Baum ISRC Progress Report December 2021
Mechanisms of Defense Suppression by Cyst Nematode Effectors

Introduction:

Soybean is one of the most important sources of oil and proteins for food and feed produced in the United States. In addition, this leguminous plant can be used to enrich the soil in nitrogen through its association with symbiotic nitrogen-fixing bacteria. It is therefore crucial to maintain soybean crops at their highest production levels. The soybean cyst nematode (SCN), *Heterodera glycines*, is considered the most damaging pathogen of soybean production. This organism causes soybean yield loss estimated in billions of dollars per year and threatens sustainable soybean production. SCN delivers effectors molecules to hijack the soybean plant's physiology, including complete reprogramming of cell fates and the powerful inactivation of plant defense mechanisms.

In the last decade, our laboratory has characterized several cyst nematode effectors and has shed light on their roles during plant-pathogen interactions. Using an interactomic approach (e.g., yeast-two hybrid screens) the laboratory has characterized dozens of effector/plant targets. Proteinaceous effectors are powerful keys to understanding plant cell physiology, and their co-evolution with the plant immune system leads to an incredibly complex system defining the rules for a successful infection.

Recently, our laboratory has reported nine SCN effectors that are able to suppress plant immunity. The research proposed here aims to use two out of these nine effectors that show the strongest immune suppression abilities, to guide the way to exploring uncharacterized components of the soybean immune system. We will adapt and implement the recent proximity labelling approach for the first time in a soybean hairy root composite system to identify soybean targets of those two effectors.

Identified plant targets could be further engineered to create broad resistance to plant pathogens in a wide sense. Therefore, the outcome of this project will provide significant impact on our understanding fundamental mechanisms of immunity in soybean and will have great potential to create sustainable agronomic production systems throughout the world.

Selection of two soybean cyst nematode effectors that we have shown to suppress plant defenses:

Among the nine previously identified effectors able to suppress plant immunity, we have selected two effectors showing the strongest Effector Triggered Immunity (ETI) suppression abilities: GLAND1 and GLAND9.

While GLAND9 is a pioneer protein without recognizable domains or similarity to other proteins in the NCBI NR databases, GLAND1 comprises three distinct domains: (i) a previously undescribed N-terminal domain; (ii) an N-acetyltransferase domain reported as being acquired from soil bacteria (*Streptomyces sp.*) through Horizontal Gene Transfer; and (iii) a C-terminal domain carrying six tandem repeats of unknown function. GLAND1 and GLAND9 both are soluble proteins and exhibit secretory signal peptides and no transmembrane domains, strongly suggesting that they are secreted from the nematode salivary glands. The presence of a Dorsal Gland Motif (DOG box) in their respective promoters is consistent with the elevated expression of GLAND1 and GLAND9 during plant infection as emphasized by RNA-seq data (**Figure 1a**) and *in situ* hybridization. Indeed, this DOG box constitutes a well-known signature of effectors produced in the dorsal salivary gland of cyst nematodes, the most active gland during late infection stages.

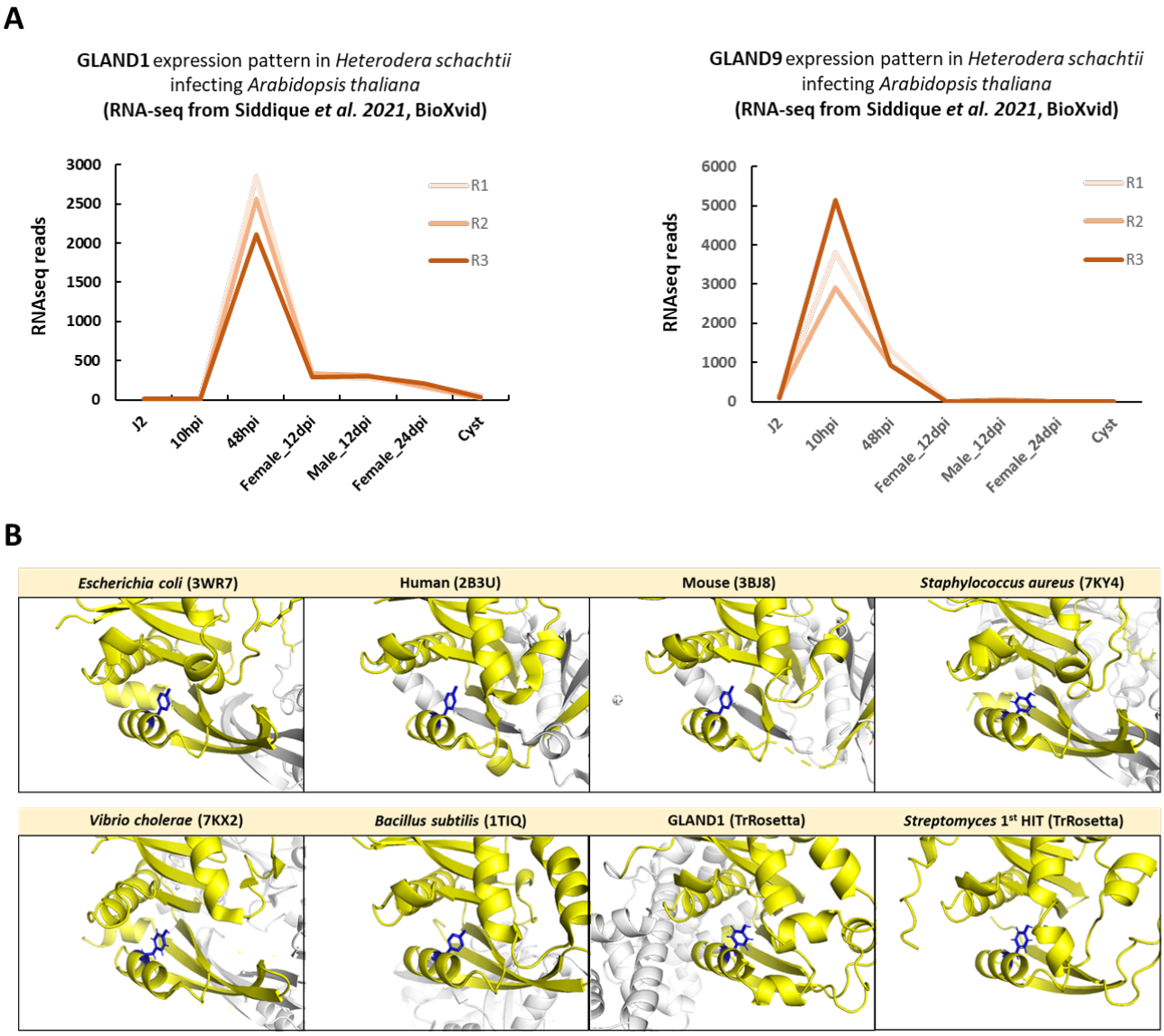

Figure 1. **(A)** Expression pattern of GLAND1 and GLAND9 throughout the life cycle of *Heterodera schachtii* (RNA-seq from Siddique *et al*. 2021, BioXvid). **(B)** Structural alignment of acetyl-transferase domain from *E. coli*, Human, Mouse, *S. aureus*, *V. cholera*, *B. subtilis*, GLAND1 and *Streptomyces* spp. The acetyl-transferase domain is colored in yellow and the tyrosine residue (GLAND1 Y231) involved in the acetyl transfer is highlighted in blue.

We used recently published expression data to monitor at which time point GLAND1 and GLAND9 are expressed during the life cycle of *H. schachtii*, another species of cyst nematode for which seven time points of its life cycle have been subjected to RNA-seq analyses. We observed that GLAND1 and GLAND9 show elevated expression very early on at 48 and 10 hours post infection (hpi), respectively (**Figure 1A**). This expression timing corroborates their being involved in plant defense suppression because plant defense has to be suppressed as soon as the nematodes enter the plant.
We previously showed that GLAND1 and GLAND9 are able to suppress the ETI response induced by *Pseudomonas syringae* in *N. benthamiana* leaves. GLAND1 strongly delays the hypersensitive response

(HR) triggered by the recognition of *P. syringae* by the host, whereas GLAND9 is a more moderate suppressor of the ETI response induced by *P. syringae*

Functional analysis of the GLAND1 domains and their involvement in ETI suppression:

Since GLAND1 comprises three distinct domains, we produced several truncated versions of this protein: A ΔN-terminal version (G1N); a ΔC-terminal version (G1C); a partial ΔC-terminal version where the three last repeats have been deleted (G1Gr 3R); a partial ΔC-terminal version where the five last repeats have been deleted (G1Gr 1R) and a ΔN-terminal version + ΔC-terminal version (AcT) (thus only the N-acetyl-transferase domain remains). Moreover, to test if the N-acetyltransferase domain is involved in ETI suppression, we first predicted the 3D structure of the acetyltransferase domain of GLAND1 (using the TrRosetta webserver) and we performed a structural alignment with other acetyl-transferase domains for which the 3D structures have been resolved by crystallography. This analysis pointed out a conserved tyrosine residue described in the literature to be essential for the acetyltransferase activity, which is conserved in the GLAND1 acetyltransferase domain. Subsequently, we used directed-site mutagenesis to introduce point mutations on the conserved catalytic Tyrosine residue (G1 Y231F) involved in the transfer of the acetyl group from Acetyl-CoA to the potential plant targets of GLAND1 (**Figure 1B**).

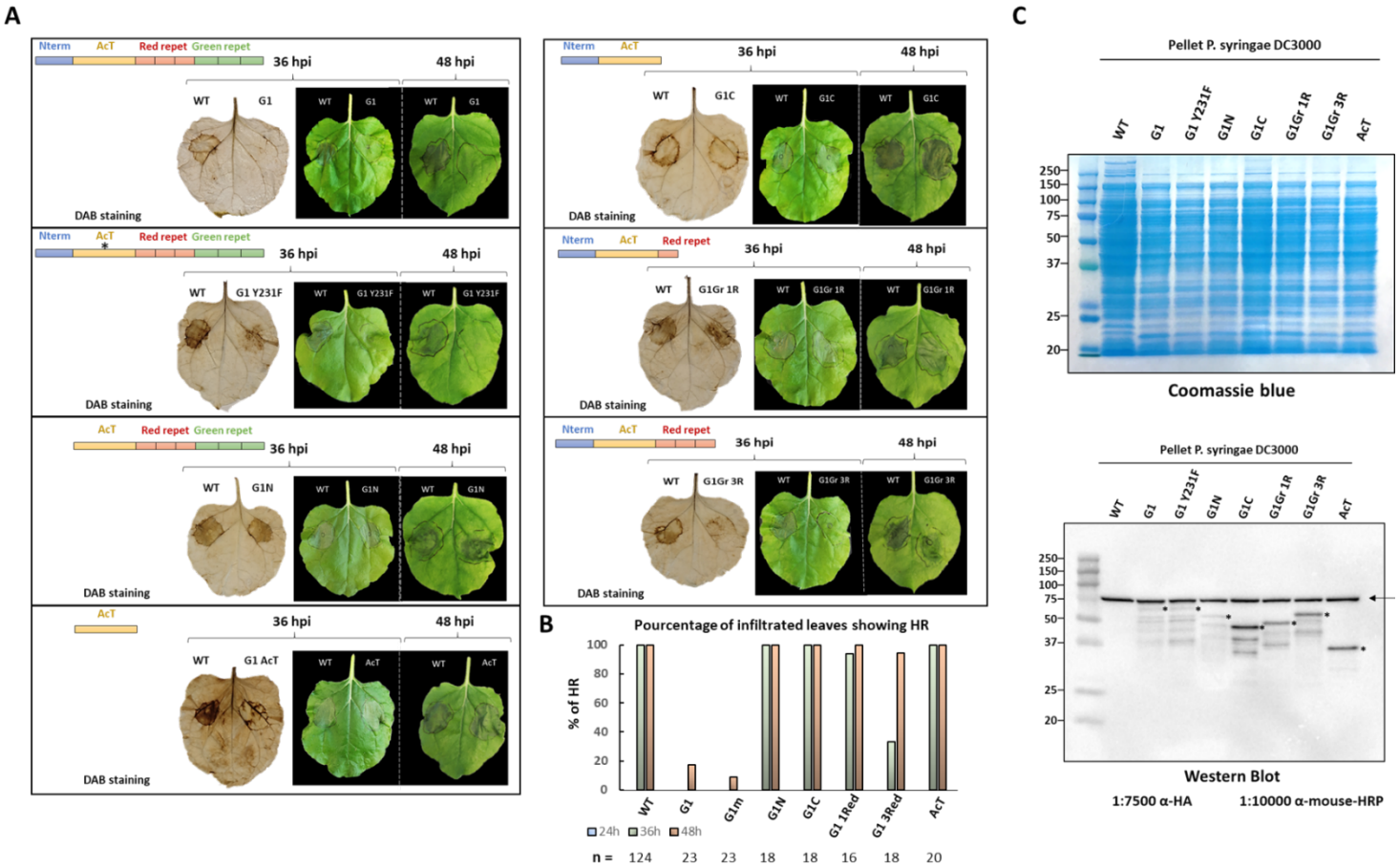

Figure 2. **(A)** GLAND1 suppresses hypersensitive response induced by *P. syringae* in *N. benthamiana*. Truncated versions of GLAND1 are expressed and secreted by *P. syringae* into *N. benthamiana* leaves. Each *P. syringae* strain has been infiltrated at O.D $_{600}$= 0.005 and HR has been scored at 24, 36, or 48 hpi. Leaves have been cleared and stained with DAB to identify the production of ROS species released during the HR. **(B)** Percentage of hypersensitive response observed among the different GLAND1 constructions. **(C)** Protein extraction from *P. syringae* pellets that have been separated on SDS-PAGE (12%) and stained with Coomassie blue (upper panel). Western blot of protein extracts using

α-HA antibody (lower panel). *= expected sizes for GLAND1 full-length or truncated versions. Arrow = non-specific band.

All GLAND1 mutants have been secreted into *N. benthamiana* leaves by *P. syringae*, which allowed us to test which domains are important for ETI suppression. We observed that the N-terminal and C-terminal domains of GLAND1 are essential for its ETI suppression ability (**Figure 2A, 2B**). Since the C-terminal domain contains multiple repeated motifs, we produced truncated versions of GLAND1 for which the three or five last repeats were deleted. The three-repeats-deleted versions still show an ability to delay the HR cause by *P. syringae*, but the HR is less delayed compared to the wild-type version of GLAND1, suggesting that those three last repeats are not essential but support the ETI suppression ability of GLAND1 (**Figure 2A, 2B**). The five-repeats-deleted version of GLAND1 could not suppress the HR as observed for the full ΔC-terminal version of GLAND1, suggesting that at least two or three repeats are essential to the ETI suppression ability of GLAND1. When the acetyltransferase domain is expressed alone, we did not observe any HR suppression ability, suggesting that the N and C-terminal domain have to be present to make GLAND1 functional. We confirmed that all those full-length point-mutated or truncated versions of GLAND1 are indeed expressed and produced in *P. syringae* as shown by Western Blot (**Figure 2C**).

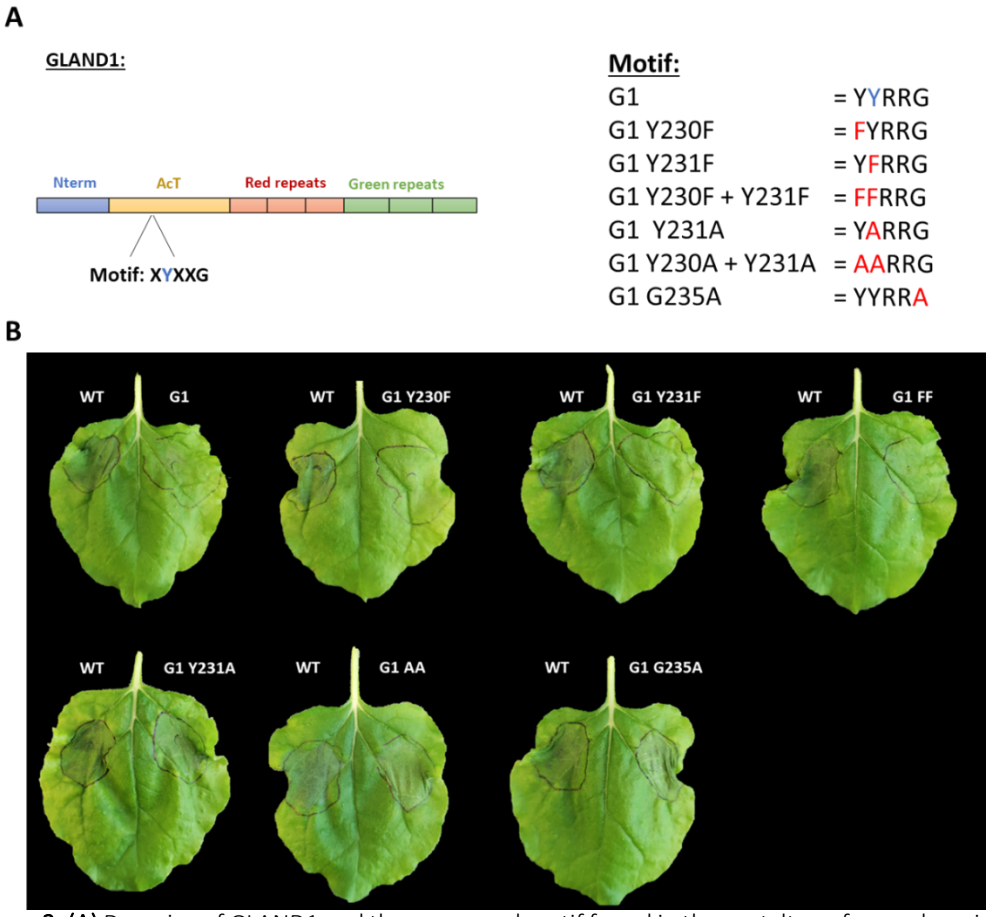

Figure 3. **(A)** Domains of GLAND1 and the conserved motif found in the acetyltransferase domain (left panel). Different point mutated versions produced into the GLAND1 acetyltransferase domain (right panel). **(B)** Infiltration of P. syringae carrying the different GLAND1 point mutations were infiltrated at O.D $_{600}$= 0.005 and HR has been scored at 36 hpi.

We reasoned that if the acetyltransferase domain is involved in the ability of GLAND1 to suppress the ETI response, then introducing a point mutation to modify the conserved tyrosine, involved in the catalytic transfer of the acetyl to its unknown plant substrate, into phenylalanine should impair the ETI suppression ability of GLAND1. We discovered that modifying the conserved tyrosine into phenylalanine does not impair the ETI suppression ability of GLAND1 (**Figure 2A, 2B**).

Because tyrosine and phenylalanine differ only in one hydroxyl group and in order to further scrutinize if this amino acid plays a central role in the acetyl-transferase domain and could be involved in the suppression of plant immunity, we subsequently modified this tyrosine into alanine, a much more distant amino acid. Also, since another tyrosine is located right next to the tyrosine of interest, we have produced multiple point mutations aimed at mutating one or both tyrosines, into phenylalanine or alanine (**Figure 3A**).

We again used *P. syringae* to secrete those point-mutated versions of GLAND1 into *N. benthamiana* leaves and we observed that constructs carrying an alanine instead of the conserved tyrosine are not able to suppress ETI anymore, whereas the phenylalanine-mutated versions are still able to suppress ETI. This result demonstrates that the acetyltransferase activity of GLAND1 is important for ETI suppression (**Figure 3B**). One can hypothesize that GLAND1 interacts with one or more plant targets through its N-terminal and/or C-terminal domains and acetylates those targets to impair or stabilize their function involved in ETI using its acetyl-transferase domain.

To gain a better insight into how GLAND1 might function inside plant cells, we produced GFP fusions with full-length or truncated versions of GLAND1. Those constructs were transiently expressed in *N. benthamiana* leaves using *Agrobacterium tumefaciens*, and the fluorescence signals were monitored using a confocal microscope at 48 hpi (**Figure 4**). GLAND1 exhibits a nucleocytoplasmic subcellular localization which is excluded from the nucleolus in all the cells observed, whereas the N-terminal, full C-terminal or C-terminal repeats-truncated versions lead to a nucleolar accumulation in some cells or show the same pattern as the full-length GLAND1 in other cells from the same infiltrated leaf area. More interestingly, the acetyltransferase domain alone also localized to the nucleolus and particularly in nuclear speckles. Since histones are located in the nucleus, and knowing that actively transcribed chromatin is localized in nuclear speckles, we hypothesize that GLAND1 could target chromatin-associated proteins, such as histones, to acetylate those and regulate the expression of genes involved in the ETI pathway. A validation of these subcellular localizations will be performed in soybean roots to confirm our observations.

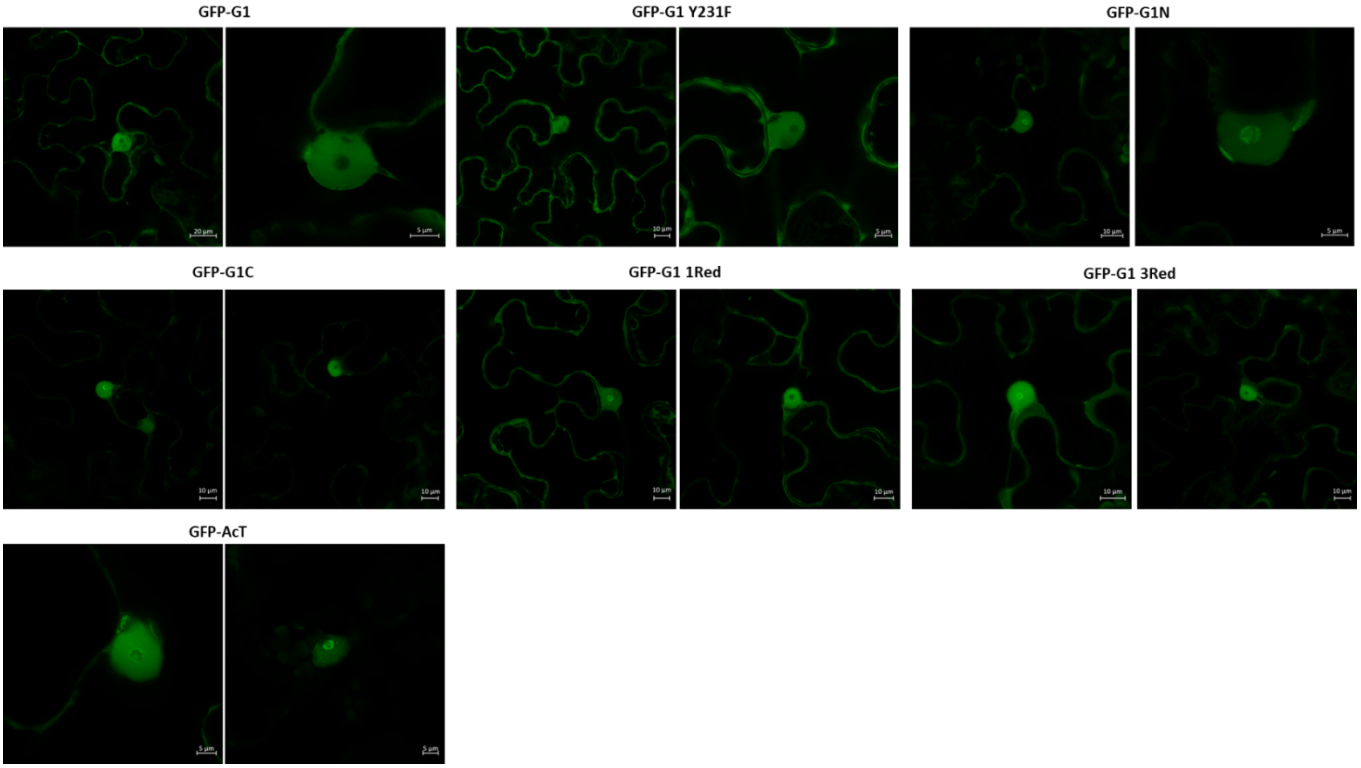

Figure 4. Subcellular localization of GFP-GLAND1 full-length and truncated versions in *N. benthamiana* leaves 48 hpi with *A. tumefaciens* GV3101.

Outlook

Concerning GLAND1, we will be conducting analysis of its expression pattern in *H. glycines* infecting soybean roots at 4, 7, 12 and 30 dpi using RT-qPCR.
We are currently constructing vectors that will allow us to perform:

- Subcellular localization of GLAND1 in soybean roots and syncytia.
- Proximity-labelling of GLAND1 to identify plant targets in soybean roots (using miniTurbo-ID).
- Overexpression of GLAND1 fused with a 3xHA epitope to monitor if its overexpression could impact *H. glycines* infection of soybean roots.
- Perform immunoprecipitation of 3xHA-GLAND1 in soybean roots and verified by Western blot if GLAND1 autoacetylates itself, using the alanine point mutation versions of GLAND1 as a control.

Concerning the 2nd effector GLAND9, we will be conducting analyses of its expression patterns in *H. glycines* infecting soybean roots at 4, 7, 12 and 30 dpi using RT-qPCR.
Similar approaches as used for GLAND1 will be conducted, including:

- Production of truncated versions of GLAND9 and testing of their abilities to suppress ETI using *P. syringae*.
- Scrutinize its subcellular localization in *N. benthamiana* leaves and soybean roots.
- Identification of its potential soybean target using proximity labelling assays.