DEVELOPING *AGROBACTERIUM TUMEFACIENS*-MEDIATED TRANSIENT EXPRESSION FOR COWPEA

By

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Abstract

The cowpea plant (Vigna unguiculata) is a major source of nutrition in drought-ridden areas such as sub-Saharan Africa. However, cowpea is plagued by numerous pests, including the cowpea aphid (Aphis craccivora), which causes serious crop damage. To understand cowpea-cowpea aphid interactions and identify aphid proteins involved in parasitism (also known as effectors), it is necessary to functionally characterize the proteinaceous aphid effectors secreted into cowpea during feeding. To functionally characterize these effectors, aphid proteins need to be expressed in planta and their effects on aphid behavior analyzed. Transiently expressing proteins by the bacterium Agrobacterium tumefaciens is typically used to functionally characterize proteins in plants and evaluate their effects on pathogens and pests. However, such an expression system has not been described for cowpea. We developed a transient expression system for cowpea by screening seven A. tumefaciens strains for expression of the marker gene green fluorescent protein (GFP), fused to a nuclear localization signal. Recombinant bacteria were syringe infiltrated into cowpea cotyledons and true leaves. Two days after infiltration, fluorescence was visualized in the treated tissue using a confocal microscope. As a positive control for GFP expression, the A. tumefaciens strains were also infiltrated into leaves of Nicotiana benthamiana, a plant commonly used for transient expression. Fluorescence was detected in the nuclei of leaves infiltrated with each of the seven A. tumefaciens strains tested, albeit at different levels. The strongest and most uniform fluorescence was detected using A. tumefaciens strain AGL01 in the cotyledon and the true leaves.

Introduction:

The cowpea plant, *Vigna unguiculata*, is a major nutritive food resource in sub-Saharan Africa and other drought-stricken areas in the world. Unfortunately, cowpea plants are threatened by numerous pathogens and pests. One pest which causes a tremendous amount of damage is the cowpea aphid, *Aphis craccivora*. Symptoms caused by the cowpea aphid include chlorosis and necrosis of above ground tissues, stunted growth, and eventually plant death[1].

Cowpea aphids feed on their host plants by inserting their needlelike stylets through the plant tissues in order to reach the phloem sap[2]. During penetration and feeding, the cowpea aphid deposits saliva into the plant. Aphid saliva has been found to be the main interface between aphids and their host plants with recent studies finding that aphid saliva is composed of proteins and effectors which disrupt the plant's defense and alter plant metabolism to facilitate aphid feeding[3]. Previous work characterizing aphid-plant interactions has identified candidate salivary proteins using mass spectrometry and overexpression *in planta* through transient expression by *Agrobacterium tumefaciens*[4]. Transient expression by *A. tumefaciens* takes advantage of the bacterium's natural machinery to express a foreign gene for a limited period of time. In nature, *A. tumefaciens* infects a wide range of plants and causes crown gall disease[5]. It requires a large plasmid to infect and cause disease in plants[6], and part of this tumor-inducing (Ti) plasmid, the T-DNA, is transferred from the Ti plasmid and inserted into the plant's genome[7]. This machinery has been engineered for plant genetic modifications. Foreign genes can be cloned into the T-DNA so that, when T-DNA is inserted into the plant's genome during infection, the foreign gene is expressed instead of the native genes that cause crown gall disease[8]. However, cloning foreign genes directly into the T-DNA region of a normal Ti plasmid is difficult[8]. As a result, several laboratories developed different versions of the binary vector approach, such as Gateway®

cloning, which makes cloning foreign genes into the T-DNA region easier[8]. Through the binary vector approach, in the engineered Ti plasmid, the T-DNA (which normally contains genes that cause tumors in plants) and *vir* (virulence) regions have been separated into at least two different replicons, and are sometimes even separated into two separate plasmids[8,9]. Although the Ti region is replaced with foreign genes, because the *vir* region is left intact, the *A. tumefaciens* strain still has the ability to infect plant cells and cause them to transiently express genes of interest, but it no longer causes gall formation[9]. Because transient expression by *A. tumefaciens* is a well-established technique that has been used in several plant species such as *Nicotiana benthamiana*[10], *Arabidopsis thaliana*[11], *Medicago truncatula*[12], tomato (*Solanum lycopersicum*)[13], and lettuce (*Lactuca sativa*)[13], it is a possible method by which foreign genes could be transiently expressed in the cowpea leaves.

Over the summer of 2017, I was involved in profiling the cowpea aphid salivary proteome to identify its protein composition. The next step is to identify the effectors among these candidate proteins, which could lead to the afflicted phenotypes in cowpea, by expressing them transiently in cowpea and monitoring their effects on the aphid. Currently, a system for transient expression does not exist for cowpea. Therefore, my project's objective was to develop an *A. tumefaciens*-mediated transient expression system for cowpea by expressing a green fluorescent protein (GFP) using different strains of *A. tumefaciens*. The success of transient expression for each *A. tumefaciens* strain will be determined by the level of GFP expression detected using a confocal microscope. However, confocal microscopy of leaves produces a certain amount of autofluorescence, making it difficult to distinguish the GFP signal from autofluorescence. Trichomes, which are small "hairs" that are found on plant appendages such as leaves[14], also show autofluorescence[15]. To decrease the interference by autofluorescence when

imaging the leaves for this experiment, the GFP gene was fused with a nuclear localization sequence (NLS), directing GFP expression only inside the nucleus of the cell. Here, I report the identification of an *A. tumefaciens* strain that could be used for successful transient expression of foreign genes in cowpea leaves.

Methodology:

Agrobacterium tumefaciens strains

Seven strains of the bacterium *A. tumefaciens* were obtained from the UCR Plant Transformation Center for screening for the expression of the marker gene GFP. These strains were GV3101, MP90, Agro 2760, AGL01, EHA105, C58C1, and LB4404.

Table 1: Antibiotic selection and media used for the *A. tumefaciens* strains

Strain	Resistance	Antibiotic Concentration (mg/l)	Solid/liquid medium
AGL01	gent	15	YEP/YEP
Agro 2760	rif	15	LB/YEP
C58C1	rif	10	YEP/YEP
EHA105	rif	10	LB/YEP
GV3101	rif/gent	10/30	LB/LB
LB4404	rif	15	LB/YEP
MP90	rif/gent	10/25	LB/LB

rif = rifampcin, gent = gentamicin

Cloning of the 35S overexpression promoter into the pBGGN plasmid

We obtained a vector pBGGN (Figure 1) which contained the gene for GFP and a nuclear localization signal but lacked the 35S overexpression promoter. To introduce the 35S promotor, we amplified this promoter from pEarleyGate 100 vector. The PCR product was prepared using

Q5® High-Fidelity DNA Polymerase. Each tube contained 25 µL Q5® High-Fidelity 2X Master Mix, 2.5 µL 10 µM 35S F primer, 2.5 µL 10 µM 35S R primer, 1 µL template DNA, and 19 µL ddH20, for a total reaction volume of 50 µL. The PCR conditions were set as follows: 1) 98 ˚C for 30 seconds, 2) 98 ˚C for 7 seconds, 3) 56 ˚C for 15 seconds, 4) 72 ˚C for 1 minute, 5) 72 ˚C for 2 minutes. Steps 2-4 were repeated for 30 cycles.

35S R primer Gateway sequence:

GGGGACCACTTTGTACAAGAAAGCTGGGTCccaaatgaaatgaacttccttatat

35S F primer Gateway sequence:

GGGGACAAGTTTGTACAAAAAAGCAGGCTttggaatagaaacagaatacccgcg

The amplified product was purified using a GeneJET PCR Purification Kit® according to the kit's protocol. After purification, the 35S promoter was cloned into pDONR207 according to Gateway® cloning protocols[16]. The BP reaction had a 3:1 ratio of concentrations of purified 35S PCR product to pDONR207 vector with 1 µL of BP Clonase™ II enzyme. The reaction was incubated at room temperature for 3 hours and transformed into chemically competent DH5α *E. coli* cells using heat shock. The heat-shocked DH5α cells were then recovered for 55 minutes while shaking in 950 µL of SOC media at 37°C and plated on LB with gentamicin plates overnight at 37°C.

A colony from these plates was grown in 3 mL of LB with gentamicin overnight shaking at 37 °C. The plasmid was then extracted from the *E. coli* cells using a GeneJET Plasmid Purification Kit® according to the kit's protocol. The recombinant plasmid was isolated from the *E. coli* cells using a GeneJET Plasmid Purification Kit® according to the kit's protocol and sequenced at the UCR Genomics Core. After confirmation of the correct sequence, the LR reaction was performed with a 3:1 ratio of concentrations of pDONR207:35S to pBGGN empty

vector along with 1 µL LR Clonase™ II enzyme. The reaction was incubated at room temperature for 3 hours and transformed into chemically competent DH5α *E. coli* cells using heat shock. The heat-shocked DH5α cells were then recovered as described earlier and the cells were plated on LB with spectinomycin plates overnight at 37 °C. Then, a single colony was grown in 3 mL of LB with spectinomycin overnight shaking at 37 °C and the recombinant plasmid was isolated from the *E. coli* cells as described earlier and sequenced at the UCR Genomics Core. The final clone was named pBGGN:GFP-NLS.

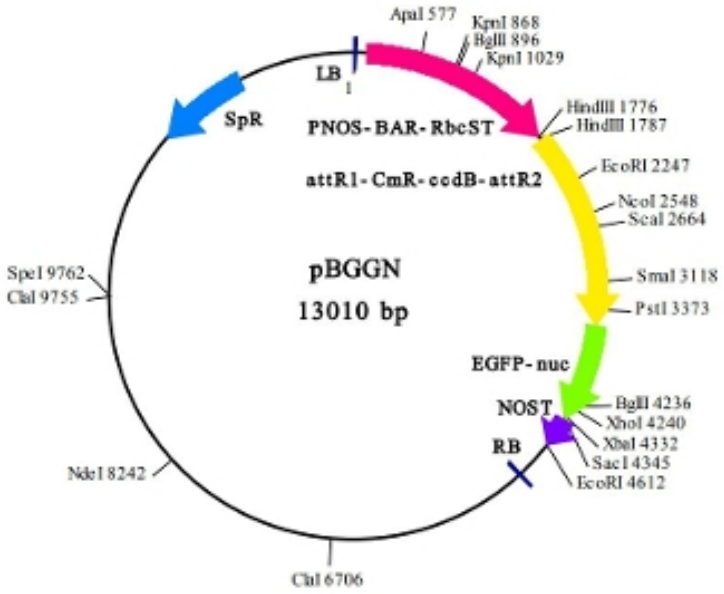

Figure 1: Map of the pBGGN vector.

Preparation of Competent Cells

For each *A. tumefaciens* strain, a 5 mL culture containing LB and the appropriate antibiotic selection as indicated in Table 1, was started from a freshly streaked plate, and incubated overnight shaking at 28˚C. The next morning, 100 mL of the appropriate media and

antibiotic selection, as indicated in Table 1, was inoculated with 1 mL of an *A. tumefaciens* strain and incubated while shaking at 28˚C until the OD_{600} reached an absorbance of 1.0. Cells were pelleted by spinning in a chilled (2˚C) Beckman J20 centrifuge with a prechilled rotor, in sterile reusable bottles, for 10 minutes at 4000 rpm. Then, the supernatant was poured off and the pellet was resuspended in 5 ml of chilled sterile water by pipetting up and down and centrifuged as before. The pellet was resuspended in 95 mL of cold, sterile water and centrifuged at 4000 rpm at 2˚C for 20 minutes. The pellet was resuspended in 40 mL of cold, sterile 10% glycerol and centrifuged for 15 minutes at 3500 rpm. The pellet was resuspended in an 0.5 mL of 20% cold, sterile glycerol and aliquoted into 100 μL aliquots in microcentrifuge tubes, flash frozen in liquid N_2 and stored at -80˚C for use at a later time.

Transformation of the A. tumefaciens strains

Competent cells of the *A. tumefaciens* strains were transformed with a pBGGN:GFP-NLS plasmid. For each strain, 100 μL of frozen electrocompetent cells in a 1.5 mL microcentrifuge tube were thawed on ice for 10 minutes. Once the electrocompetent cells were thawed, 2 μL of the pBGGN:GFP-NLS plasmid was added to the cells, on ice, in a laminar flow hood. The tube was gently mixed by stirring with the pipette tip, and kept on ice for 20 minutes. The cells with the plasmid were pipetted into an ice-cold 1 mm electroporation cuvette and electroporated at 1800 V. After 2 minutes of incubation on ice, 950 μL of SOC media was added into the electroporation cuvette, the cells were pipetted out of the cuvette into a fresh 2 mL microcentrifuge tube and the tube was incubated for two hours at 28˚C with gentle shaking. Cells were then plated on either YEP or LB agar media with the appropriate antibiotic selection as indicated in Table 1.

Bacterial culture and Agroinfiltration

For each *A. tumefaciens* strain, bacteria were streaked on a plate containing the appropriate solid medium and antibiotic selection (see Table 1) and grown for 2 days at 28˚C. A single colony was picked from the plate and inoculated into 5 mL of the appropriate liquid medium and antibiotic selection in a polystyrene culture tube. The culture was grown by shaking overnight at 30˚C. The next day, 1 mL of the overnight culture was used to inoculate 25 mL of the appropriate liquid medium and antibiotic selection along with 150 µM acetosyringone. Acetosyringone was used to increase bacterial virulence and plant transformation efficiency[17]. The culture was grown by shaking overnight at 30˚C. The next day, the bacteria were harvested by centrifugation at 4000 rpm at room temperature for 10 minutes, the supernatant was discarded and the pellet was resuspended in 200 mL of 10 mM $MgCl_2$ and 10 mM MES. The culture was centrifuged at 4000 rpm for 10 minutes at room temperature. This washing step with $MgCl_2$ and MES was repeated for a total of three washes, and the pellet was resuspended in infiltration buffer containing 10 mM $MgCl_2$, 10 mM MES and 150 µM acetosyringone. The bacterial titer was measured through spectrophotometry and the culture volume was adjusted to obtain an OD_{600} = 0.5. The tube containing the culture was then placed in the dark and incubated while shaking slowly at room temperature for 3 hours.

Due to the ease of transient expression in *N. benthamiana,* this plant was screened first with the different strains of the *A. tumefaciens* with the GFP construct to ensure the constructs' expression *in planta*. Bacteria were infiltrated (Agroinfiltration) into leaf tissues of 4-week-old *N. benthamiana* plants using a needless syringe. Next, cotyledons of 10-day-old cowpea plants and cotyledons and trifoliate leaves of 19-day-old cowpea plants (Figure 2) were infiltrated with the different *A. tumefaciens* strains with the GFP construct. Infiltrated plants were maintained under 16 h low light at room temperature.

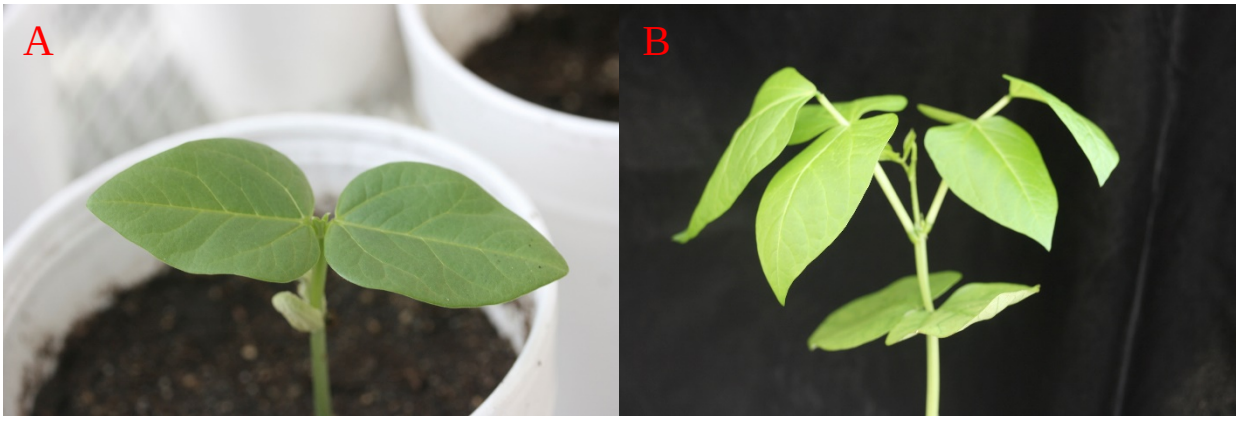

Figure 2: Cowpea plants, where (A) is a 10-day-old plant with cotyledons, and (B) is a 19-day-old plant with first trifoliate leaves and cotyledons.

Screening for GFP expression

Leaf areas infiltrated with the different *A. tumefaciens* strains were evaluated 48 hours after agroinfiltration using a Leica SP5 confocal microscopy. To visualize the GFP in the nuclei, the GFP was excited with a 488 nm laser line and GFP fluorescence was collected with emission filters of 498-550 nm.

Results:

pBGGN:GFP-NLS construct viability in each A. tumefaciens strain

The success of expression varied across the seven different *A. tumefaciens* strains, though all strains caused at least medium expression of GFP in *N. benthamiana* leaves. Strains C58C1, EHA105, and LB4404 were able to transiently express GFP at a high level, while all other strains transiently expressed GFP at a medium level (Figure 3 and Table 2). Even with the different levels of GFP expression, my results showed that each *A. tumefaciens* strain/clone was viable.

Screening different cowpea tissue types with A. tumefaciens strains for GFP expression

The success of the GFP expression varied across the seven different *A. tumefaciens* strains tested and across different cowpea leaf type and age, with all strains resulting in some level of GFP expression in at least one type of cowpea leaf. In 10-day-old cowpea cotyledon leaves, strains GV3101 and AGL01 were able to transiently express GFP at a medium level, while all other strains expressed GFP at low levels (Figure 4 and Table 2). Among the low GFP expressing *A. tumefaciens* strains, EHA105, C58C1 and LB4404 resulted in the lowest amount of GFP expression since GFP was detected only in a single nucleus of the infiltrated tissue (Figure 4). In 19-day-old cowpea trifoliate leaves, AGL01, C58C1 and MP90 strains expressed GFP at a medium level, while all other strains did not express GFP at all (Figure 5 and Table 2). In 19-day-old cowpea cotyledon leaves, C58C1, GV3101, and LB4404 strains expressed GFP only in a single nucleus of the infiltrated tissue (Figure 6 and Table 2).

Table 2: Qualitative Fluorescence Evaluation of *A. tumefaciens* strains Transiently Expressing 35S:GFP-NLS by Confocal Microscopy.

Strain	Expression in *N. benthamiana*	Expression in 10-day-old cowpea cotyledon leaf	Expression in 19-day-old cowpea cowpea trifoliate leaf	Expression in 19-day-old cowpea cotyledon leaf
AGL01	+++	+++	+++	-
Agro 2760	+++	++	-	-
C58C1	++++	+	+++	+
EHA105	++++	+	-	-
GV3101	+++	+++	-	+
LB4404	++++	+	-	+
MP90	+++	++	+++	-

++++ = high expression, +++ = medium expression, ++ = low expression, + = expression in a single nucleus and - = no expression.

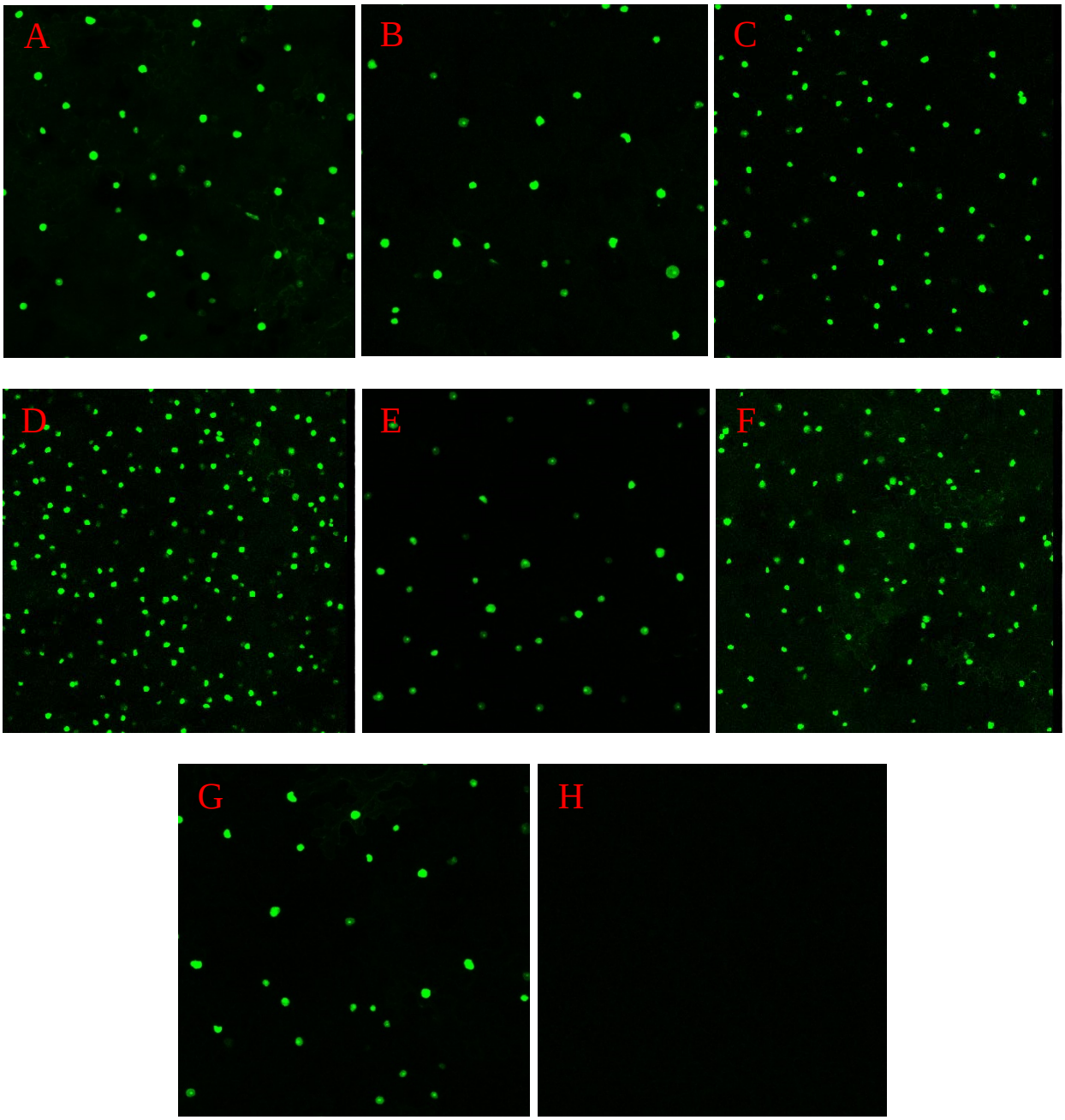

Figure 3: Confocal Microscopy Images of Transient Expression of 35S:GFP-NLS in *N. benthamiana* leaves. Images were taken 2 days after agroinfiltration. Expression with **A)** AGL01, **B)** Agro 2760, **C)** C58C1, **D)** EHA105, **E)** GV3101, **F)** LB4404, **G)** MP90, and **H)** infiltration buffer control.

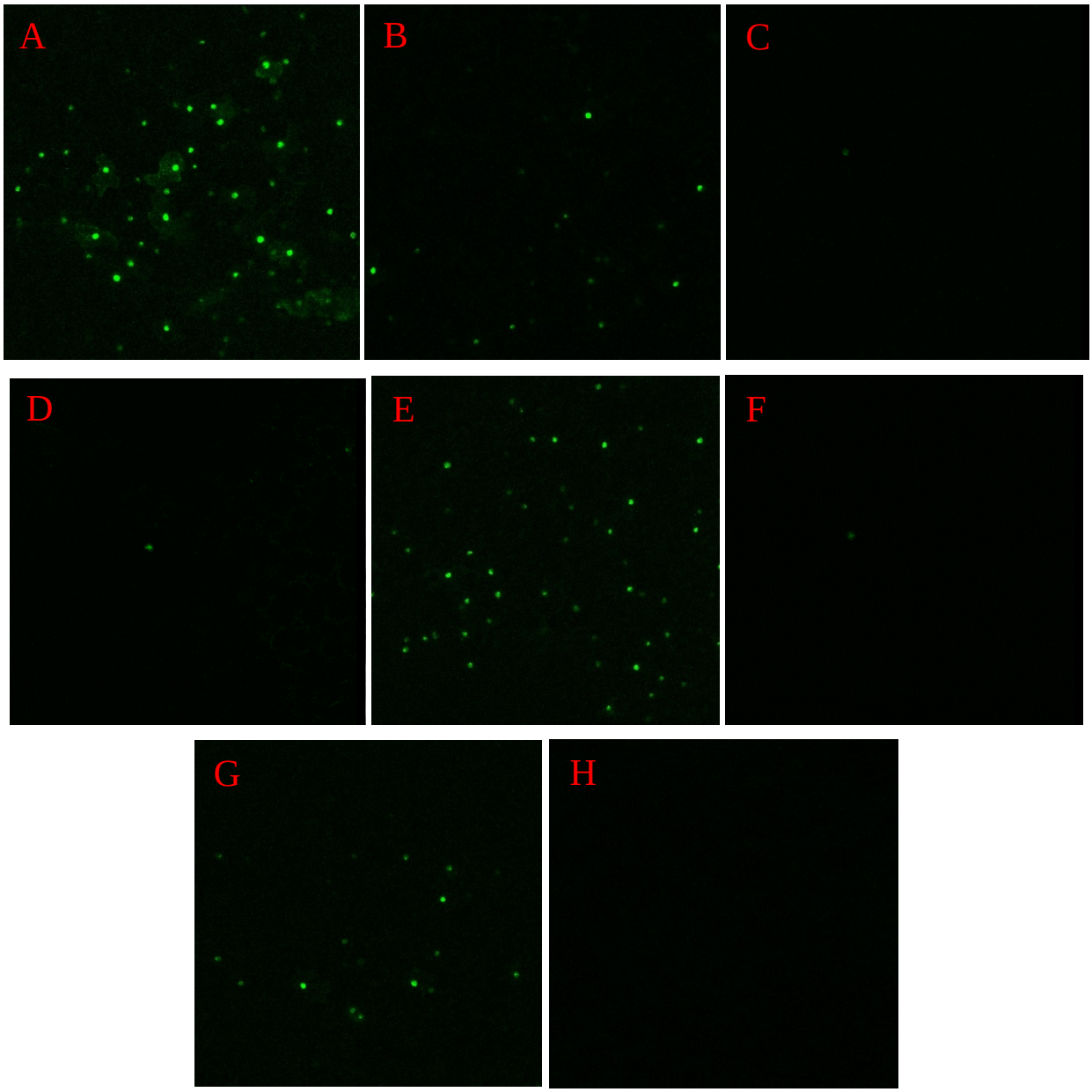

Figure 4: Confocal Microscopy Images of Transient Expression of 35S:GFP-NLS in young (10-day-old) cowpea cotyledons. Images were taken 2 days after agroinfiltration. Expression with **A)** AGL01, **B)** Agro 2760, **C)** C58C1, **D)** EHA105, **E)** GV3101, **F)** LB4404, **G)** MP90, and **H)** infiltration buffer control.

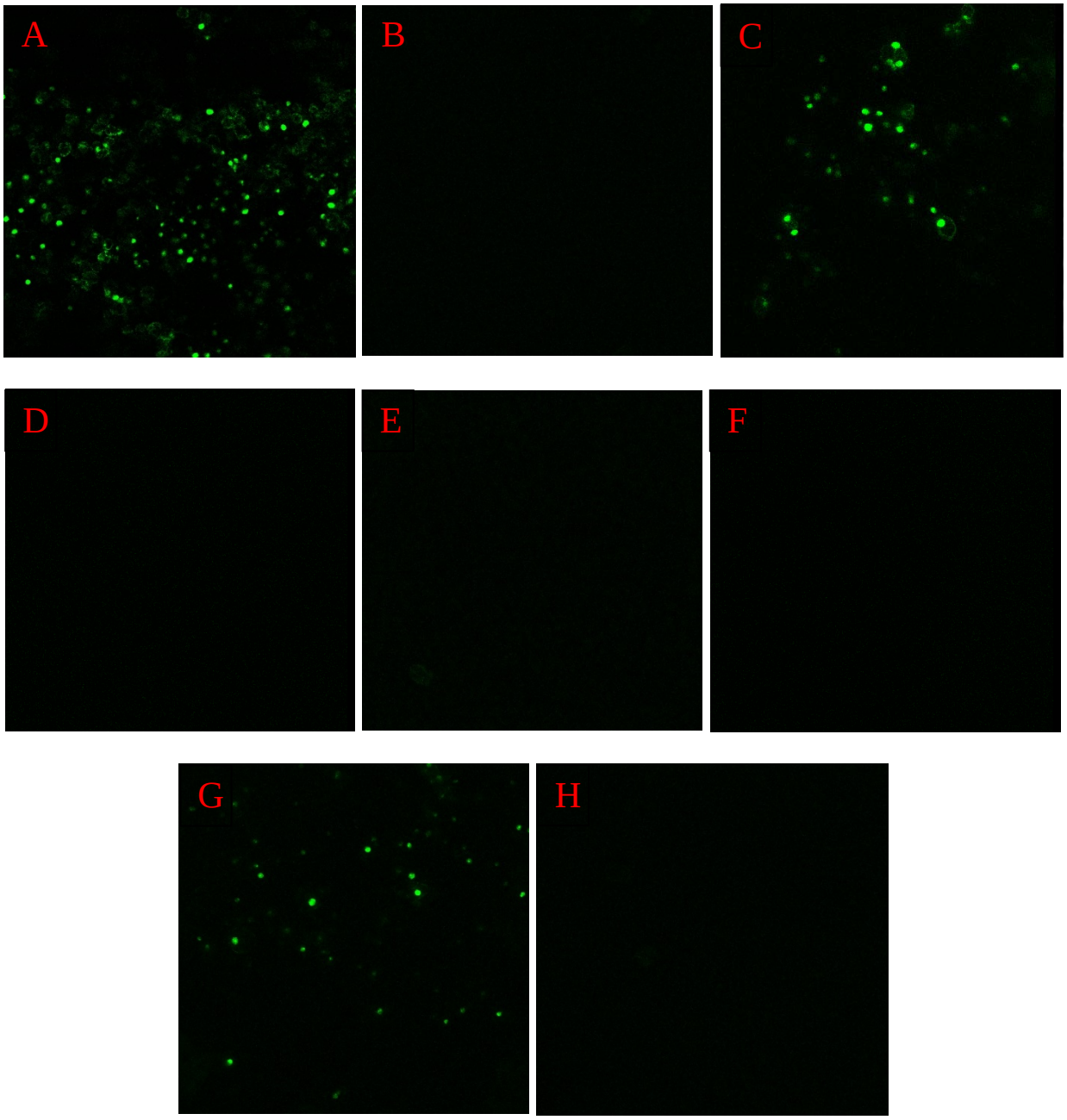

Figure 5: Confocal Microscopy Images of Transient Expression of 35S:GFP-NLS in the first trifoliate leaves of cowpea. Images were taken 2 days after agroinfiltration. Expression with **A)** AGL01, **B)** Agro 2760, **C)** C58C1, **D)** EHA105, **E)** GV3101, **F)** LB4404, **G)** MP90, and **H)** infiltration buffer control.

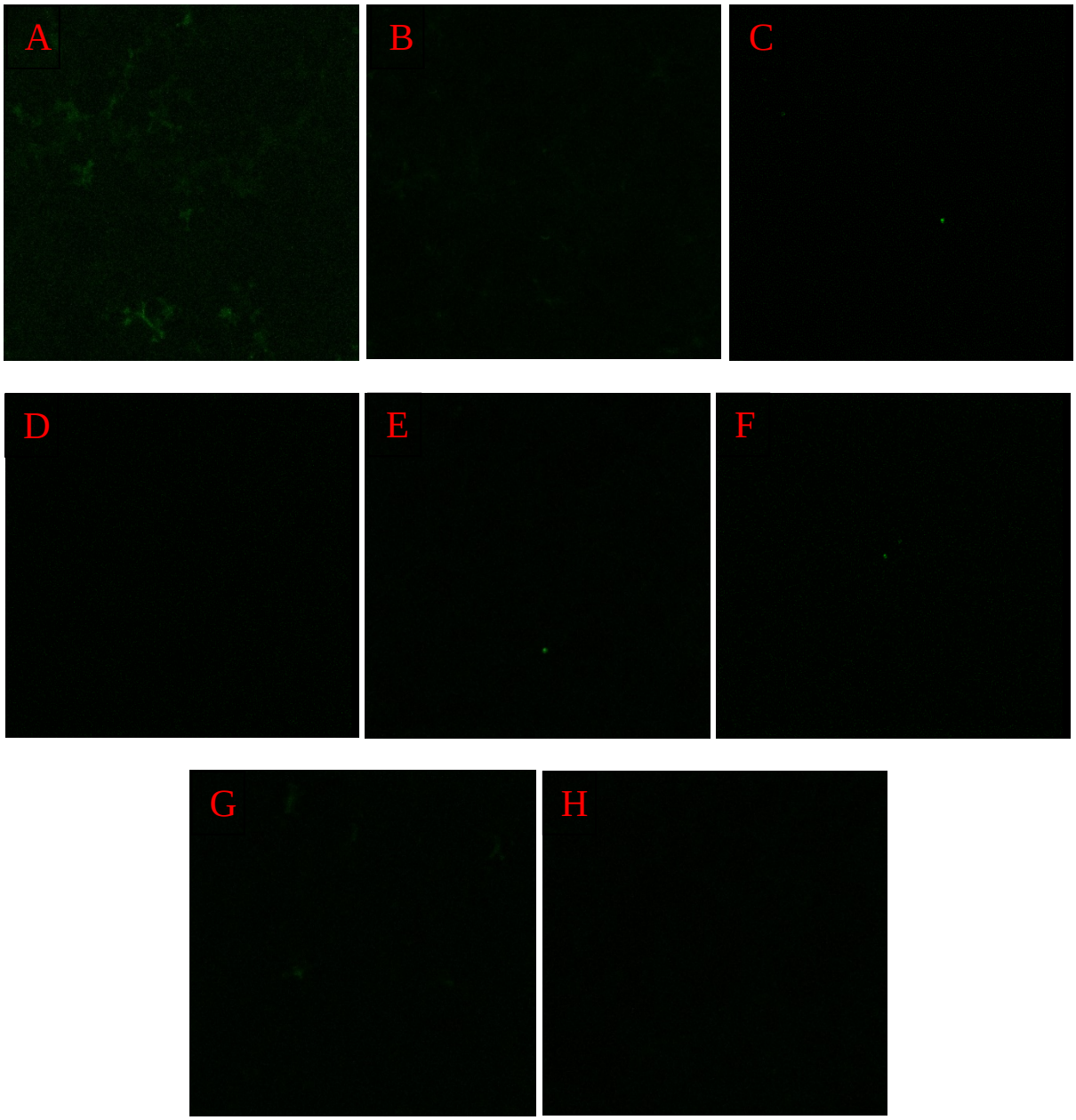

Figure 6: Confocal Microscopy Images of Transient Expression of 35S:GFP-NLS in 19-day-old cowpea cotyledons. Images were taken 2 days after agroinfiltration. Expression with **A)** AGL01, **B)** Agro 2760, **C)** C58C1, **D)** EHA105, **E)** GV3101, **F)** LB4404, **G)** MP90, and **H)** infiltration buffer control.

Discussion:

All *A. tumefaciens* strains were viable constructs as determined by successfully expressing GFP in *N. benthamiana* leaves. However, only some of the *A. tumefaciens* strains tested could transiently express GFP in cowpea (Table 2). Although the same concentration (OD) of *Agrobacterium* was used for infiltration of *N. benthamiana* and cowpea leaves, different expression levels and patterns appeared in cowpea compared to *N. benthamiana*. The level of GFP expression for most strains was mostly reduced in cowpea compared to the level of GFP expression in *N. benthamiana*.

A. tumefaciens strains C58C1, EHA105, and LB4404 resulted in the highest expression of GFP in *N. benthamiana*. In contrast to *N. benthamiana,* these three *A. tumefaciens* strains expressed GFP in only a single nucleus in 10-day-old cowpea cotyledon leaves. The most successful GFP expression in 10-day-old cowpea cotyledon leaves was detected for *A. tumefaciens* strains AGL01 and GV3101.

In cowpea first trifoliate leaves, only *A. tumefaciens* strains AGL01, MP90, and C58C1 caused a medium level of GFP expression. None of the other *A. tumefaciens* strains were able to cause any detectable level of GFP expression in cowpea first trifoliate leaves. It was more difficult to do infiltrations in the first trifoliate leaves, so the infiltration site for all strains had a smaller area than the infiltrations in the cowpea cotyledon or *N. benthiamana* leaves. Therefore, it is likely that the low expression of GFP in this leaf tissue type is caused by a combined low level of cell transformation and expression of the construct.

In 19-day-old cowpea cotyledon leaves, *A. tumefaciens* strains C58C1, GV3101, and LB4404 caused GFP expression in only one cell. None of the other *A. tumefaciens* strains were able to express GFP in any of the cells of the 19-day-old cowpea cotyledon leaves. This lack of

GFP expression is mostly likely due to the age of these cotyledon leaves. There seems to be an age-related issue in the cotyledon leaves where transient expression is no longer efficient. More screens need be done to monitor this age-related phenomenon of the cotyledon leaf for best timing of GFP expression.

Through the work of the experiment, it was determined that *A. tumefaciens* strain AGL01 is the best candidate for use for transient expression in cowpea. What set AGL01 apart from the other *A. tumefaciens* strains was that it was able to cause a medium level of GFP expression in 10-day-old cowpea cotyledon and first trifoliate leaves, which none of the other strains were able to accomplish. One strain, GV3101, was able to cause a medium level of GFP expression, but only in the 10-day-old cotyledon leaves. Other strains were able cause GFP expression in multiple types of leaves, but only at a low level. My results indicate that AGL01 strain could be used for transient expression of foreign genes in cowpea. Future experiments should test the duration and level of the transient expression to better define the use of this important tool in plants.

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