ANALYZING TAN1 AND AIR9 COLOCALIZATION TO REVEAL THEIR GENETIC RELATIONSHIP

By

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Abstract

In plants, an antiparallel array of microtubules, called the phragmoplast, guides positioning of the vesicles required for the new cell wall during cell division. TANGLED1 (TAN1) and AUXIN-INDUCED-IN-ROOTS9 (AIR9) are microtubule-binding division site marker proteins that orient the phragmoplast toward the cortical division site. In Arabidopsis, no single mutant of these proteins produces a discernible phenotype. However, the tan1air9 double mutant displays misoriented divisions as a result of phragmoplast guidance defects, suggesting that wild-type TAN1 and AIR9 function together during phragmoplast guidance. Whether this functional redundancy is due to the corresponding genes acting in the same pathway (in the same protein complex, for example) or two separate genetic pathways converging to perform a common function, was examined by determining whether TAN1 and AIR9 co-localize. I used confocal microscopy to analyze mitotic and cytokinetic TAN-YFP, CFP-AIR9 localization in Arabidopsis thaliana, Nicotiana benthamiana, and tobacco bright yellow (BY) 2 suspension cells.

Contents

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Introduction

Plant cells are surrounded by cell walls that prevent migration, so their relative positions are fixed. Consequently, proper orientation of cell division is critical for plant cell patterning and the plane of division is "programmed" before the onset of mitosis. In most plants, this is accomplished by a ring of microtubules and F-actin positioned on the cytoplasmic side of the plasma membrane surrounding the nucleus, called the preprophase-band (PPB). This cortical array is assembled during the G_2 phase of the cell cycle and disintegrates before metaphase, as the nuclear envelope breaks down and the spindle forms orthogonal to the previous location of the PPB. After the spindle has properly segregated newly duplicated daughter chromosomes, another microtubule and F-actin array, termed the phragmoplast, forms in its place and expands centrifugally toward the cortex, guiding vesicles toward the growing cell plate. Finally, its leading edges make contact at the site previously occupied by the PPB. Along the way, the vesicles fuse and form the cell plate, later maturing into the new cell wall.

The faithful return of the phragmoplast leading edge to the former site of the PPB led to the idea that the PPB leaves behind some "memory" the phragmoplast may later recognize, but very few of the components that made up this memory were yet known, though negative markers of the cortical division zone such as the local depletion of actin and KCA1 were observed (Mineyuki and Plaevitz 1990 and Vanstraelen et al. 2006). This hypothesis was supported by the discovery of a microtubule-associated protein (MAP) whose maize mutant displayed aberrant phragmoplast positioning and resulted in defective division plane orientation: TAN1. Maize *tan1* mutants have short stature and misoriented cell divisions (Smith et al. 1996), and AtTAN1-YFP was later the

first positive division site marker protein discovered in *Arabidopsis thaliana,* colocalizing with the PPB and persisting at the cortical division zone throughout mitosis and narrowing to the cortical division site during cytokinesis *(*Walker et al. 2007*).* TAN1 remains at this location even when microtubules are depolymerized, suggesting that it may be recruited in a microtubule-dependent manner, but anchored to the division site by an unidentified membrane-associated protein. Additionally, TAN1 is similar to the adenomapolyposis coli (APC) microtubule binding domain (Smith et al. 2001). Since APC is a protein that promotes correct division plane orientation in animal cells (Sugioka et al. 2018), . However, *tan* mutants in *Arabidopsis thaliana* produce only a mild phenotype, unlike their maize counterparts, complicating efforts to gather functional data.

While AtTAN has no recognizable domains, a deletion study found that the first 132 amino acids (Region 1) are necessary for colocalization with PPBs, but TAN missing Region 1 was not found at the cortical division site after prophase. AtTAN missing amino acids 126-473 (Region 2) always localized to the cortical division site, except during prophase. Together, this data suggests that while AtTAN is involved in proper phragmoplast guidance, a different PPB-mediated molecule marks the cortical division site, since AtTAN is recruited to this site even when it was not previously colocalized with the PPB. Additionally, localization to the cortical division site is at least partially mediated by Phragmoplast Orienting Kinesin 1 (POK1), which was shown to bind Region 1 (Rasmussen et al. 2011).

AIR9 was characterized as another MAP with a unique localization pattern (Buschmann et al. 2006). AIR9 colocalizes with interphase cortical microtubules and PPBs, only to be downregulated during mitosis. Once the leading edge of the expanding phragmoplast contacts the "programmed" cortical division site, as defined by the PPB, AIR9 reappears at the cortical division

site, then migrates inward, directly following the path of phragmoplast disassembly. At this stage, AIR9 is observed as a cortical ring around the new cross-wall, just before it is re-established at interphase cortical microtubules. AIR9 has a highly basic N-terminal microtubule binding domain, a C-terminal set of A9 repeats that confer localization to the site of cell plate insertion, and a leucine-rich repeat domain. The A9 domains are of particular interest because they have significant sequence homology with immunoglobulins, suggesting that they function in protein-protein and/or protein-ligand interactions. Since AIR9 does not decorate cell plates induced to insert at cortical locations separate from the "programmed" division site, and that these cell plates remain rich in callose (a non-rigid polymer characteristic of immature cell walls), AIR9 is thought to function in cell plate attachment at the cortical division site and mediate cell wall maturation. However, like TAN1, air9 single mutants have no discernible phenotype (Buschmann et al. 2015).

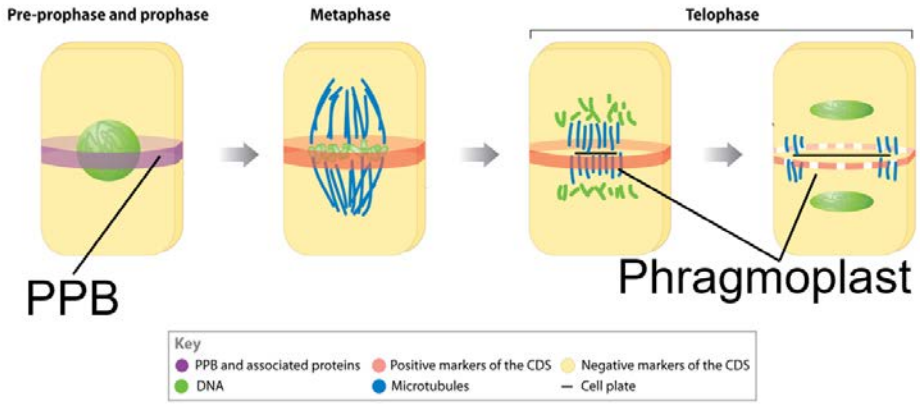

Figure 1. **Schematic representation of plant cell division. (Rasmussen et al. 2011)**

At the end of G2 the preprophase band (PPB) is assembled. PPB disappears during metaphase while the spindle is formed. During telophase microtubules organize in a structure called the phragmoplast that guides the formation of the new cell wall at the site that the PPB initially occupied. Division marker proteins localize at the division site and are important for proper guidance of the phragmoplast.

Additionally, AIR9 is required for proper recruitment of Kinesin-like Calmodulin Binding Protein (KCBP), which can be found at the plant cortical division site throughout cell division (Buschmann et al. 2015). KCBP is a microtubule binding protein with a motor domain at its C terminus that confers movement toward the minus end of microtubules that it is associated with, and it has been proposed that microtubules emanating from the centrifugally expanding phragmoplast may interact with KCBP. This would result in minus end directed movement of KCBP, which is anchored to the cortex by its MyTH4-FERM domain, leading to the phragmoplast being "reeled in" towards the programmed cortical division site. This model draws parallels to the action of dynein in animal cells that mediates the placement of the cleavage furrow during cytokinesis, and since flowering plants lack dynein heavy chain it is suggested that minus end directed motor proteins like KCBP have taken over some of this function in plants.

There are several interesting parallels between TAN1 and AIR9; each protein has separate domains which permit localization to the PPB and cortical division site, and both require an unknown signal from the PPB to localize to the cortical division site. Mir and colleagues found that tan1air9 double mutants have a synergistic phenotype (defects in division plane orientation, root growth, and cell file rotation) that is reminiscent of the maize *tan1* phenotype (Mir et al 2018). A yeast-two hybrid assay, which tests protein-protein interaction by splitting a transcription activator and fusing those fragments to the two proteins of interest, did not show TAN1-AIR9 interaction. This relationship is an example of genetic buffering, which can occur when two proteins function in separate pathways which later combine to fill a common role, or when the proteins both act in the same pathway.

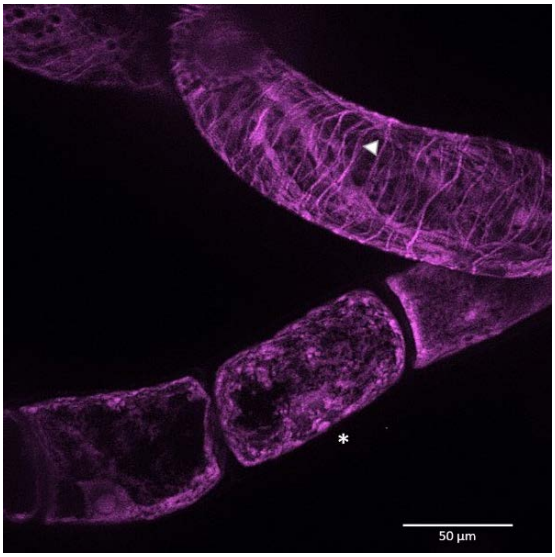

Figure 2. **CFP-AIR9 localization in tobacco BY-2**

Interphase cortical microtubules (arrowhead) labeled by CFP-AIR9.
(*) indicates damaged cell

Results

CFP-AIR9 localization in tobacco BY2 cells

BY2 cells are convenient for localization studies because of their large size and lack of chloroplasts. To investigate TAN-YFP and CFP-AIR9 localization in this model system, lines expressing both proteins were generated by co-culturing with appropriate Agrobacterium strains. CFP-AIR9 labeled interphase cortical microtubules that were transverse to the long axis of the cell, as expected (Figure 1). YFP signal indicative of TAN-YFP expression was not observed (some cells showed signal that was characteristic of cellular damage; disorganized or

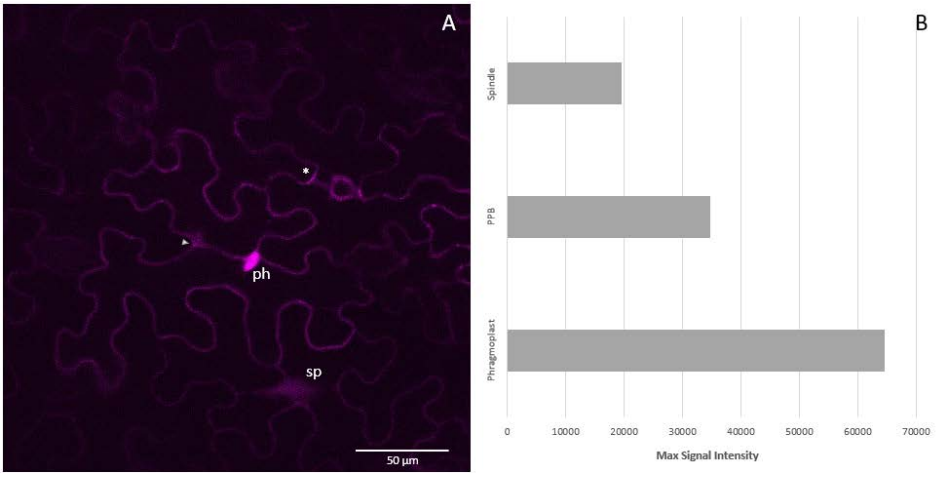

Figure 3. **CFP-AIR9 localization in mitotic *Nicotiana benthamiana* cells**

(A) Preprophase band (*), spindle (sp), phragmoplast (ph) and site of cell-plate insertion(arrowhead)
(B) Max fluorescence intensity of selected microtubule structures as measured by imageJ software

depolymerized cortical microtubule array, shrunken cytoplasmic volume, Figure 1). Mitotic cells expressing either fluorescent protein fusion were not observed. Future BY2 experiments will be repeated, varying Agrobacteria co-culture concentrations and BY2 subculture periods to observe mitotic BY2 co-expressing CFP-AIR9 and TAN-YFP.

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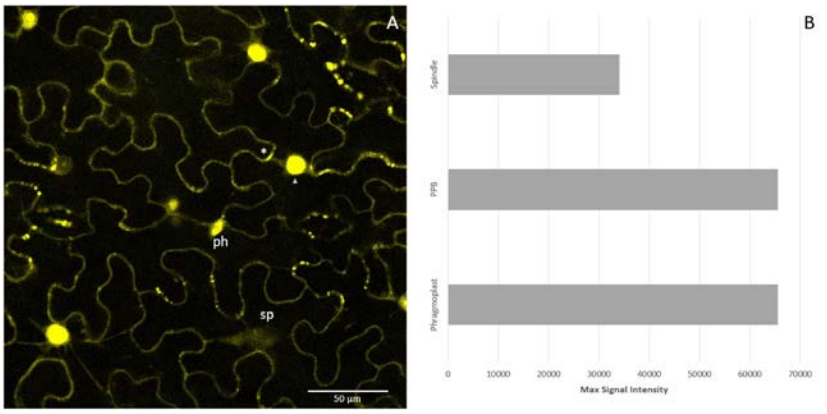

Figure 4. **TAN-YFP localization in mitotic *Nicotiana benthamiana* cells**

(A) Preprophase band (*), spindle (sp), phragmoplast (ph) and nuclei(arrowhead)
(B) Max fluorescence intensity of selected microtubule structures as measured by imageJ software

CFP-AIR9, TAN-YFP localization in mitotic *Nicotiana benthamiana* cells

We hypothesized that if TAN1 and AIR9 functioned in the same pathway, they would colocalize at the cortical division site when the phragmoplast contacts the cortex and begins to disassemble (TAN1 forms punctate rings at the cortical division site until phragmoplast disassembly, AIR9 forms an inwardly moving torus as the phragmoplast disassembles). To test this hypothesis, *Nicotiana benthamiana* leaves were transformed with constructs containing TAN-YFP and CFP-AIR9 and imaged these transformants using a confocal microscope. Normally, adult leaf pavement cells do not undergo cell division, so CYCLIND;3-1 was co-transformed. CYCLIND;3-1 is a protein that targets CDKA to Retinoblastoma (Rb), an inhibitor of the transcription factor E2F. Once Rb is phosphorylated, E2F promotes the transcription of proteins that propel the cell into mitosis (Polyn et al., 2015). In this way, non-dividing cells were induced to

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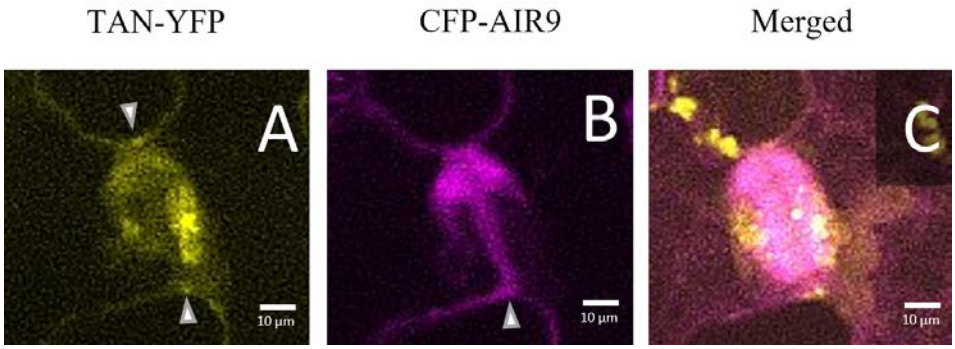

Figure 5. **TAN-YFP, CFP-AIR9 localization during phragmoplast disassembly**

(A) Disassembling phragmoplast (top). Characteristic TAN-YFP puncta at cortical division site (arrowheads)
(B) Disassembling phragmoplast (top). Cell plate insertion site and presumed AIR9 torus (arrowhead)
(C) Merged Z-projection

divide so that localization of TAN-YFP and CFP-AIR9 at mitotic division structures and the cortical division site could be studied.

CFP-AIR9 localized to PPBs, spindles, phragmoplasts and the cortical division site after phragmoplast contact (signal intensity was generally lower in spindles than PPBs and phragmoplasts, Figure 2). TAN-YFP localized to nuclei, spindles, phragmoplasts and PPBs and remained at the cortical division zone until just after phragmoplast disassembly (Figure 3A), meanwhile sharpening into a punctate ring that marked the cortical division site during cytokinesis. Like CFP-AIR9, TAN-YFP had much weaker signal intensity in spindles than in PPBs and phragmoplasts (Figure 3B). To assess whether TAN-YFP remains at the cortical division site during cytokinesis at the same time as CFP-AIR9 forms a torus at this location, we focused on cells that were nearing the point of cell plate insertion and phragmoplast disassembly. In one cell, the phragmoplast is beginning to disassemble on one side, and the TAN-YFP puncta are still

present at the cortical division site (Figure 5A). Simultaneously, CFP-AIR9 appears to have formed the characteristic torus (Figure 5B), though there were not early phragmoplast examples that could be used as reference. However, published images of GFP-AIR9 in Arabidopsis thaliana show cells with early phragmoplasts that do not have their midlines labeled by AIR9.

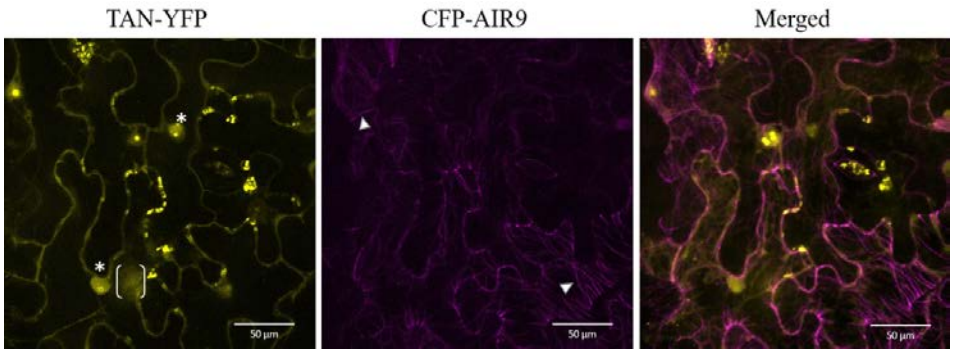

Figure 6. **CFP-AIR9, TAN-YFP localization in interphase *Nicotiana benthamiana* cells**

All are Z-projections of interphase *N. benthamiana* cells co-expressing CFP-AIR9, TAN-YFP.
(*) indicate nuclear localization, brackets indicate cytoplasmic localization, arrowheads show cortical microtubules labeled by CFP-AIR9.

TAN-YFP, CFP-AIR9 localization in interphase *Nicotiana benthamiana* cells

TAN-YFP localized to nuclei/nucleoli and the cytoplasm in interphase cells; CFP-AIR9 labeled interphase cortical microtubules (Figure 4). No colocalization was observed.

Discussion

TAN1 and AIR9 both have separate domains which are necessary for their early and late localization to cortical division site during mitosis and cytokinesis. TAN1 remains at the cortical division site until phragmoplast disassembly, while AIR9 is recruited to the cortical division site in a pattern that directly follows phragmoplast disassembly. Localization patterns of these two proteins have not been evaluated in the same organism at the same time, so whether there is overlap between these patterns as the cell plate is inserted is thus far unknown.

Here, I transiently expressed a D-type cyclin to induce cell division in mature leaf pavement cells and transformed them with TAN-YFP and CFP-AIR9 using Agrobacterium. I found that TAN-YFP and CFP-AIR9 appeared to colocalize at the cortical division site just after cell plate insertion, as well as in PPBs, spindles and phragmoplasts. Conclusions, however, should not be drawn from this data due to the low sample size and relatively low resolution of the 40x and 60x objectives used to capture images. Additionally, the inclusion of z-stacks that included the extremely bright TAN-YFP nuclear accumulation resulted in images that were under-exposed for the weaker TAN-YFP signal that is present at the cortical division site. Future experiments will include repeating the transient transformation of *Nicotiana benthamiana* and imaging a greater

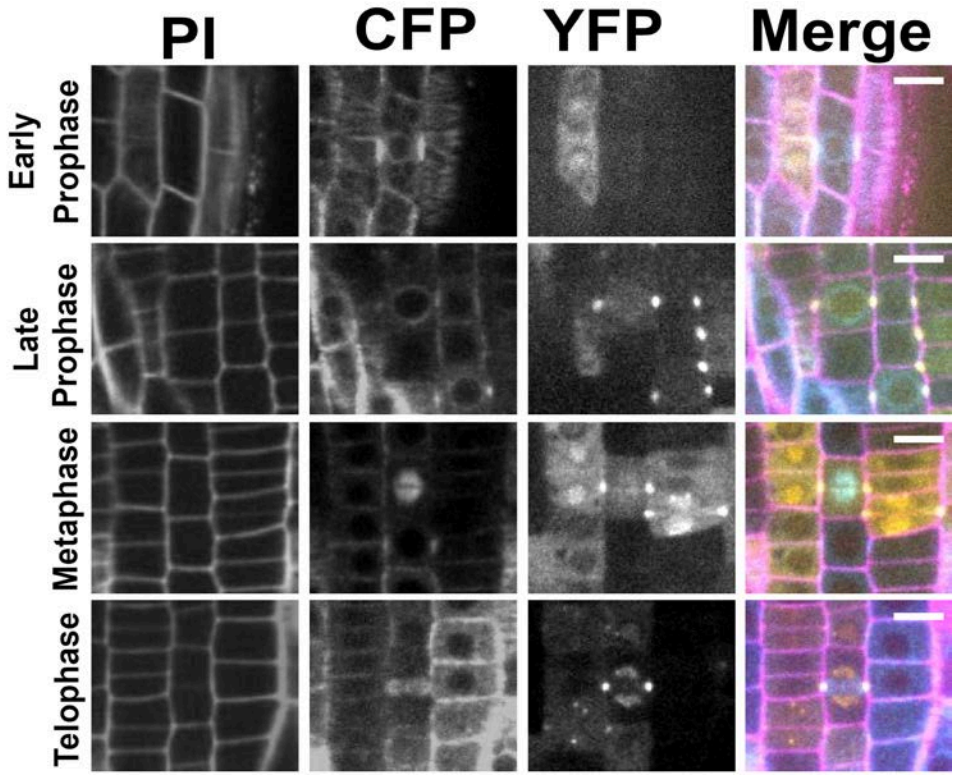

Figure 7. **TAN-YFP localization in dividing *tan1air9* double mutant cells. (Alison Mills, unpublished)**

Left panel- Propidium Iodide (PI) stained cells to show cell walls
Middle panels- CFP-tubulin shows microtubule-based division structures. YFP channels shows TAN-YFP localization. Note that TAN-YFP colocalizes with mature PPB and the division site throughout mitosis and cytokinesis, as in wild-type plants. Non-dividing cells displayed nuclear and nucleolar localization, as expected (not shown).

number of samples with the higher-resolution 100x objective and images taken with shallower z-stacks that only include the cell cortex.

While these data seem to suggest that TAN-YFP and CFP-AIR9 may colocalize at the cortical division site, colocalization would not necessarily imply interaction. Yeast-two hybrid

experiments have failed to demonstrate TAN1-AIR9 interaction, and recently transformation of TAN-YFP expressed from the KNOLLE promoter into *Arabidopsis thaliana tan1air9* double mutants resulted in normal localization of TAN-YFP (unpublished data, Alison Mills 2020). Since the absence of functional AIR9 did not alter TAN-YFP localization, it is not likely that AIR9 aids in TAN1-YFP recruitment to the division site.

Future studies on TAN1 and AIR9 colocalization in *Arabidopsis thaliana*, along with bimolecular fluorescence complementation experiments will help elucidate the precise nature of the interaction (or lack thereof) between TAN1 and AIR9 that result in reliable guidance of the phragmoplast to the "programmed" cortical division site specified by the PPB.

Methods

Agrobacterium infiltration of tobacco leaves

To observe colocalization patterns in N benthamiana, plants were grown in a growth chamber (22°C and 16 hour light: 8 hour dark photoperiods). 4-week old wild-type plants were used. Prior to infiltration, GV3101 agrobacteria transformed with TAN-YFP, CFP-AIR9 and CYCLIND;3-1 were grown until reaching stationary phase in antibiotic-containing liquid LB. They were centrifuged, then the pellet was resuspended in 10mM MES (pH 5.6), 10mM $MgCl_2$, and 150 μM acetosyringone to OD_{600}. Agrobacterium cultures containing different constructs were mixed at equal volumes and allowed to equilibrate for 2 hours. Fully expanded leaves were then pricked

with a 1000µL micropipette tip to form a lesion on the abaxial side, then the prepared agrobacterium mixture was delivered into the mesophyllar space using a blunt-end syringe. Plants were placed back in the growth chamber until imaging.

Confocal Microscopy

Images were captured using a Nikon Ti equipped with a motorized stage and spinning-disk confocal microscope (Yokogawa W1) running Micromanager software, built by Solamere Technology. Obis Solid-state lasers and Chroma Technology emission filters were used: excitation, 445; emission. 480/40 (CFP-AIR9); excitation, 514; emission, 540/30 (TAN-YFP). 40x/60x and 100x objectives were used with Cargille perfluorocarbon immersion liquid (water and oil-based, respectively) and with 1.15 and 1.20 numerical aperture, respectively.

Transformation of Tobacco BY-2 by Agrobacterium co-culture

Liquid Agrobacteria cultures harboring constructs coding for proteins of interest were grown for 2-3 days at 28◦C with appropriate antibiotics. This culture was mixed with a liquid BY-2 culture (10 µL Agrobacteria per 10 mL BY-2 cells) and grown for 2 days. 8 mL of this co-culture was plated, the plate sealed and covered in foil and grown at room temperature for 2 days. The culture was transferred to a 50 mL Falcon tube, which was then filled to 40 mL with BY-2 media. Cells were collected by centrifugation (8000 rpm, 3 minutes; 9 acceleration, 5 brake). The cells were then washed 3x by filling the Falcon tube with BY-2 media and decanting. Appropriate antibiotics were added and 5 replicates per strain were plated (3 mL per plate), making sure to break the pipet tip before suction. After spreading uniformly on the plates, colonies were picked at 7 days.

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