STSM Scientific Report

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MS Vernerey

Institut National de Recherche Agronomique

UMR BGPI (Biologie et Génétique des

Interactions Plantes Parasites)

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Sub-cellular localization of rice blast resistance proteins RGA4 and RGA5

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Applicant: Marie-Stéphanie VERNEREY

Host: Sophien Kamoun and Yasin Dagdas,TSL, UK

- **Background:**

The rice CC-NB-LRR proteins RGA4 and RGA5 confer together resistance to strains of the rice blast fungus *M. oryzae* expressing the effectors AVR-Pia or AVR1-CO39 (Césari et al. (2013), Plant Cell). RGA4 acts as a constitutive cell death activator which is repressed in its activity by RGA5 (unpublished). In addition, RGA4 and RGA5 interact via their CC domains. RGA5 recognizes the AVR effectors by direct physical binding leading to the de-repression of RGA4 and cell death induction (Césari et al. (2013), Plant Cell and unpublished).

RGA5 carries in its C-terminus a farnesylation site indicating that it could be tethered to the plasma membrane by this lipid modification. Preliminary RGA4 and RGA5 localization studies in *N. benthamiana* and rice protoplasts suggested cytoplasmic localization but the results were not conclusive due to lack of good markers for the plasma membrane and conflicting results from independent inoculation experiments which could not exclude localization to the plasma membrane or other membrane systems. In addition, RGA5 was observed in some cells in vesicle-like structures and RGA4 localized to the similar structures when co-expressed with RGA5. Due to the lack

of appropriate markers, it was not possible to decide whether these structures were indeed vesicles or rather protein aggregates formed as a consequence of strong overexpression.

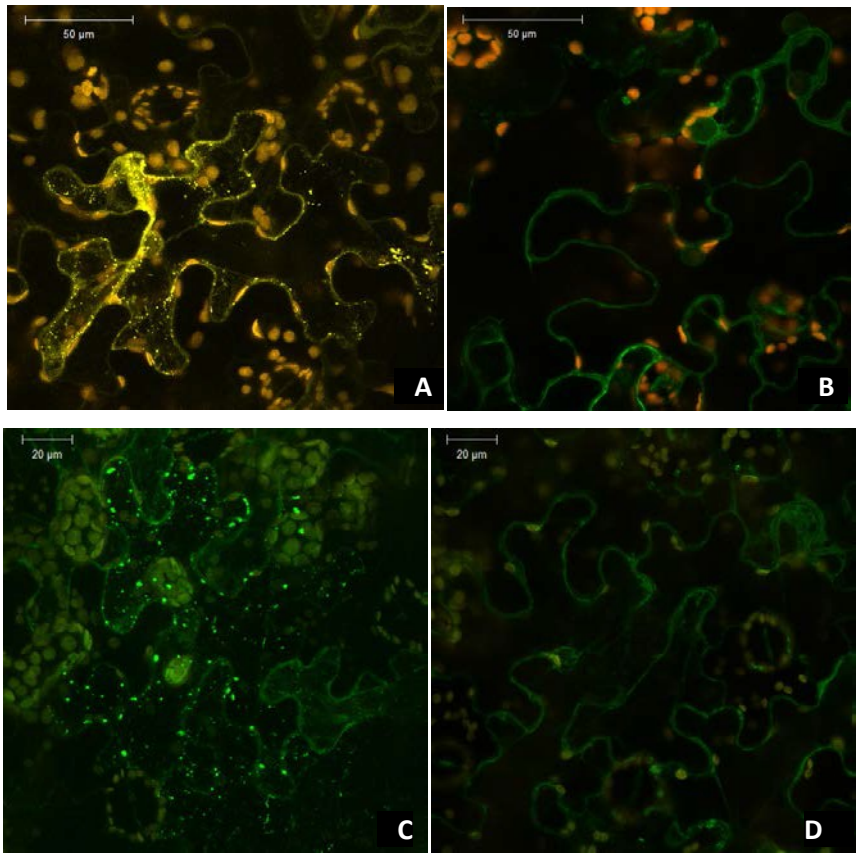


Figure 1: Representative pictures of sub-cellular localization of YFP-RGA5 and RGA4-GFP in *N. benthamiana* leaves

A: YFP-RGA5 signal, localization in cell periphery and in vesicle-like structures

B: RGA4-GFP signal, localization in cell periphery

C: RGA4-GFP signal when co-expressed with HA-RGA5, localization in cell periphery and vesicle-like structures

D: RGA4-GFP signal when co-expressed with HA-RGA5 and AVR:Pia, localization in cell periphery

- **Objective:**

The objective of the STSM was to identify the sub-cellular localization of RGA4 and RGA5 to respond i.e. to two major questions:

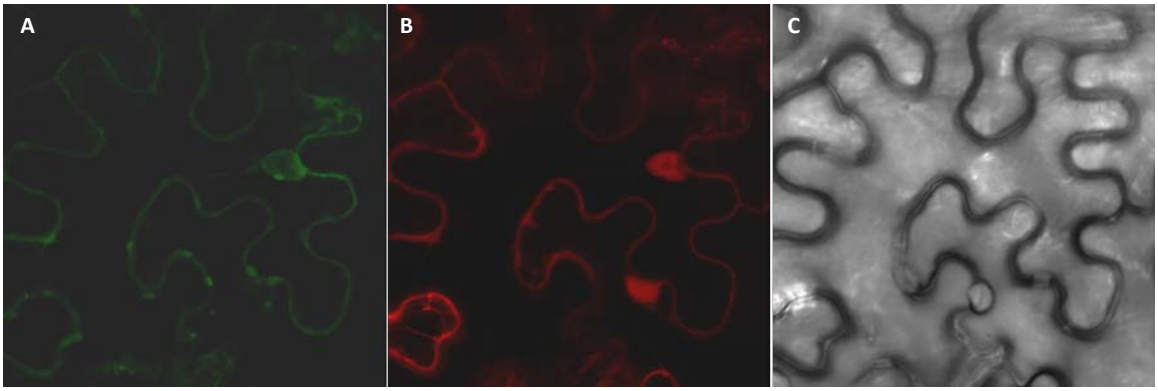
- Is RGA5 localized entirely or partially to the plasma membrane or other cellular membrane systems?
- Does RGA5 localize to vesicles? Is this localization biologically relevant or an artefact of overexpression?

- **Methodology:**

Fusions between RGA4 or RGA5 and fluorescent proteins (YFP, GFP or mRFP) and markers of different cellular compartments (cytoplasm, plasma membrane, late or early endosomes, phagosomes) labelled by mRFP, or mCherry were expressed by agroinfiltration (OD of 0.2) in leafs of 5 week old *Nicotiana benthamiana* plants. 1 to 3 days after infiltration, fluorescence was analyzed by laser scanning confocal microscopy using ga Leica SP5.

- **Main results:**

To address the **subcellular localization of RGA4 and RGA5**, RGA4-GFP and YFP-RGA5 were co-expressed in *Nicotiana benthamiana* leaves with mRFP which labels the cytoplasm and the nucleus. In both cases, green or yellow fluorescence co-localized with red fluorescence in the cytoplasm (Figure 2 and 3) indicating cytoplasmic localization of both proteins. However, the nucleus was not or only weakly stained by RGA4-GFP and YFP-RGA5 confirming previous and preliminary results obtained in rice protoplasts and *N. benthamiana*.



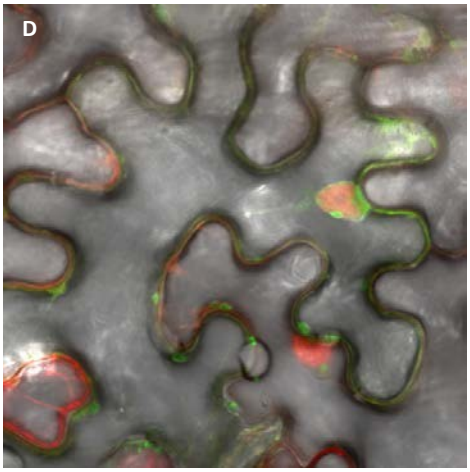


Figure 2: Co-expression of RGA4-GFP and RFP in *N.benthamiana*.

A: RGA4-GFP signal: localization in cell periphery, excluded from the nucleus

B: mRFP: localization in cytoplasm and nucleus

C: Bright Field image

D: Merged image: red and green signals colocalize in the cell periphery

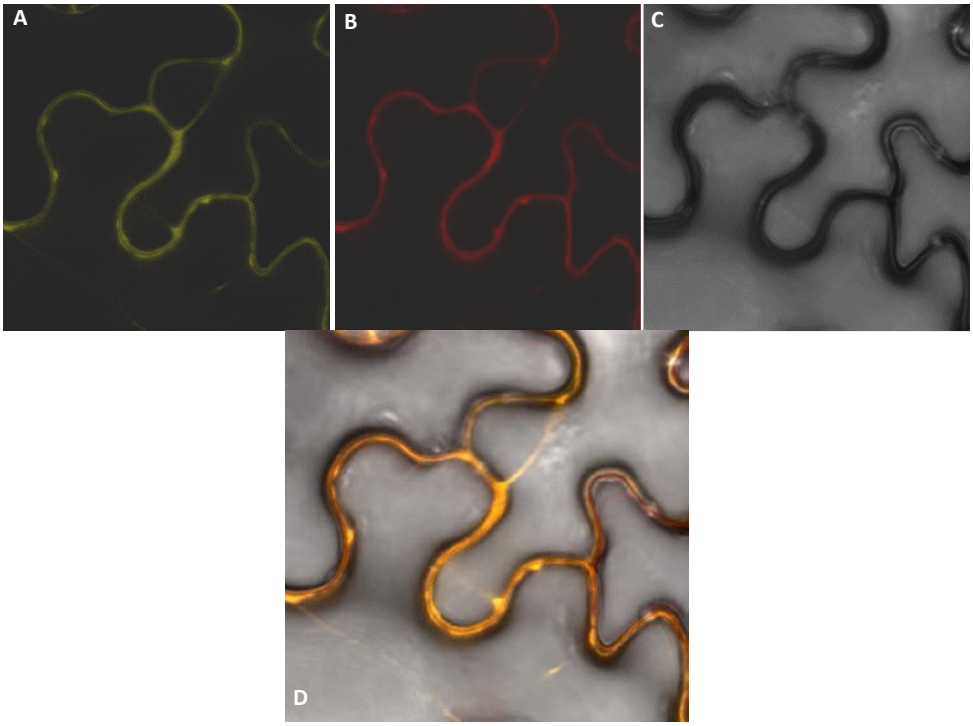


Figure 3: Co-expression of YFP-RGA5 and mRFP in *N.benthamiana*.

A: YFP- RGA5 signal: localization in the cell periphery

B: mRFP signal: localization in cytoplasm

C: Bright Field image

D: Merged image: red and yellow signals colocalize in cytoplasm

Since the cytoplasm is very narrow in *N. benthamiana* epidermal cells and cannot unambiguously be distinguished from the plasma or vacuole membrane, plasmolysis was performed. This further supported co-localization of RGA4-GFP and YFP-RGA5 with mRFP in the cytoplasm since green or yellow and red fluorescence perfectly overlapped in plasmolyzed cells (Figure 4 and 5).

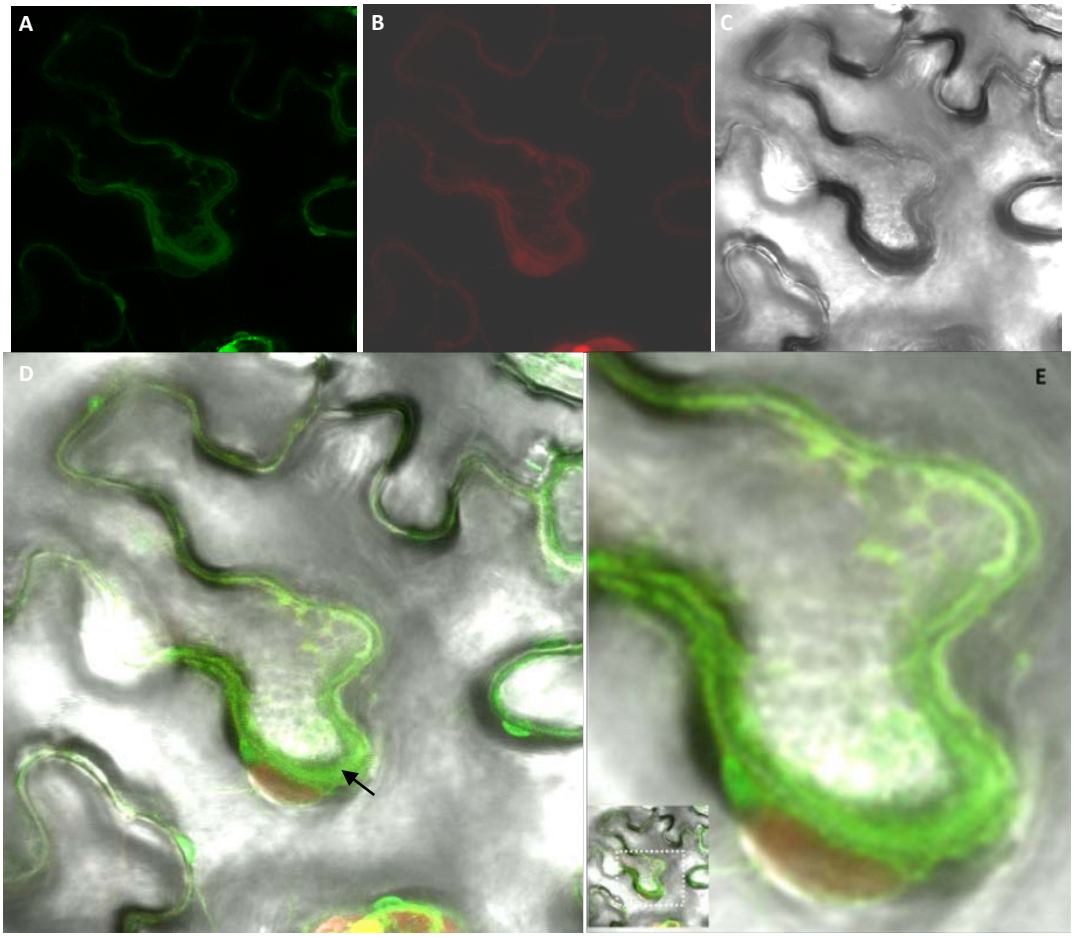


Figure 4: Co-expression of RGA4-GFP and mRFP in plasmolysed *N.benthamiana* cells.

A: RGA4-GFP signal

B: mRFP signal: localization in cytoplasm

C: Bright Field image

D: Merged image: both red and green signals colocalize in the cytoplasm of the plasmolysed cell (arrow)

E: Crop of image 4D (dashed square)

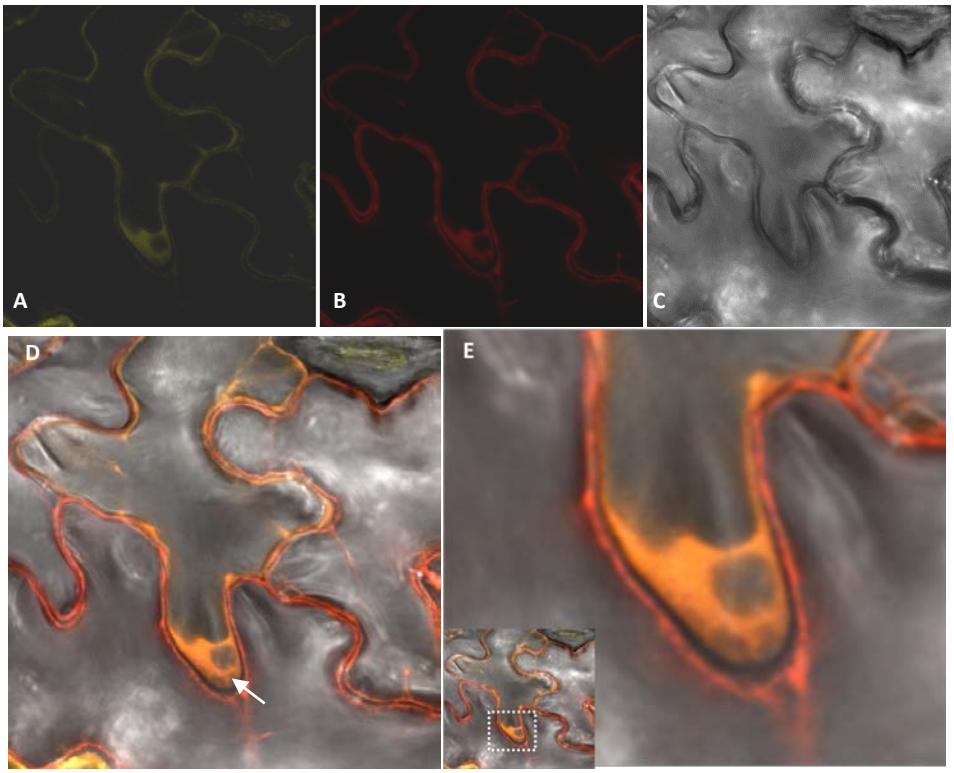


Figure 5: Co-expression of YFP-RGA5 and mRFP in plasmolysed *N.benthamiana* cells.

A: YFP-RGA5 signal

B: mRFP signal, localization in cytoplasm

C: Bright Field image

D: Merged image: both red and yellow signals colocalize in the cytoplasm of the plasmolysed cell (arrow)

E: Crop of image 5D (dashed square)

To further exclude plasma membrane localization of RGA5, YFP-RGA5 was co-expressed with the red fluorescent membrane markers PIP1.4-Cherry and AVRblb2-mRFP. The plasma membrane aquaporin PIP1.4 is a well-known and frequently used plasma membrane marker (Lu et al., 2012) and the *Phytophthora infestans* effector AVRblb2 has been shown to localize to the plant plasma membrane by the host laboratory (Bozkurt et al., 2011).

PIP1.4-mCherry localization proved to be slightly different from YFP-RGA5 localization (Fig. 6). However, with PIP1.4-mCherry, not only the cell periphery but also cytoplasmic strands were labelled suggesting that PIP1.4-mCherry labeling is, at least under conditions of strong overexpression, not really specific for the plasma membrane (Fig6B). AVRblb2-mRFP gave more precise labeling of the plasma membrane. Only the outline of the cells was labelled and no cytoplasmic strands were marked. No co-localization with the fluorescence from YFP-RGA5 was detected (Fig7).

Taken together, these results indicate that RGA4 and RGA5 are localized exclusively to the cytoplasm.

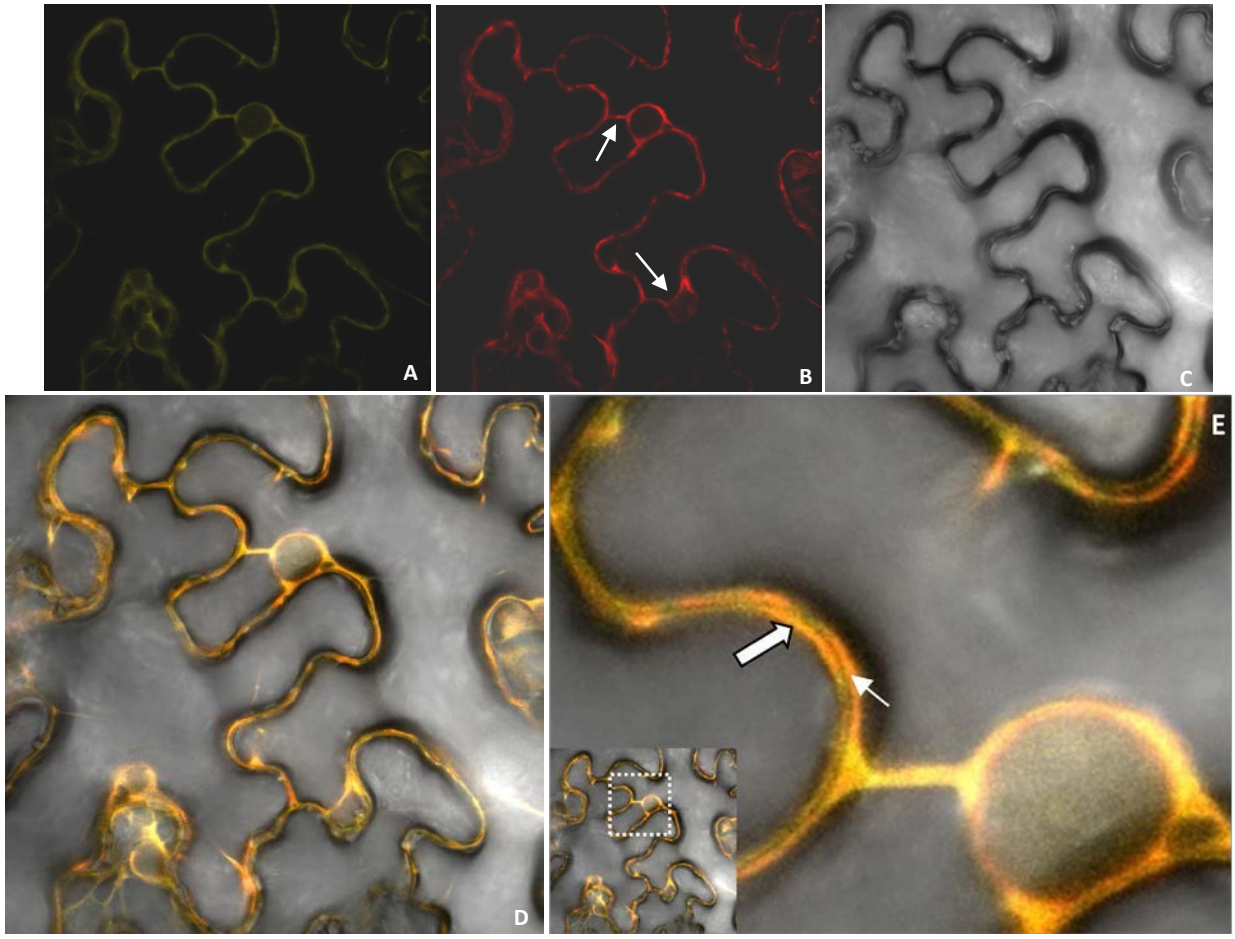


Figure 6: Co-expression of YFP-RGA5 and PIP1:4-mCherry

in *N.benthamiana* leaves.

A: YFP-RGA5 signal

B: PIP1;4-mCherry signal, localization mainly in the plasma membrane. However, cytoplasmic strands were also stained (arrows)

C: Bright Field image

D: Merged image

E: Crop of image: slight difference between red (PIP1;4-mCherry) (thin arrow) and yellow signal (YFP-RGA5) (thick arrow)

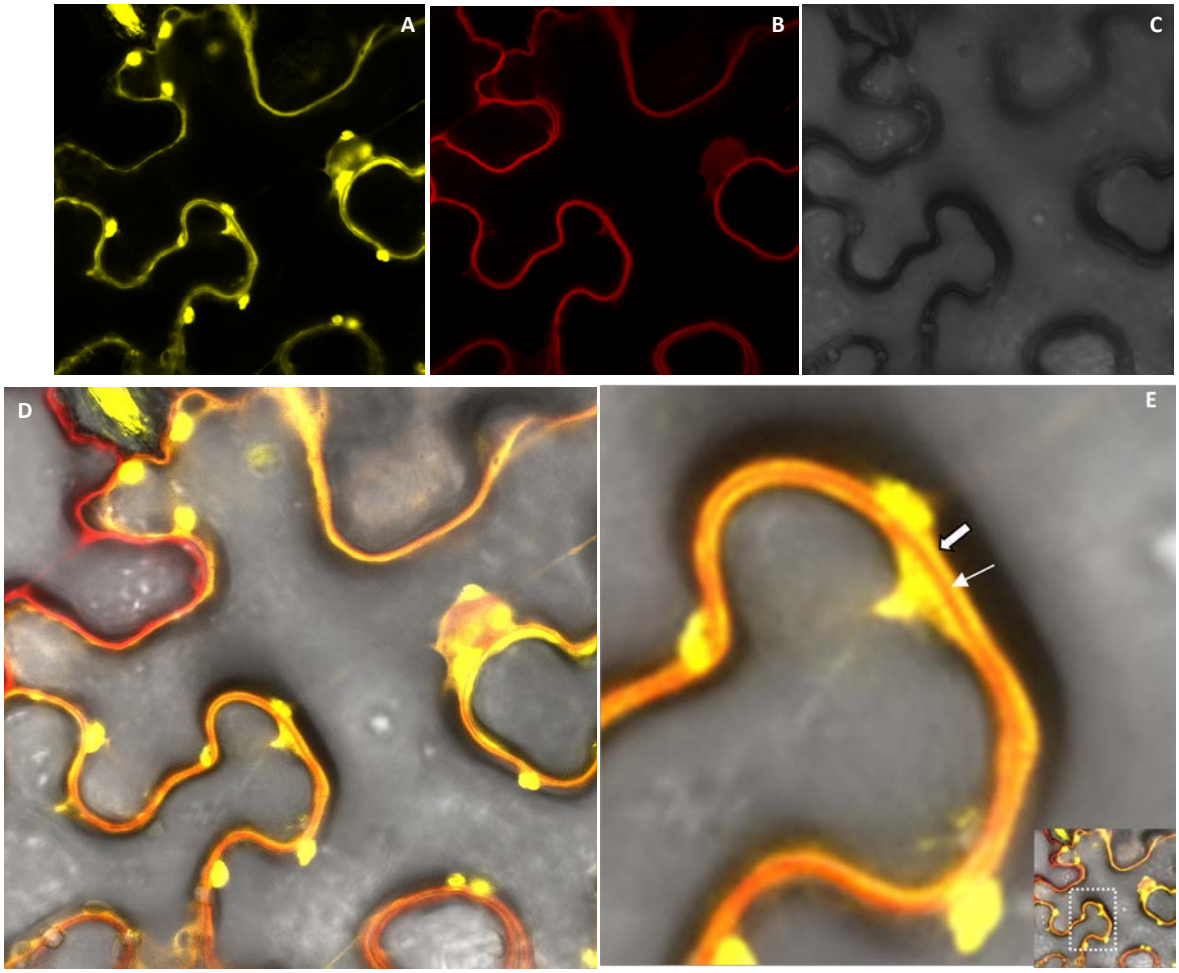


Figure 7: Co-expression of YFP-RGA5 and AVRblb2-mRFP

in *N.benthamiana* leaves.

A: YFP-RGA5 signal

B: AVRblb2-mRFP signal, localization in plasma membrane

C: Bright Field image

D: Merged image

E: Crop of image 5D (dashed square): red (AVRblb2-mRFP) (thin arrow) and yellow signal (YFP-RGA5) (thick arrow) do not superpose.

To address the localization of RGA5 in vesicle-like structures in certain cells, YFP-RGA5 was co-expressed with Ara6-RFP, a marker of early endosomes or Ara7-RFP, a marker of late endosomes (Figure 8 A and B respectively). No colocalisation of these markers with YFP-RGA5 spots was observed suggesting that RGA5 does not accumulate in endosomes

When YFP-RGA5 was co-expressed with the aggregates marker JOKA2:RFP, co-localization was observed (Figure 8C). JOKA2 is a hybrid homolog of two mammalian selective autophagy cargo receptors labelling phagosomes and accumulating in cytoplasmic aggregates. This suggests that the spot-like structures with strong yellow fluorescent signal from YFP-RGA5 are phagosomes or protein aggregates and probably an artefact caused by strong overexpression.

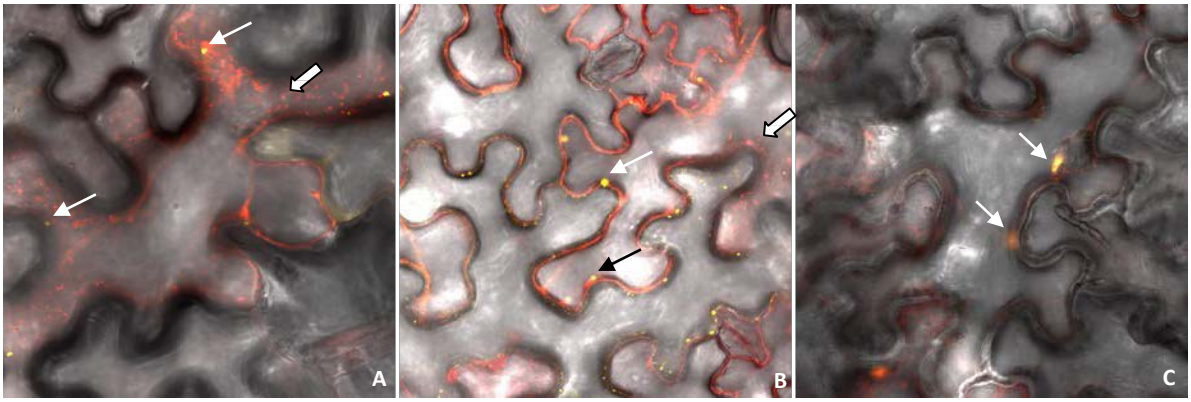


Figure 8: Co-expression of YFP-RGA5 and markers of the endosome and the aggregates

A: YFP-RGA5 localized to vesicle-like structures (thin arrows) and the early endosomes marker Ara6-mRFP (thick arrow) do not colocalize.

B: YFP-RGA5 localized to vesicle-like structures (thin arrows) and the late endosome marker Ara7-mRFP do not colocalize..

C: YFP-RGA5 localized in vesicle-like structures and the aggregates marker JOKA2-mRFP colocalize (arrows).

Conclusion and perspectives:

The STSM generated evidence that RGA4 and RGA5 are exclusively localized to the cytoplasm and that RGA5, even if it should be farnesylated, does not localize to the plasma membrane or other membrane compartments. To further confirm this result, cell fractionation (by ultracentrifugation of protein extracts) coupled to immune-blot experiments will be performed allowing to determine if RGA4 and RGA5 are detected exclusively in the cytoplasmic fraction or also in the membrane fraction. To address the potential farnesylation of RGA5, immune-precipitated RGA5 will be analyzed in immune-blot experiments with anti farnesyl antibodies to determine if it contains indeed this post translational modification.

The STSM generated also evidence that the spots seen for YFP-RGA5 are a consequence of over expression and biologically not relevant. Further experiments where YFP-RGA5 agro stains are infiltrated at much lower OD (0.01 instead of 0.2) are planned to determine if under such conditions vesicle-like localization is still detected.

- **Future collaboration with the host institution (if applicable):** Some Immunoprecipitation-Mass Spectrometry with RGA4 and RGA5 so as to identify the components of the immune complex could be envisaged.

- **Projected publications/articles related to or resulting from the STSM:** Additional experiments are needed before publication

References:

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