

Adapting the Split GAL4 RUBY assay for Golden Gate cloning: applications in *Nicotiana benthamiana* protein interaction studies



Julie Lintz, Jiorgos Kourelis, Mark Youles, Sophien Kamoun
The Sainsbury Laboratory, University of East Anglia, Norwich Research Park, NR4 7UH, UK

@lintz_julie1

Introduction

Investigating protein–protein interactions (PPIs) in plant cells is essential for understanding signalling pathways, immune receptor complexes, and effector targets, but remains technically challenging. Here, we have adapted the recently developed split GAL4 RUBY assay (Chen et al., 2023) for use with Golden Gate cloning, a widely adopted modular assembly system in plant molecular biology.

Goal

To optimize and validate a Golden Gate-compatible version of the split GAL4 RUBY assay for plant PPI studies, enabling visual detection via betalain pigment production in *Nicotiana benthamiana*.

Method using the Golden Gate cloning

Proof of concept using mCherry and its LaM-4 nanobody

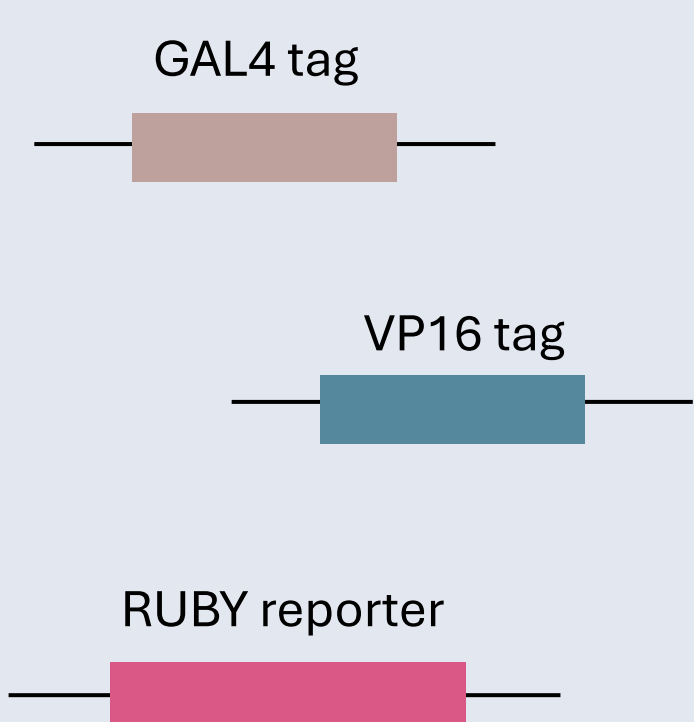
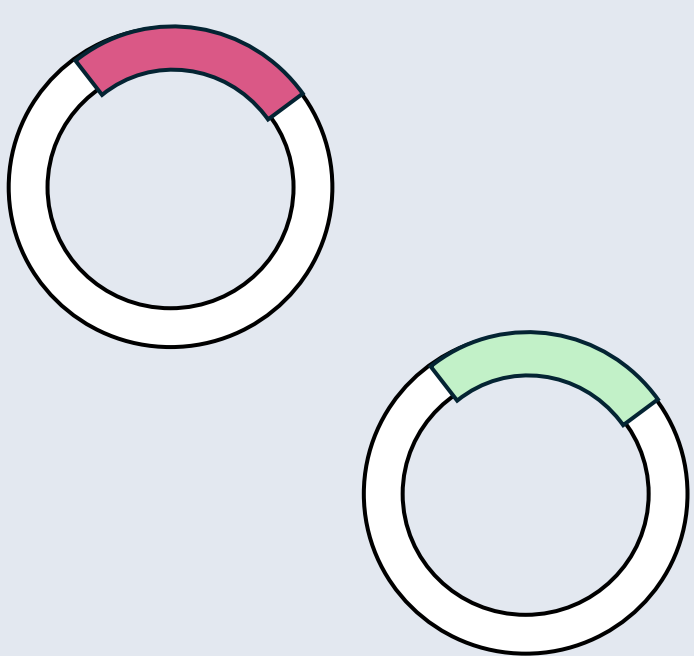
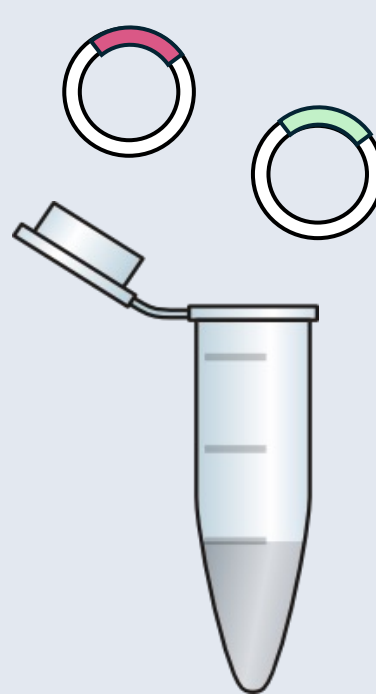
Modular design

Cloning

Validation system

Transient expression

Detection



Split GAL4 domains and RUBY reporter were adapted for Golden Gate cloning

Assembly of interaction constructs using the Golden Gate MoClo system

Known interacting pair mCherry/LaM-4 nanobody used as proof of concept

Agrobacterium-mediated infiltration in *N. benthamiana*

Visual scoring of betalain accumulation as PPI readout

Result

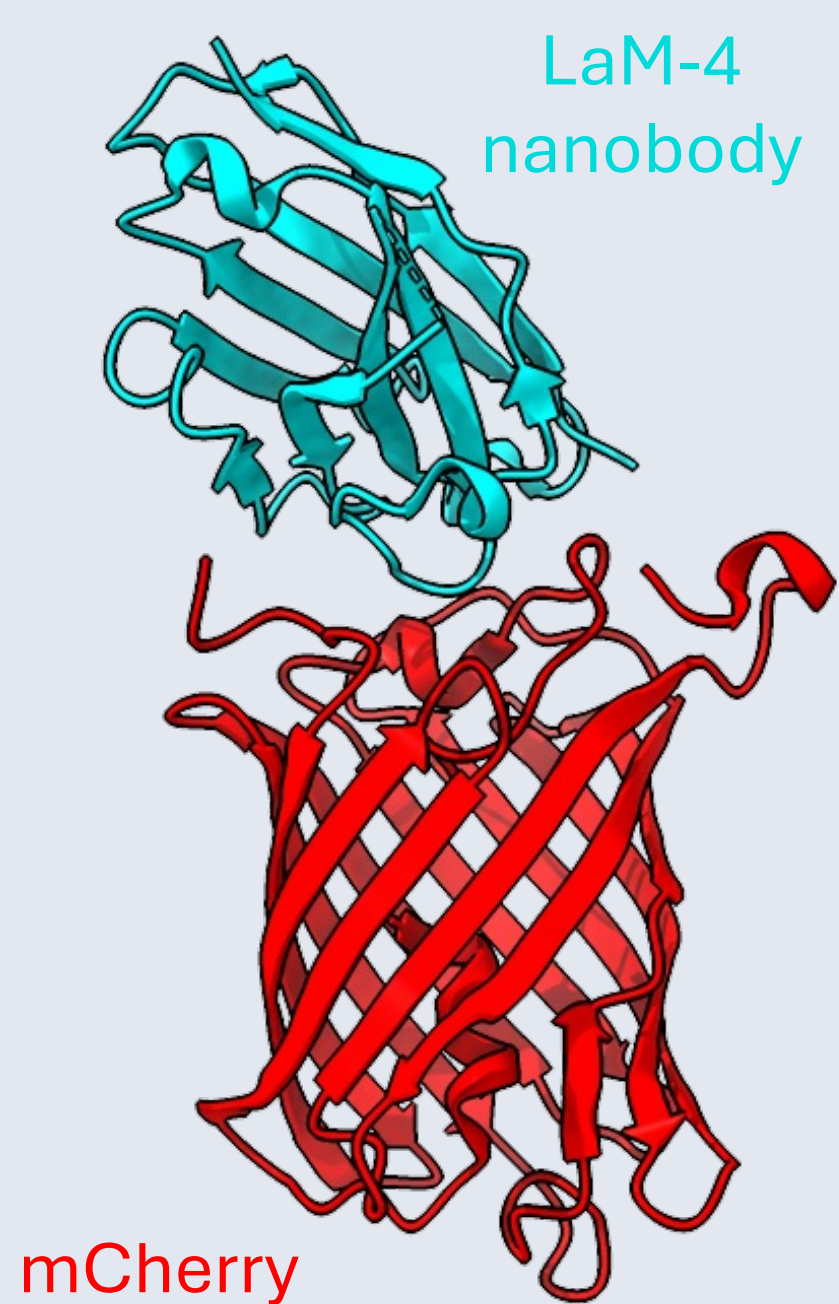
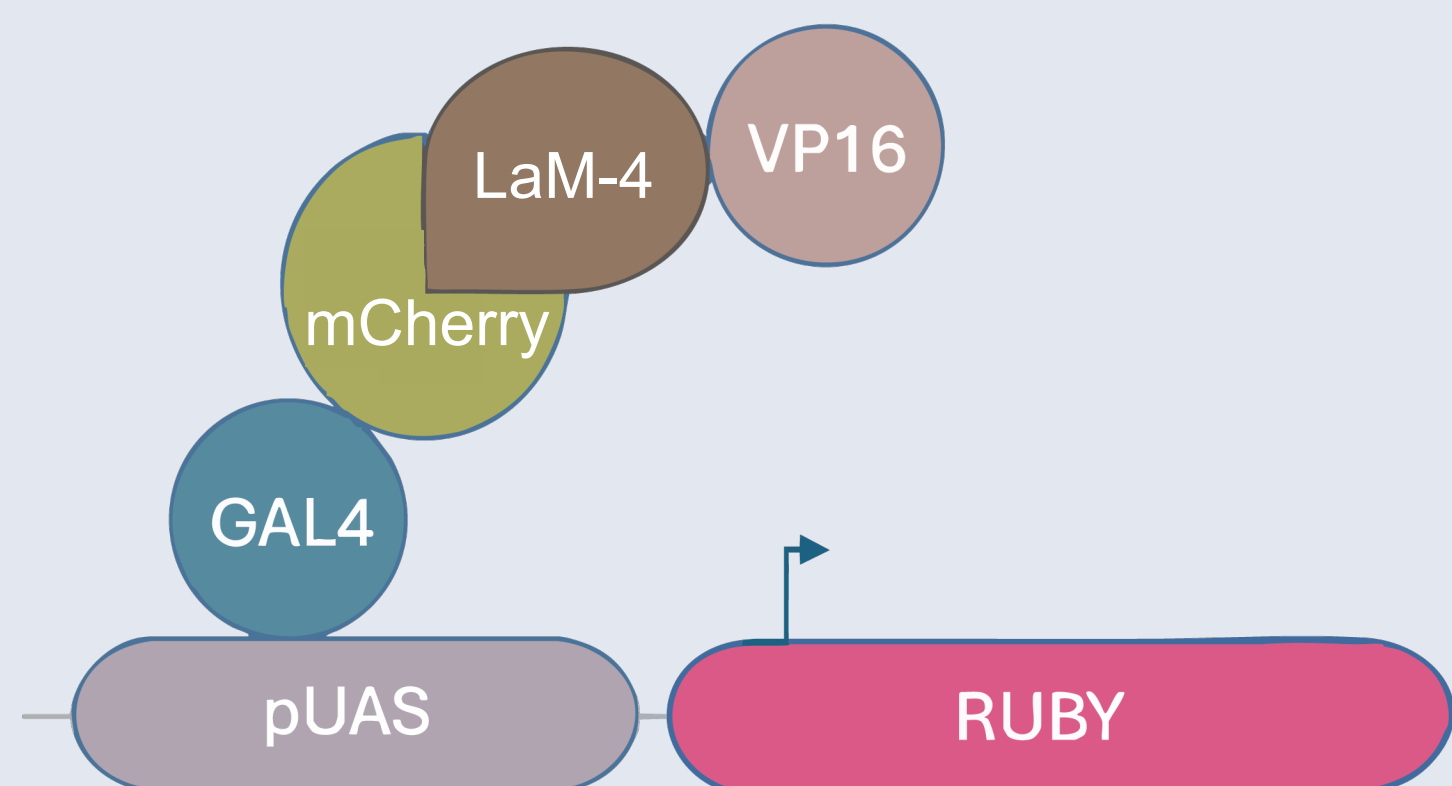
mCherry/LaM-4 nanobody interaction leads to betalain pigment accumulation in *N. benthamiana*

Known interactors

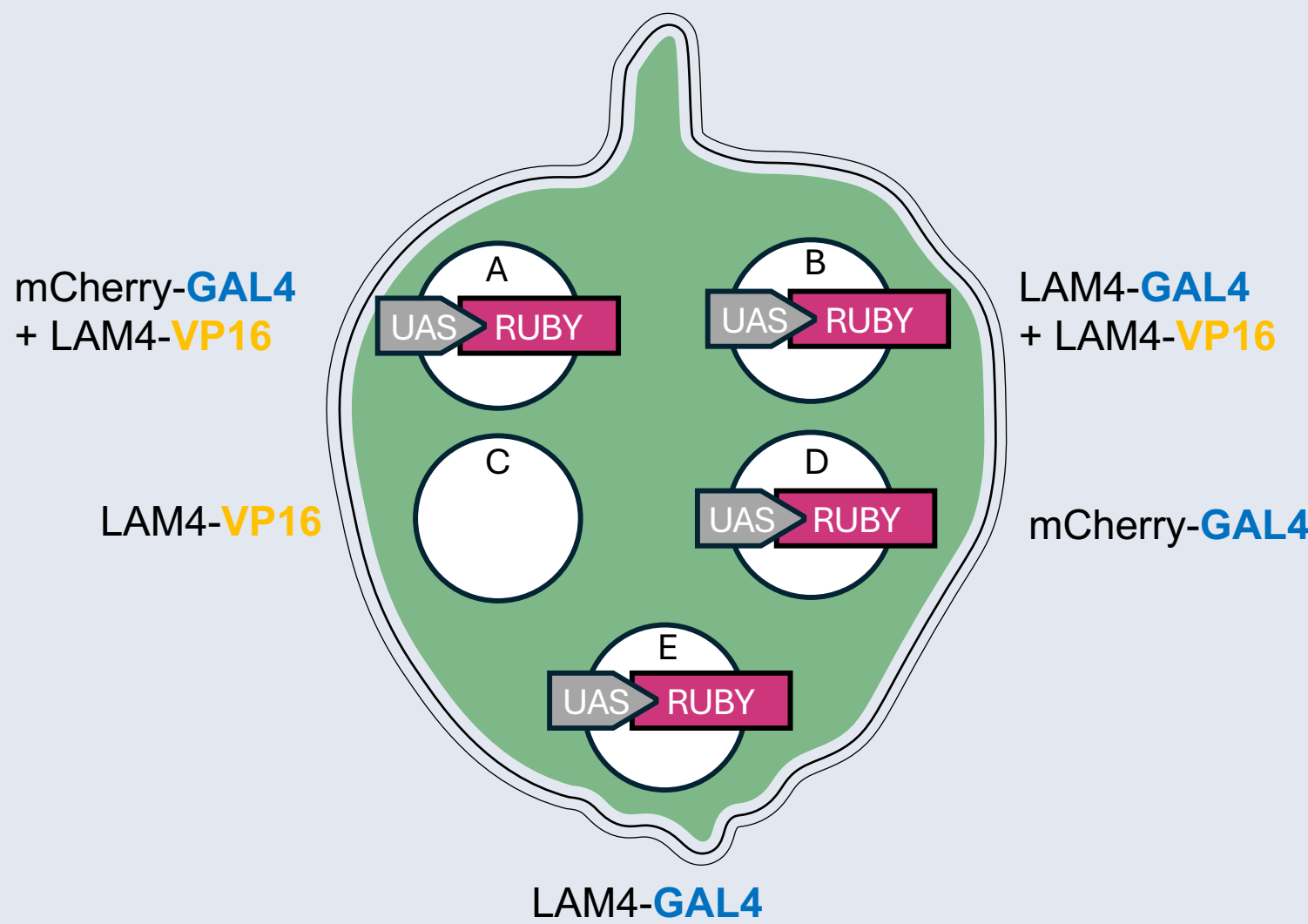
Golden Gate cloning

Transient expression in *N. benthamiana*

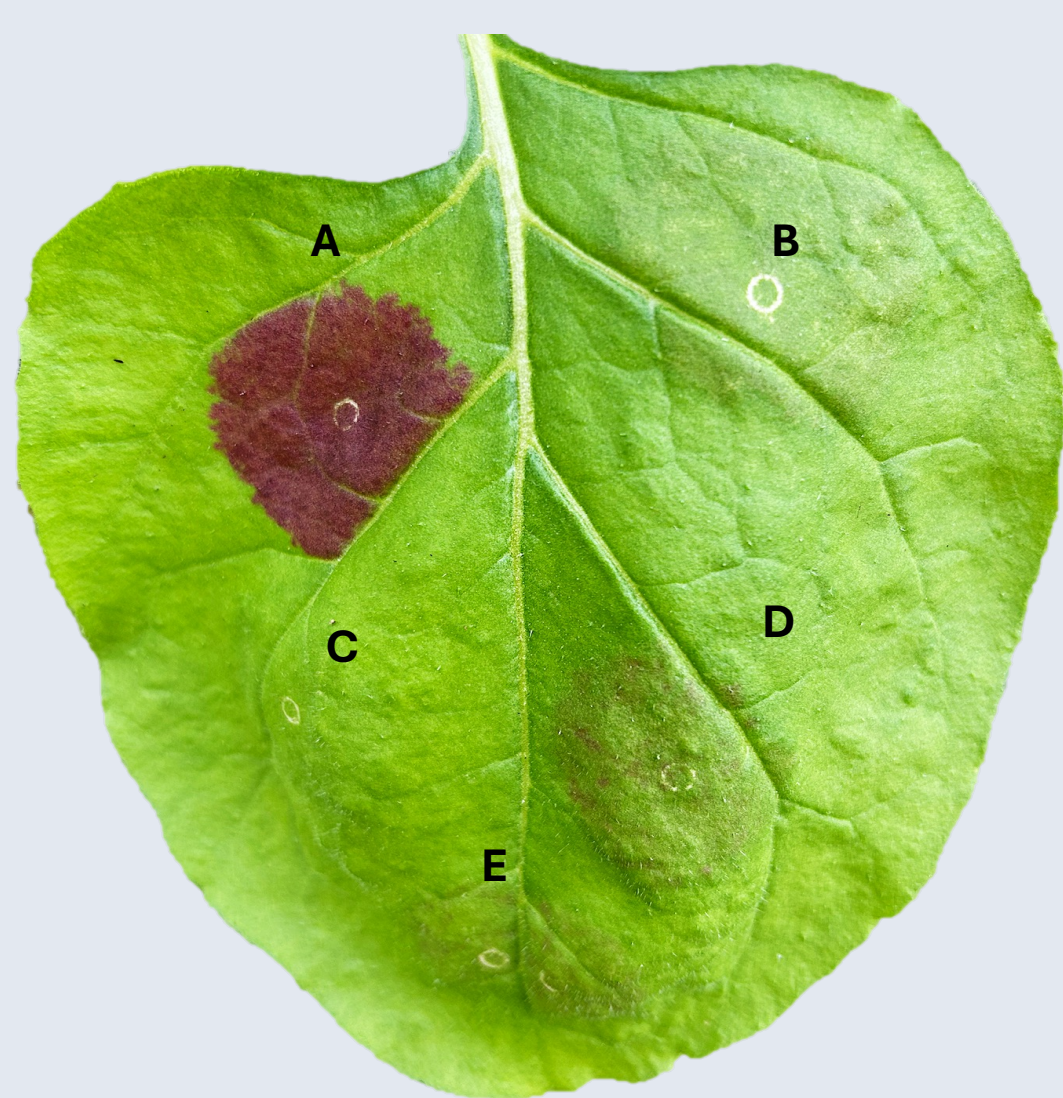
Visual readout 3 dpi



Successful assembly of test constructs using the Golden Gate system



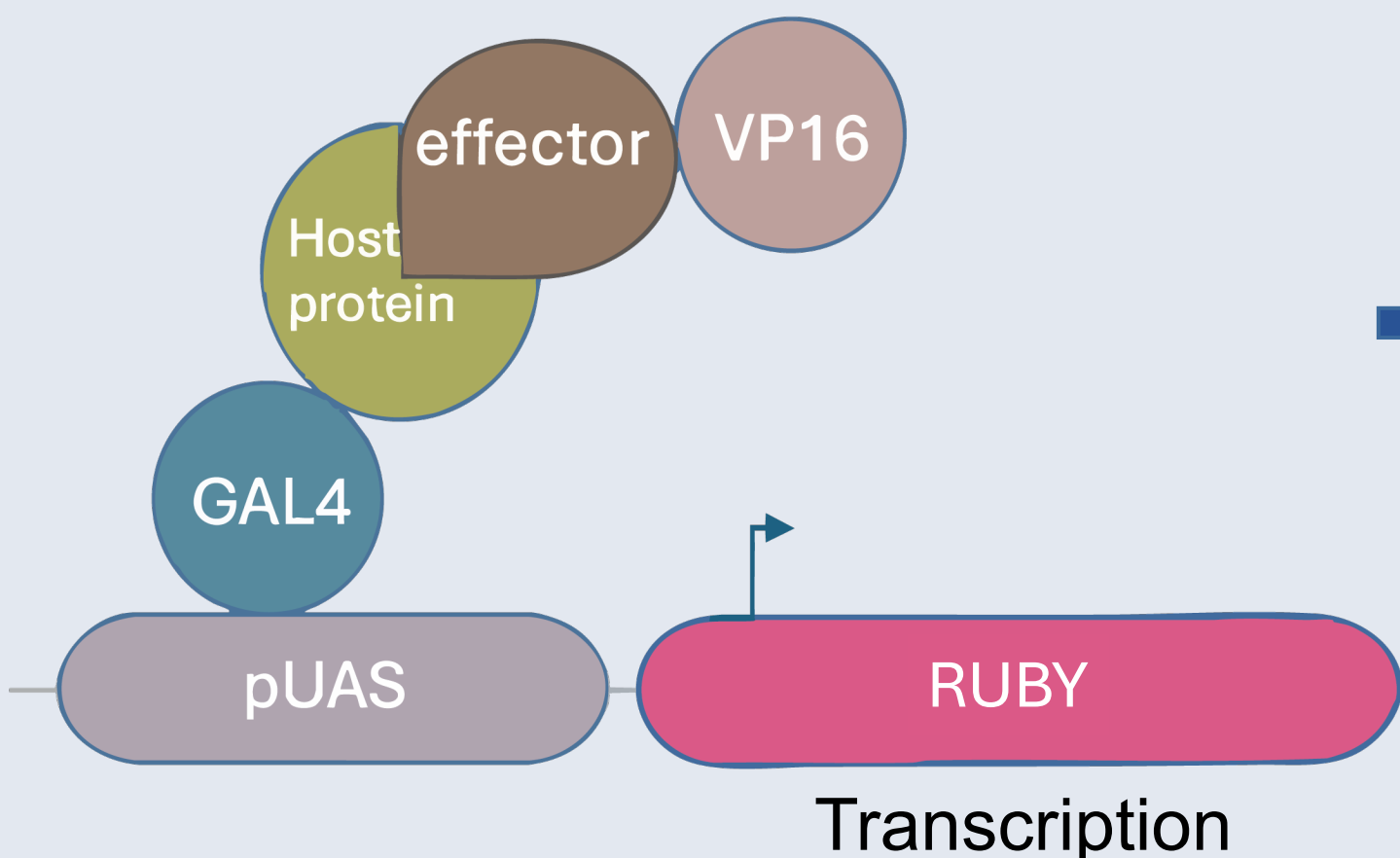
4 negative controls to demonstrate assay specificity (B, C, D, E)



Betalain pigment accumulation observed in A co-expressing mCherry/ LaM-4, confirming interaction

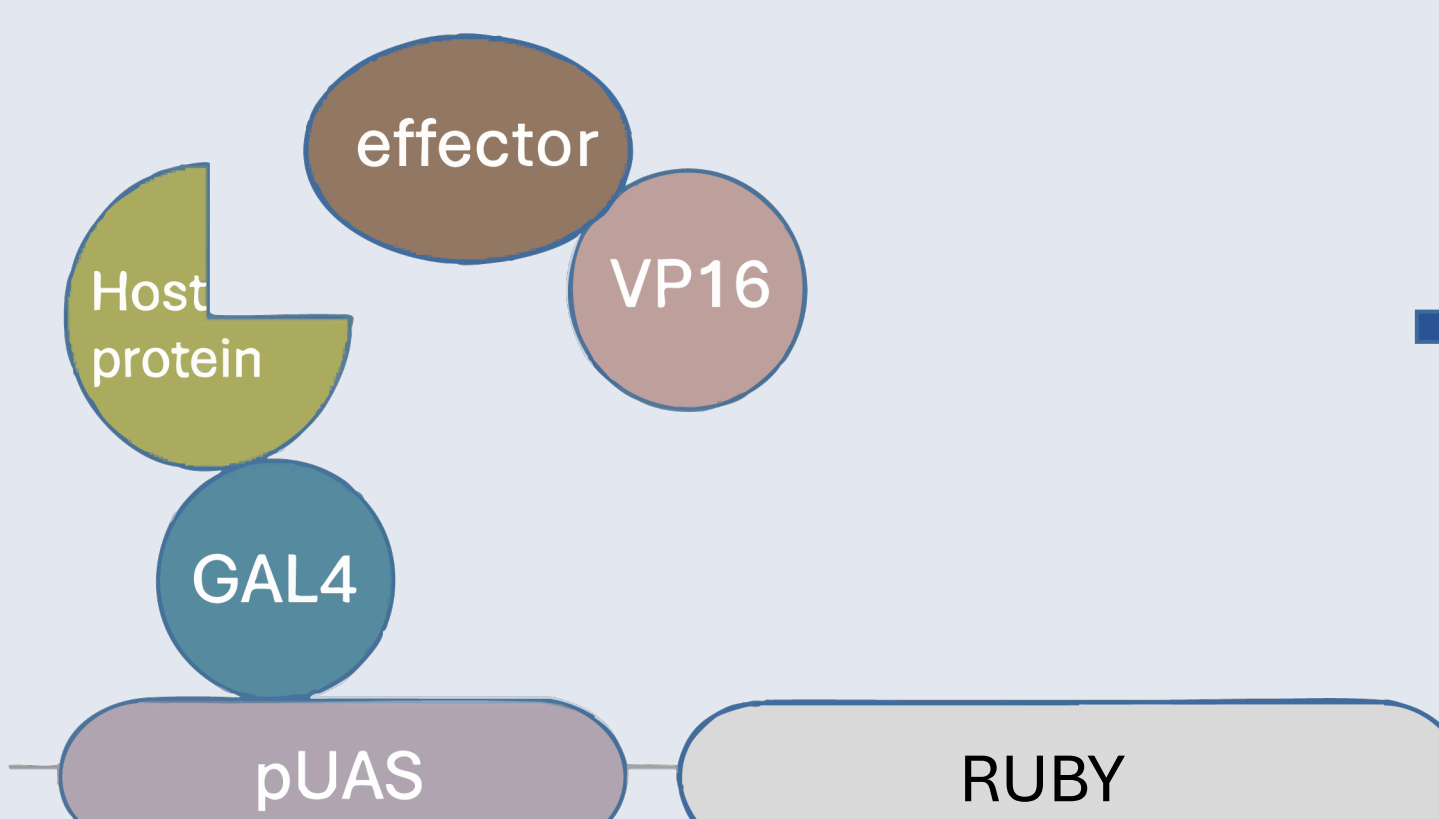
Future applications in plant immunity research

Interaction



Betalain accumulation

No interaction



No betalain accumulation

Pros and Cons of the Split GAL4 RUBY assay

PROS



In planta relevance
Visual and non-destructive
Adaptability (from Gateway to Golden gate cloning)
Easy and quick experiment to set-up in the lab
Low cost and requirement basic equipments
Betalain accumulation can be quantify by spectrometry

CONS



Auto-activity of the system (with P19 or TFs)
Dependency on protein expression
Leakiness of the RUBY reporter
Only one to one interactions can be tested

TIPS

If the system is auto-active, avoid using a 35S promoter, you can use another promoter such as AtUBQ10. You can also play with the position of the GAL4 and VP16 tags fused to the N-ter or C-ter of your genes of interest!

References:

Chen, J. et al. (2023). A Split GAL4 RUBY Assay for Visual in Planta Detection of Protein–Protein Interactions. *The Plant Journal*, 114 (6), 1209–1226. <https://doi.org/10.1111/tpj.16234>.
Engler, C., Kandzia, R., & Marillonnet, S. (2008). A one pot, one step, precision cloning method with high throughput capability. *PloS one*, 3(11), e3647. <https://doi.org/10.1371/journal.pone.0003647>

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