was visualized (excitation at 364 nm, detection at 450 nm) and images were captured with a Q Imaging RetigaRX digital camera using Open Lab software.

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