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I am submitting herewith a thesis written by Bryce N. Trull entitled "A robust agroinfiltration method." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

Charles N. Stewart Jr., Major Professor

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A robust soybean agroinfiltration method

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Bryce N. Trull

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Abstract

Stable transformation of soybean (*Glycine max*) is a markedly slow and laborious process. Thus, a tool that enables rapid evaluation of genetic elements in planta is critical to advance complex research and genetic engineering in soybean. To that end, a substantially robust agroinfiltration method was innovated in this work. Agroinfiltration is a technique that leverages *Agrobacterium*-mediated transient transformation to deliver genetic elements to the cells of whole plant tissues, usually leaves. Several factors were found to be relevant to successful soybean leaf agroinfiltration, including genotype, surfactant, developmental stage, and *Agrobacterium* culture medium. This research represents not only a new research tool for soybean biotechnology, but also indicates critical parameters for guided agroinfiltration optimization that could be used for other crop species.

Table of Contents

Chapter 1 Introduction	1
Background.....	2
<i>Agrobacterium</i> -mediated transformation.....	3
Transient expression strategies	3
Challenges.....	4
References.....	6
Chapter 2 A robust soybean agroinfiltration method.....	10
Abstract.....	11
Introduction.....	12
Materials and methods	13
Plant materials and growth conditions.....	13
Vector construction	13
Optimized <i>Agrobacterium</i> growth, preparation, and agroinfiltration.....	13
Parameters tested.	14
GUS staining, GUS/Ruby imaging, and image analysis.....	15
Fluorescence spectroscopy, compound microscopy, and confocal microscopy	15
qRT-PCR transcript abundance assay	16
Statistical analysis.....	16
Results.....	16
Soybean genotype	16
Wounding and surfactants.....	17
<i>Agrobacterium</i> culture media.....	18
Plant developmental stage.....	19
Genotype rescreen / <i>Agrobacterium</i> strain.....	19
Co-infiltration / RUBY reporter.....	20
Conclusion	20

References.....	23
Chapter 3 Conclusions and recommendations.....	28
List of References.....	31
Appendix.....	38
Vita.....	68

List of Tables

Table 1 Soybean genotypes tested. Most of germplasm tested are breeding lines developed at Virginia Tech (those beginning with “V”) and the University of Tennessee (those beginning with “T”).....	58
Table 2 Description of variations of infiltration procedure as it relates to the infiltration medium suspension, surfactant, sonication, and time of vacuum exposure. Throughout the study, the infiltration medium, as well as antioxidant additives, baring variations in the <i>Agrobacterium</i> strain and/or its culturing, was consistently maintained. The initial genotype screen (Fig. 1) was conducted with sonication treatments beforehand, Tween-20 as a surfactant and two rounds of 30-minute vacuum exposure. Once we established Silwet L-77 as the surfactant of choice and ruled out sonication as a useful treatment (Fig. S1 & S2) the remainder of optimization experiments were performed without a wounding treatment, using Silwet-L77 as the surfactant and a single round of 5-minute vacuum exposure.	59
Table 3 Plant developmental stage/leaf ages and morphologies tested. Soybean leaves were infiltrated in the vegetative emergence (VE) stage and vegetative compound (VC) stage. Both VE and VC are the first true leaves of a germinated soybean plant with VE being the leaves when unexpanded immediately after germination and VC being defined as fully unfurled and expanded first true leaves. Soybean leaves were also infiltrated in the vegetative 1 st -trifoliate leaf stage. Both the first unifoliate leaves and first trifoliate leaves were tested.	60
Table 4 <i>Agrobacterium</i> strains tested and chromosomal backgrounds.	61
Table 5 Transcript analysis primer sets. Target genes and the respective forward and reverse primers used for qRT-PCR experiments.	62

List of Figures

Figure 1 Vectors used in optimization experiments. **a** Vector plasmid pTF101.1 with a CaMV35Ss (35S) promoter driving a GUS reporter with an intron and a nopaline synthase terminator (NOS) 35S:GUSplus:NOS. **b** Vector plasmid pAGM4723 with a *Glycine max* ubiquitin (Gmubi) promoter driving a red fluorescent protein (turboRFP) reporter and an *Agrobacterium* octopine synthase terminator (OCS) Gmubi:turboRFP:OCS. **c** Vector plasmid pAGM4723 with a Gmubi promoter driving a green fluorescent protein (mGFP5-ER) and an OCS terminator. **d** Vector plasmid pAGM4723 with a Gmubi promoter driving a three-gene betalain biosynthetic pathway linked by 2A peptides: CYP76A1, DODA1 and glucosyltransferase as a single open reading frame and an OCS terminator (He et al. 2020). 39

Figure 2 Schematic of plant preparation and optimized soybean agroinfiltration process. **a** Sterile seeds are imbibed for 24 hours in water. **b** Paper towel is thoroughly moistened and cut into approximately 5 cm × 7.5 cm rectangles. Imbibed seeds are placed individually on moistened towels, rolled up, and placed upright in a beaker. **c** The paper towel rolled seeds are placed in a growth chamber on 16-h day/8-h night cycle (~90-120 μmol intensity) at 24 °C and grown for 10-15 days to the VC or V1 stage. **d** *Agrobacterium* cultures are streaked on a YEP plate supplemented with antibiotics 4 days prior to infiltration (PTI) and grown for two days. **e** A colony from the YEP plates is used to inoculate a liquid starter culture of 50 ml YEP supplemented with antibiotics 2 days PTI, and grown for 16-24 hr on a shaker at 225 rpm at 28 °C. **f** The evening PTI, the starter culture is centrifuged at 4100 × g for 15 minutes and resuspended in AB:MES induction media to an OD₆₀₀ of 0.3, supplemented with 200 μM AS and selection antibiotics, and grown for 16 hr at 225 rpm at 28 °C. **g** The day of infiltration, the AB:MES induction media is centrifuged at 4100 × g for 15 minutes and the *Agrobacteria* are resuspended in SIB to an OD₆₀₀ of 0.6, supplemented with 200 μM AS and incubated in the dark on a shaker at 50-75 rpm for 2 hr. **h** After incubation, SIB-*Agrobacterium* suspension is supplemented with 400 mg/L L-

cysteine, 78 mg/L DTT, and 1% v/v Silwet L-77. SIB-*Agrobacterium* suspension beaker is placed in the vacuum chamber. Plants are then removed from the growth chamber and placed in the beaker with abaxial leaf surface facing up. **i** Plants are subjected to 5 minutes of vacuum at -84.65 kPa. The air intake valve is swiftly released to rapidly apply positive pressure and infiltrate leaf tissue. **j** Infiltrated plants are removed from the vacuum chamber, dunked in a beaker of DI water to wash off excess suspension and surfactant, patted dry with paper towel, placed back in their original growing container, lightly sprayed with water and placed under a plastic dome to maintain humidity. Finally, plants are placed in a dark cabinet for 16 hr before being placed back in the growth chamber for evaluation 72 hr post infiltration.40

Figure 3 Initial soybean genotype screen for amenability to agroinfiltration. Soybean lines infiltrated with *Agrobacterium* strain EHA 105 harboring 35S:GUSplus:NOS. Mixed VE and VC stage plants were subjected to 30 seconds of sonication at 42 kHz and vacuum infiltrated at -84.65 kPa for two 30-minute intervals using SIB supplemented with 400 mg/L of L-cysteine, 78 mg/L of DTT and 1% v/v Tween-20. **a** Mean leaf area stained of infiltrated leaves. Each soybean line was infiltrated with 3 negative controls (SIB only) of the same line, but all negative controls were included as a single negative in the graph. Lines with differing letters considered significantly different. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD. **b** Representative images of all infiltrated soybean lines stained for GUS expression.41

Figure 4 Soybean line 'V17-0799DT' infiltrated with *Agrobacterium* strain EHA 105 harboring 35S:GUSplus:NOS incubated in AB:MES induction media + 200 μ M AS , YEP + 200 μ M AS grown the day of infiltration, or YEP + 200 μ M AS incubated overnight. Infiltrations were conducted with a single 5-minute round of vacuum at -84.65 kPa using 0.01% v/v. Silwet-L77. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD.43

Figure 5 Assessment of plant developmental stage. **a** Soybean line 'V17-0799DT' infiltrated during various developmental stages (VE-V1) with *Agrobacterium* strain EHA 105

harboring 35S:GUSplus:NOS grown in AB:MES induction media and subjected to a single 5-minute round of vacuum at -84.65 kPa using 0.01% v/v. Silwet-L77. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD. **b** Examples of line 'V17-0799DT' in the VE, VC, and V1 stage of development.44

Figure 6 Reassessment of soybean lines with a fully refined infiltration protocol using *Agrobacterium* strain EHA 105 harboring Gmubi:tRFP:OCS cultured and prepared as described in Fig. 2. VC stage plants were subjected to one 5-minute round of vacuum at -84.65 kPa in SIB supplemented 400 mg/L of L-cysteine, 78 mg/L of DTT and 1% v/v Silwet L-77. **a** Fluorescence spectroscopy of 26 infiltrated plant lines. Fluorescence emission intensity measured in counts per second (CPS). Excitation at 540 nm, emission measured from 560 nm-640 nm. Plants infiltrated with buffer and additives, but no *Agrobacterium* were used as negative controls. **b** Line 'TN16-5004' was the top performing genotype with a mean intensity of 476,296 cps. Peak emission (574 nm) was used for evaluation. Lines with differing letters considered significantly different. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD. **c** qRT-PCR of lines 'TN16-5004' and 'V170799DT' for transcript abundance of tRFP relative to soybean polyubiquitin (Gmubi3). Plants infiltrated with a Gmubi:RUBY:OCS construct as well as wild type plants were used as negative controls. Line 'TN16-5004' showed a relative expression level of 14.16% and line 'V17-0799DT' showed a level of 9.5% of the reference gene, Gmubi3. Error bars represent standard error of the mean. $P < 0.05$, $n = 20$, one-way ANOVA.45

Figure 7 *Agrobacterium* strain screen. Fluorescence spectroscopy of line 'TN16-5004' infiltrated as described in Fig. 2 with *Agrobacterium* strains EHA 105, LBA4404, GV3101 and AGL1 harboring Gmubi:tRFP:OCS. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD.47

Figure 8 VC stage soybean line 'V17-0799DT' co-infiltrated as described in Fig. 2 using *Agrobacterium* strain harboring Gmubi:tRFP:OCS and Gmubi:mGFP5-ER:OCS at a 1:1 ratio. **a** Compound microscopy of co-infiltrated plants. Scale bar = 500 μm . **b** Compound microscopy of negative control. Excitation for tRFP 540 nm, emission

measured from 560 nm-580 nm. Excitation for mGFP5-ER 480nm, emission measured from 500 nm-520 nm **c** Confocal microscopy of co-infiltrated leaves (pseudo coloration). Scale bar =50 μ m. Excitation for tRFP 553 nm and detected at peak emission of 574 nm. Excitation for mGFP5-ER at 490 nm and detected at peak emission of 509 nm. **e** Fluorescence spectroscopy of co-infiltrated plants. Excitation for tRFP 540 nm, emission measured from 560 nm-580 nm. Excitation for mGFP5-ER 480 nm, emission measured from 500 nm-520 nm. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, T-test. **f** qRT-PCR of lines ‘TN16-5004’ and ‘V170799DT’ for transcript abundance of tRFP and mGFP5-ER relative to soybean polyubiquitin (Gmubi3). Plants infiltrated with a Gmubi:RUBY:OCS construct as well as wild type plants were used as negative controls. Line ‘TN16-5004’ showed a relative expression level of tRFP and mGFP5-ER of 7.8% and 6%, respectively. Line ‘V17-0799DT’ showed a relative expression level of tRFP and mGFP5-ER of 20.8% and 17%, respectively. Error bars represent standard error of the mean. $P < 0.05$, $n = 24$, one-way ANOVA48

Figure 9 VC stage soybean line ‘V17-0799DT’ infiltrated as described in Fig. 2 using *Agrobacterium* strain EHA 105 harboring Gmubi:RUBY:OCS. **a** Images showing infiltrated soybean leaves transiently expressing betalain. **b** Negative control infiltrated with *Agrobacterium* strain EHA 105 harboring Gmubi:mGFP5-ER:OCS. **c** Total leaf area showing betalain pigmentation vs. negative control. Error bars represent standard deviation. $P < 0.05$, $n = 18$, Student’s T-test. 52

Supplementary Figure 1 **a** Soybean line ‘V17-0799DT’ infiltrated after sonication. **b** Soybean line ‘V17-0799DT’ infiltrated without sonication and 0.01% v/v Silwet L-77.53

Supplementary Figure 2 Wounding treatments and surfactant test. **a** Soybean lines ‘Williams 82’ and ‘V17-0799DT’ subjected to infiltration with or without sonication using the method from Fig 2. No significant GUS expression difference between sonication and infiltration using surfactant alone was found. $P < 0.005$, $n = 18$, Student’s T-test. **b** Comparison of surfactants Silwet L-77 and Tween-20 using soybean line ‘V17-0799DT’ subjected to one five-minute round of vacuum or 2-4 rounds, respectively.

P>0.05, n=18, Student's T-test. **c** Comparison of differing concentrations of Silwet L-77 in SIB. A concentration of 0.01% v/v was found to be optimal. **d e f** Shows examples of soybean line 'V17-0799DT' after two 5-minute rounds of vacuum and release at -84.65 kPa with no surfactant, 0.01% v/v Tween-20, and one 5-minute round with Silwet L-77, respectively. P<0.005, n=18, one-way ANOVA.....54

Supplementary Figure 3 Expression differences between line 'V17-0799DT' infiltrated with Gmubi:mGFP5-ER:NOS and Gmubi:mGFP5-ER:OCS.....57

Supplementary Notes

Supplementary Note 1 ImageJ macro code for isolation of total leaf area.....63

Supplementary Note 2 ImageJ macro code for isolation of leaf area stained.....65

Chapter 1
Introduction

Background

Soybean (*Glycine max*), the most widely grown genetically engineered (GE) crop in the world (ISAAA 2019), is a staple in world agriculture because of its many uses ranging from oil, fuel and feedstock to human consumption and industrial applications. Soybean was among the first crops for which genetic transformation strategies were developed, with the first reports of successful stable transformation occurring in 1988 (Hinchee et al. 1988.; McCabe et al. 1988.) In the mid-1990s the development of glyphosate resistant (Roundup Ready[®]) transgenic soybeans (Padgett et al. 1995) led to the overwhelming adoption of GE soybean that continues to this day (Voorra et al. 2020). However, in the decades since these innovations, progress towards complex genetic manipulation and improvement in soybean has been sluggish and lackluster, especially in terms of yield improvement (Ainsworth et al. 2012; Xu et al. 2013). A significant obstacle affecting the production of GE soybeans is the frustratingly slow stable transformation pipeline which can take six months to a year to produce a stable transgenic plant suitable for evaluation (Li et al. 2017; Rech et al. 2008; Yamada et al. 2011; Yang et al. 2016). In the relative early days of plant biotechnology, when the pinnacle of applied crop improvement was single gene insertions conferring single traits such as glyphosate resistance (Padgett et al. 1995) or insecticidal proteins (Stewart et al. 1996), this slow pipeline from build to test was somewhat tolerable. However, future agronomic improvement of soybean using GE technology demands more complex strategies centered on whole metabolic pathway alterations, concurrent expression and tight control of genes of interest (GOI) and gene products, and targeted gene edits, among other intricate strategies (Kamthan et al. 2016). Clearly, the time and resource investment required for stable transformation is the most significant obstacle to studying, validating, and implementing GE soybean innovations. Thus, there is a critical need for tools that enable rapid evaluation of GOI in soybean before proceeding with the investment of producing stable engineered plant lines (Altpeter et al. 2016; Pouvreau et al. 2018). The aim of this work is to address that need through the development of a robust and repeatable transient expression assay in planta.

***Agrobacterium*-mediated transformation**

Typically, soybean is transformed using either particle bombardment or through *Agrobacterium*-mediated transformation. Particle bombardment involves the coating of micro projectiles, usually gold or tungsten, with DNA, which is then shot into plant tissues at high velocity and the DNA is integrated into the cell's genome or expressed transiently (Ozyigit & Yucebiligili Kurtoglu, 2020). *Agrobacterium*-mediated transformation instead utilizes “nature's genetic engineer,” *Agrobacterium tumefaciens*. *A. tumefaciens* is the crown-gall disease pathogen, whereas hairy root disease is caused by *Agrobacterium rhizogenes*. These bacteria naturally deliver a single stranded T-DNA (transfer DNA) cleaved from the TI (tumor inducing) plasmid (which is usually altered to replace native *Agrobacterium* genes with GOI) to plant cells (Gelvin, 2003).

Transient expression strategies

There have been some significant developments in transient gene expression assays in soybean such as using protoplasts for rapid testing of promoters (Sultana et al. 2019). However, while amenable to transfection, and easy to evaluate, protoplasts still require sterile conditions, maintenance of cell cultures (in addition to all the associated reagents), and still yet, a pipeline of several weeks to months. Alternatively, a protocol utilizing the soybean mosaic virus as a gene-delivery vector delivered by aphids has been developed (Seo et al. 2016). However, it is still limited by the inability to deliver genetic elements larger than a few kilobases and is dependent upon virus-based vectors, the fidelity of which is still debated (Khakhar & Voytas, 2021). There has also been developed a transient assay utilizing soybean callus in tissue culture (Xu et al. 2017). Nevertheless, it still suffers from many of the same limitations as protoplasts; that being sterility requirements, a relatively long pipeline, maintenance of explants, and plant tissue which is physiologically much different than it would be in a field environment. Therefore, a more attractive strategy which has been utilized by plant geneticists for the study and manipulation of gene expression, regulatory elements, protein-protein interactions, gene edits, and metabolic pathways of various plant species is agroinfiltration. Agroinfiltration is a relatively simple, non-sterile, method where *A. tumefaciens* containing a GOI is forcibly introduced into plant tissues. Once the bacteria are in close proximity to amenable cells, they deliver T-DNA containing GOIs into the cell's cytoplasm to either be integrated into the genome, transiently expressed, or both (Gelvin, 2003; Lee & Yang, 2006.;

Zottini et al. 2008). Using this method, transient gene expression can be observed in a matter of hours and persist for days. The method is useful for researchers interested in transgene function/interactions/characteristics, promoter function, cellular/whole physiological processes, protein-protein interactions, plant-pathogen interactions, and a variety of other applications (Deguchi et al. 2020; Seo et al. 2016; Vaghchhipawala et al. 2011; Zhang et al. 2020). Agroinfiltration is regularly utilized in model species such as *Nicotiana benthamiana* and *Arabidopsis thaliana* (Norkunas et al. 2018; Wroblewski et al. 2005) as well as other non-model species (Wroblewski et al. 2005; Zhang et al. 2020). However, a robust agroinfiltration procedure in soybean has yet to be widely adopted (King et al. 2015).

Challenges

The current lack of progress in soybean agroinfiltration is a reflection of the many obstacles which have long plagued stable soybean transformation: plant recalcitrance to *Agrobacterium* infection, genotype dependencies, barriers to *Agrobacterium* delivery, such as wax and hairs on leaf surfaces, and innate plant defense (Xu et al. 2022). The difference in developing a successful agroinfiltration protocol as opposed to a stable transformation method, is that a robust agroinfiltration protocol requires the majority of plant tissue exposed to an *Agrobacterium* suspension to be transformed (transiently) in order to easily study the outcome over whole leaf or plant area. Whereas stable transformation, with the aid of selectable markers such as antibiotic resistance, only requires the successful transformation of a small number of cells which can be multiplied and later regenerated into a whole plant. Thus, it is imperative that the mechanical and biological factors which make a robust soybean agroinfiltration procedure so difficult be understood and overcome. The main impediments facing successful transient expression of *Agrobacterium*-delivered GOI is first selecting genotypes that are most amenable to *Agrobacterium* infection, as well as selecting tissue to be infiltrated, which is not only at the most optimal state for susceptibility to *Agrobacterium* but also for high expression of delivered GOI (Song et al. 2013; Villemont et al. 1997; Xu et al. 2022). Once that is established, a solution for delivering *Agrobacterium* suspension past the leaf epidermis/stomata into the intercellular space and saturating the entire leaf area must be determined (Li et al. 2017; Opabode 2006; Song et al. 2013). Additionally, special attention must be paid to ensure the most suitable quantity and quality (strain) of *Agrobacterium* are delivered,

and care must be given to culturing *Agrobacterium* in such a way as to maximize the bacterial competence to transfection of the greatest number of cells after infiltration (Cody et al. 2023; De Saeger et al. 2021; Wu et al. 2014). Additionally, mitigations must be put in place to combat plant defense mechanisms such as reactive oxygen species as well as RNAi and gene silencing (Dan, 2008; Felippes et al. 2020; Norkunas et al. 2018).

Thus, I investigated these parameters with the goal of developing a robust and repeatable methodology for soybean agroinfiltration. Besides some preliminary experiments to determine starting points for *Agrobacterium* strain, infiltration buffer, and vacuum specifics, my main effort was focused on first identifying the soybean genotypes most susceptible to *Agrobacterium* through a screen of 26 genotypes of diverse germplasm. Once the optimal genotype was selected, I further refined the protocol to narrow down wounding and vacuum parameters, as well as optimal *Agrobacterium* culture conditions and leaf/plant developmental stages. Once the protocol was sufficiently refined, I tested its efficacy through co-infiltrations of fluorescent proteins as well as a pigment-based reporter: RUBY.

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Chapter 2

A robust soybean agroinfiltration method

This chapter will be submitted to the journal Plant Cell Reports for publication.

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Key message

A robust agroinfiltration-mediated transient gene expression method for soybean leaves was developed. Plant genotype, developmental stage and leaf age, surfactant, and *Agrobacterium* culture conditions are critical to successful agroinfiltration.

Abstract

Agroinfiltration of *Nicotiana benthamiana* has emerged as a workhorse transient assay for plant biotechnology and synthetic biology to test the performance of gene constructs in dicot leaves. While effective, it is nonetheless often desirable to assay transgene constructs directly in crop species. To that end, we innovated a substantially robust agroinfiltration method for *Glycine max* (soybean), the most widely grown dicot crop in the world. Several factors were found to be relevant to successful soybean leaf agroinfiltration, including genotype, surfactant, developmental stage, and *Agrobacterium* strain and culture medium. In young soybean leaves we show high expression of the GUS reporter gene, co-expression of two fluorescent protein genes, and nearly 100% expression production efficiency of the RUBY reporter product, betalain. The research represents not only a new research tool for soybean biotechnology, but also indicates critical parameters for guided agroinfiltration optimization that could be used for other crop species.

Introduction

Genetically engineered (GE) soybean (*Glycine max*) accounts for over 92 million hectares of planted farmland and is among the most widely grown crops across the globe (ISAAA 2019). Despite advances in soybean tissue culture, transformation, and regeneration, production of GE soybean still requires an abundance of time, resources, and capital; often requiring a time investment of 6-12 months to produce a stable transgenic plant suitable for evaluation (Li et al. 2017; Rech et al. 2008; Yamada et al. 2011; Yang et al. 2016). Increasingly, the pressures on modern agriculture require complex approaches focused on enhancing crop yield and resilience by altering whole metabolic pathways, tightly controlling multiple transgenes and gene products, as well as generating targeted gene edits (Altpeter et al. 2016; Anjanappa & Gruissem, 2021; Kamthan et al. 2016; Kumar et al. 2020). Certainly, fundamental molecular research of soybean and subsequent genetic improvement is hindered by the stable transformation bottleneck. Thus, it is imperative to develop high throughput techniques that enable rapid screening of transgene characteristics, native/synthetic promoters, protein-protein interactions, gene edits, and pathway alterations in soybean (Altpeter et al. 2016). To this end, agroinfiltration is a particularly useful tool for rapid transient gene expression in leaves because of its simplicity in procedure and that its results can be observed in a matter of hours and persist for days. While agroinfiltration is well established in model species such as *Nicotiana benthamiana* and *Arabidopsis thaliana* (Norkunas et al. 2018; Wroblewski et al. 2005), and has been reported in species such as tobacco, tomato, *Arabidopsis* and lettuce (Wroblewski et al. 2005; Zhang et al. 2020), little has been published on soybean agroinfiltration. In 2015, King et al. (2015) broke ground by demonstrating a successful soybean agroinfiltration protocol for the first time. However, while that study marked significant progress in soybean agroinfiltration, only one reporter gene was used for validation and transient expression levels were relatively low compared to model species. Moreover, few other groups (Bao et al. 2017) have reported successful use of the King et al. (2015) protocol in soybean agroinfiltration. Indeed, a well-established procedure for tobacco agroinfiltration required years of work and many contributing publications. Therefore, we sought to develop a robust soybean agroinfiltration procedure. Here, using multiple reporter genes, we detail a substantially improved soybean agroinfiltration protocol with refined parameters

including genotype, surfactant, developmental stage and leaf age, and *Agrobacterium* culture medium.

Materials and methods

Plant material and growth conditions

Seeds obtained from the University of Tennessee, University of Kansas, and Virginia Polytechnic Institute were grown in the field for seed-bulking. Seeds from the field-grown plants were collected and lightly sprayed with 70% ethanol. Once dry, seeds were surface sterilized with chlorine gas [100 mL of commercial bleach (NaClO) + 3.5 mL of 12 N hydrochloric acid (HCl)] in a desiccator for 12 h (Paz et al. 2006). Seeds were then imbibed in non-sterile water and left in the dark overnight. Once imbibed, seeds were rolled in damp paper towel, placed in a beaker with a shallow layer of water, and put in a growth chamber under 16-h day/8-h night (~90-120 μmol intensity) at 24 °C for germination/growth.

Vector Construction

Plant transformation vectors used in the study were constructed via Golden Gate assembly. All constructs were driven by constitutive promoters driving a fluorescent, enzymatic, or pigment-based reporter gene (Fig.1).

Optimized *Agrobacterium* growth, preparation, and agroinfiltration

The parameters for *Agrobacterium* growth, preparation and the agroinfiltration procedure itself evolved with the study as variables were optimized with each experiment (Fig. 2). For clarity, the final optimized procedure is described here while factors that were tested are noted under the parameters tested sub-section.

All *Agrobacterium* strains utilized were transformed with constructs driven by constitutive promoters driving a fluorescent, enzymatic, or pigment-based reporter gene (Fig.1) and grown for two days on yeast extract peptone (YEP) plates (5 g/L NaCl, 10 g/L peptone and 10 g/L yeast extract) and supplemented with 50 mg/L rifampicin and kanamycin for two days.

The evening of two days prior to agroinfiltration, a streak of *Agrobacterium* from the YEP plates were used to inoculate a 25-50 mL YEP liquid culture supplemented with antibiotics and grown for no more than 24 hours.

Then, the evening prior to agroinfiltration, the YEP liquid culture was centrifuged at $4100 \times g$ for 15 minutes and resuspended in 50-150 mL of AB:MES media to an OD_{600} of 0.3, supplemented with 200 μ M acetosyringone (AS) and 50 mg/L kanamycin and grown overnight as described in Cody et al. 2023.

The day of infiltration, the overnight grown AB:MES *Agrobacterium* cultures were centrifuged at $4100 \times g$ and resuspended in soybean infiltration buffer (SIB) (10 mM MES and 10 mM $MgCl_2 \cdot 6H_2O$ at a pH: 5.4), diluted to an OD_{600} of 0.6, supplemented with 200 μ M AS and incubated in the dark on a 50-75 rpm shaker for 2 hours at room temperature ($\sim 22^\circ C$). After incubation, immediately prior to infiltration the SIB was supplemented with 400 mg/L of L-cysteine (reducer), 78 mg/L of dithiothreitol (DTT) (reducer) and 1% v/v Silwet L-77.

The plants were then submerged in ~ 150 -200 mL of SIB *Agrobacterium* suspension in a beaker and subjected to a single 5 min period of vacuum at -84.65 kPa using a building-wide vacuum. After 5 minutes of negative pressure, the air intake valve was *swiftly* released to rapidly fill the vacuum chamber with air and push the SIB *Agrobacterium* suspension into the leaf tissues. The infiltrated plants were then removed from the vacuum chamber, dunked in water to wash off excess SIB/surfactant, and pat dried with paper towel. The plants were then misted with water and placed back in their original growing container, covered with a plastic dome to maintain humidity, and left in the dark overnight. The next day plants were placed in a growth chamber for an additional 56-60 hr after which the plants were evaluated for transient expression.

Parameters tested

The following variables were evaluated and modified throughout the course of the study:

- Soybean genotype (Table 1).
- Infiltration buffer, *Agrobacterium* culture, surfactants, and procedural alterations (Table 2).
- Plant developmental stage (Table 3).

- *Agrobacterium* strain (Table 4).

GUS staining, GUS/Ruby imaging, and image analysis

Leaves were excised from infiltrated plants 72 hours post-infiltration, and GUS histochemical assays were performed as described by Jefferson (1989) with modifications by Sultana et al. (2022). Plant tissue samples were harvested and soaked in GUS staining solution (2 mM X-Gluc, 50 mM potassium phosphate buffer, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.2% Triton X-100) for 16 hr at 37°C. After staining, the leaves were soaked in 70% ethanol prior imaging to clear the leaves of chlorophyll. Stained and cleared leaves were then imaged on a standard desktop scanner (EPSON Scan V300). ImageJ thresholding and color thresholding tools (Schindelin et al. 2012) were used to isolate the total leaf area and leaf area-stained blue in pixels, respectively. The percent area stained was then calculated by dividing the area stained in pixels by the total area of the leaf and multiplying by 100 (percent area stained / total leaf area × 100). To prevent bias and to facilitate large image sets, an automated ImageJ macro was used to determine the percent area stained for GUS-stained images (Supplemental Notes 1-2). Plants infiltrated with vectors carrying the RUBY reporter gene were imaged and analyzed in similar fashion by isolating red coloration with the color thresholding tool to determine leaf area producing betalain.

Fluorescence spectroscopy, compound microscopy, and confocal microscopy

Fluorescent protein production (specific ectopic fluorescence) was measured in leaves as described in Millwood et al. (2003) using a Fluorolog® spectrofluorometer (Horiba/Jobin Yvon). Excitation for mGFP5-ER was 480 nm and the emission was measured from 500 nm-520 nm with a peak of 509 nm. Excitation for tRFP was 540 nm and the emission was measured from 560 nm-640 nm with a peak of 574 nm. Compound fluorescent microscopy images were obtained using an EVOS FL auto microscope. Tissues infiltrated with vectors carrying mGFP5-ER were excited at 470 nm and observed at 510 nm. Tissues infiltrated with vectors carrying tRFP were excited at 531 nm and observed at 593 nm. Confocal microscopy images were obtained using an Olympus Fv1000 confocal microscope. Tissues infiltrated with vectors carrying mGFP5-ER were excited at 490 nm and detected at peak emission 509 nm while tissues

infiltrated with vectors carrying tRFP were excited at 553 nm and detected at peak emission, 574 nm.

qRT-PCR transcript abundance assay

RNA extraction from entire soybean leaves were performed using TRI reagent (Zymo Research). Extractions were performed according to the manufacturers protocol, treated with DNase I, and cleaned with an RNA Clean & Concentrator™ kit (Zymo Research). RNA quantification was determined using a nanodrop spectrophotometer ND-1000 (Thermo Fischer Scientific). cDNA of was synthesized using 2 mg of RNA per sample with ZymoScript™ RT PreMix Kit (Zymo Research) following the manufacturers protocol. qPCR was performed using PowerUp™ SYBR™ Green 2X Master Mix (Thermo Fischer Scientific) in a 5 mL reaction, using 2.5 ng of cDNA and 0.5 mM of each forward and reverse primers. The qPCR was performed using QuantStudio™ 6 Flex qPCR System (Applied Biosystems), and the settings for all primer sets were as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec, descends 1.6°C per second until 60°C, 60°C for 1 min, then ascends to 95°C at 1.6°C per second. The qRT-PCR results were analyzed by the $2^{-\Delta CT}$ method, with *GFP* and *RFP* C_T values set relative to *GmUbi3* C_T values.

Statistical analysis

All experiments were conducted using a complete randomized design with at least 9 plants (18 leaves) infiltrated per experiment. One-way analysis of variance (ANOVA) was conducted for experiments of more than two variables. Otherwise, a student's t-test was conducted. In cases where comparisons were made between all treatments, ANOVAs were followed up with a Tukey's HSD analysis. In cases where one or more treatments were compared exclusively to a negative control ANOVAs were followed by a Dunnett's test. All statistical analysis was performed using the JMP pro 15 software (SAS, Cary, NC).

Results and discussion

Soybean genotype

Identifying plant genotypes most compatible with a particular strain of *Agrobacterium* as well as the genotype's general amenability to agroinfiltration has been established as a critical parameter

in *Agrobacterium*-mediated soybean transformation (Delzer et al., 1990; Donaldson & Simmonds, 2000; Van Wordragen & Dons, 1992.). Therefore, we screened 26 soybean genotypes of diverse germplasm (Table 1) using a modified infiltration protocol based on King et al. (2015) (Fig. 3). This initial adapted protocol incorporated extended vacuum exposure, supplementation with L-cysteine and DTT, utilization of Tween-20 as a surfactant, 30 seconds of sonication at 42 kHz and employed *A. tumefaciens* strain EHA 105 harboring the constitutively expressed GUS reporter gene (Fig. 1). Of the genotypes tested, the majority (23 out of 26), displayed minimal or negligible transient GUS expression. However, lines 'V17-0799DT', 'V16-0248DI', and 'TN16-5004' exhibited potential for further evaluation with total GUS expression areas of 35%, 19% and 10%, respectively. All subsequent optimization experiments were performed using line 'V17-0799DT' (Figs. 4-5).

Wounding and surfactants

A common strategy to improve *Agrobacterium*-mediated transient transformation efficiency has been to mechanically wound tissue through micro-wounding, most often with sonication (Acanda et al., 2021; Bakshi et al., 2011; De Oliveira et al., 2009, Deguchi et al., 2020; King et al., 2015). However, through the course of our experimentation, we frequently observed excessive necrosis in the days following infiltration, and in certain instances complete plant death (Fig. S1). Therefore, we sought to test the efficacy of vacuum infiltration with surfactant alone vs our previous method of sonication plus surfactant. A comparison between 30 seconds of sonication at 42 kHz followed by vacuum infiltration and vacuum infiltration using 0.01% v/v Tween-20 without sonication found no statistically significant difference in transient GUS expression. Though, as previously observed, the sonicated plants were visibly necrosed in the days following infiltration (Fig. S1). Therefore, we chose to proceed with surfactant alone for subsequent experiments. We determined that while in certain circumstances, wounding may increase overall transient transformation efficiency, its expression increase effect was negligible in soybean and the eventual leaf necrosis would likely be deleterious to practical research goals. Indeed, other studies have demonstrated that surfactant alone can be sufficient for the delivery of *Agrobacterium* suspension during vacuum infiltration (Norkunas et al., 2018; Zhang et al., 2017). Thus, to optimize vacuum infiltration without wounding, we explored various concentrations

(ranging from 0% to 0.01% v/v) of the surfactants Tween-20 and Silwet L-77 added to the infiltration buffer. At a concentration of 0.01% v/v, both surfactants performed relatively the same (Fig. S2). However, we observed that to fully infiltrate soybean leaves, Tween-20 required two or more rounds of vacuum and release whereas Silwet L-77 required only a single round (Fig. S2). Therefore, we chose to proceed with Silwet L-77 as the surfactant for subsequent experiments to minimize the overall time under vacuum.

***Agrobacterium* culture media**

To further optimize our agroinfiltration protocol, we investigated the influence of the culture media on transient transformation efficiency. In many instances, AS alone is added to standard growth media before infiltration or transformation, but culture conditions such as carbon source are also known to significantly impact *Vir* gene induction and subsequent transient expression efficiency (Gelvin, 2003, 2014b, 2021; Gelvin, 2006.). To understand if culture condition was a critical parameter in soybean agroinfiltration, we tested strain EHA 105 harboring 35S:GUS:NOS grown in AS-supplemented YEP media incubated for 8 hr the day of infiltration, YEP incubated for 16 hr and an *Agrobacterium* induction medium (AB:MES) (derived from Cody et al. 2023) incubated for 16 hr. Strikingly, a three-fold difference was observed between the *vir* gene-inducing medium AB:MES and the standard YEP medium regardless of incubation time. It has been reported that in *Agrobacterium* media supplemented with AS, T-strand transfer rises significantly between 9 and 12 hours of incubation in and peaks at 24 hours of incubation (Xi et al., 2018). However, the large difference between the YEP grown and AB:MES grown cultures points to the influence of additional factors that impact *Vir* gene induction and T-DNA transfer besides phenolic compounds such as AS, alone. The increased expression efficiency of the AB:MES media is likely influenced by glucose (Shimoda et al., 1990), low pH (5.7 AB:MES vs. 7 YEP) (Mantis and Winans, 1992), or more likely, a combination thereof (Gelvin, 2006). Regardless, we proceeded with AB:MES as the primary *Agrobacterium* culture media in all subsequent experiments.

Plant developmental stage

Over the course of the study, we used plants in the VC stage of growth (Fehr et al. 1971) as done previously by King et al. (2015). However, we noted that there is an important and easily confused distinction between VE and VC growth stages, which encompasses the expansion of the first true leaves over an approximately two-day period. Therefore, we investigated various leaf ages, including VE stage plants at emergence, VC stage plants when the leaves had fully unfurled, the first trifoliolate leaves of V1 stage plants, and the older, unifoliolate leaves of V1 stage plants (Fig 5). Our findings revealed a two-fold difference between VE and the late VC stage leaves and the highest expression efficiency in fully expanded 1st trifoliolate leaves, which underscores the importance of maintaining consistency in leaf age throughout experiments. It has been shown that cell division/cycle plays a crucial role in a plant cells competence to *Agrobacterium* T-DNA transfer with more cell division usually equating to greater expression (Binns & Thomashow, 1988; Sangwan et al., 1992; Villemont et al., 1997). Although this may explain the difference in expression levels of the first true leaves of V1 plants vs. the expanded trifoliolate leaves, we suspect that the expression difference shown here between VE and VC stage might be the result of differences in leaf architecture; we observed that unexpanded leaves (VE) were generally less amenable to full infiltration of the SIB *Agrobacterium* suspension through the entire leaf area than fully expanded VC stage leaves. Thus, it is imperative that for successful application of this protocol, plants must be infiltrated in the VC or fully expanded V1 stage of growth.

Genotype rescreen/*Agrobacterium* strain

When we first conducted the soybean genotype screen, we were unable to reach expression levels sufficient for efficient detection of fluorescent proteins. So, once we had established the essential parameters for efficient agroinfiltration of soybean, we rescreened 26 soybean genotypes using the final procedure outlined in Fig. 2. Plants in the VC stage of maturity were infiltrated with *Agrobacterium* strain EHA 105 harboring a Gmubi:tRFP:OCS construct (Fig. 6). The results were generally consistent with the original screen. However, when measured using fluorescent spectroscopy, line 'TN16-5004' surpassed line 'V17-0799DT' as the superior expressing germline. Our qRT-PCR assay for transcript abundance also showed a higher level of

transcript abundance in ‘TN16-5004’ vs ‘V17-0799DT’ with relative expression levels compared to soybean polyubiquitin of 14.16% and 9.5%, respectively, though no statistically significant difference between the two lines were found. Regardless, our expression efficiency was sufficient to apply the protocol to screen four *Agrobacterium* strains for transient expression efficiency in this system (Fig. 7). Our results show that strain EHA 105 is critical to the success of the protocol with over 10× the mean fluorescence of the other strains LBA4404, AGL 1 and GV3101.

Co-infiltration/RUBY reporter

Because we were able to effectively reach high levels of transient expression with a single reporter and advanced plant biotechnology and synthetic biology demand the expression and/or perturbation of multiple genes simultaneously, we aimed to assess the protocol's effectiveness by employing multiple reporter genes concurrently through co-infiltration (Fig. 8). Using our final protocol as described in the Materials and Methods, which, among other parameters, includes selecting top performing genotypes, no wounding mechanism, utilizing 0.01% Silwet L-77, a single round of vacuum exposure, and AB:MES induction media, we successfully co-infiltrated soybean plants with equal portions of *Agrobacterium* strain EHA 105 harboring Gmubi:mGFP5-ER:OCS and *Agrobacterium* strain EHA 105 harboring Gmubi:tRFP:OCS (Fig 8). Line ‘TN16-5004’ showed a relative tRFP and mGFP5-ER expression level of 7.8% and 6.0%, respectively compared to soybean polyubiquitin. Interestingly, line ‘V17-0799DT’ showed a relative tRFP and mGFP5-ER expression level of 20.8% and 17%, respectively when compared to soybean polyubiquitin. In a first for soybean, we also used our protocol to infiltrate soybean line ‘V17-0799DT’ with the three-gene betalain biosynthetic pathway CYP76A1, DODA1 and glucosyltransferase (RUBY) (He et al. 2020) to better visualize the efficacy of the protocol (Fig. 9), and we were able to achieve over 70% of the mean leaf area showing betalain expression.

Conclusion

In this study, we have developed a robust agroinfiltration-mediated transient gene expression method for soybean leaves. This procedure offers a rapid and efficient means to evaluate transgene constructs and conduct GE experiments directly within soybean leaves. Our research yielded several key findings that contribute to efficient agroinfiltration. One of our primary

findings was the identification of genotypes highly susceptible to *Agrobacterium*, namely lines ‘V17-0799DT’ and ‘TN16-5004.’ Another requirement for successful agroinfiltration is the selection of leaves at the precise optimal age/developmental stage: fully expanded leaves at the VC or V1 stage. Furthermore, we show that other key elements are important, namely *Agrobacterium* culture and surfactant used for vacuum infiltration. This technique provides a meaningful reduction in the time and resources needed for assessing transgene characteristics, evaluating native or synthetic promoters, studying protein-protein interactions, performing gene edits, or modifying metabolic pathways in soybean. Through the course of the research, we have noted inherent limitations of soybean leaf agroinfiltration. Most evident is batch-to-batch variation in agroinfiltration frequency and reporter gene expression. This variability is clearly observed in Fig. 9, where some leaves exhibited nearly 100% expression efficiency while others had significantly lower expression levels, even within the same plant. Consequently, we recommend the infiltration of a substantial number of plants in each experiment to account for this variance. Fortunately, the scalability of our protocol facilitates this approach. It is also worth emphasizing that the success of this method relies on several critical factors, leaving little room for error. Specifically, the optimal culturing of *Agrobacterium* and the infiltration of plants at the precise developmental stage are paramount. Therefore, we advocate not only for meticulous attention to these parameters but also the use of a comparator group, wherein a few soybean plants are infiltrated with a constitutively expressed and easily observable reporter gene. This serves as a safeguard against potential errors in the preparation process, such as infiltrating leaves outside the optimal developmental stage. Ultimately, despite these considerations, this technique presents a significantly improved and reproducible method that overcomes previous limitations which will aid in accelerating the next generation of GE crops.

Competing interests

Authors are inventors of biotechnology innovations in which the intellectual property is assigned to their employers. CNS is editor-in-chief of Plant Cell Reports and had no editorial participation in the peer review process of this submitted manuscript.

Author contributions

BNT contributed to the design, established, and conducted all experiments, analyzed data, and drafted the manuscript. MSS contributed to the design, helped establish experiments, provided valuable experimental insight, and manuscript revisions. ACP generated plasmid constructs, provided valuable suggestions, and manuscript edits. JNS conducted and analyzed qRT-PCR experiments and contributed to drafting the manuscript. VP and BZ provided germplasm and manuscript edits. CNS conceived and coordinated the study, revised and wrote the manuscript, analyzed data, and obtained funding.

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Chapter 3
Conclusions and recommendations

Plant biotechnology and GE crops have had a profound impact on world agriculture, but the field has also struggled to fully live up to the many lofty goals that scientists have envisioned for decades, such as drastically improved yields, extreme weather adaptability, and precise crop metabolic engineering. It is not unreasonable to predict that these goals will eventually be accomplished and implemented in GE crops, but *it is* unreasonable to expect it to be accomplished with the currently available tools and methodologies alone. As the field of plant biotechnology (and increasingly, synthetic biology) evolves, faster and more reliable tools must be deployed in order to implement increasingly complex GE research goals. A small, but nevertheless important contribution to solving this need is the agroinfiltration protocol we developed. It presents a significantly improved and reproducible method that overcomes previous limitations and provides a framework for further optimization of both stable transformation and transient transformation in soybean and other crops. We have identified critical parameters, including genotype selection, infiltration method, surfactant choice, *Agrobacterium* culture conditions, and developmental stage/leaf age, that collectively contribute to the success of the method.

While we have innovated a robust agroinfiltration procedure for soybean, it is not without its limitations. There is large GOI expression variation within experiments (Figs. 3-9). This is easily seen in Fig. 9 with some leaves showing near 100% expression efficiency and others much lower expression levels, even on the same plant. Thus, it is recommended that a large number of plants be infiltrated per experiment to account for this. Luckily, this is easily facilitated because of the innate scalability of the protocol. The method also relies on several factors that leave little room for margin of error, particularly, optimally culturing *Agrobacterium* and infiltrating plants at the precise optimal age/developmental stage. Therefore, it is recommended that not only should special attention be paid to the parameters, but a comparator (i.e., a few soybean plants infiltrated with a constitutively expressed, easy-to-observe reporter) should be utilized as a check against mistakes in the preparation process (e.g., technical errors such as infiltrating leaves outside of the optimal age). Negative controls should also be included. It is important to note that 5' and 3' UTRs will affect the transient transformation efficiency as certain sequences can trigger post transcriptional gene silencing after infiltration or otherwise affect transcript stability (Pfothner et al. 2022) (Fig S4). So researchers must also validate constructs they plan on

using by fusing them to a reporter gene and comparing the expression levels with a known value (e.g., Gmubi:mGFP5-ER:OCS) after infiltration. If carefully performed, this soybean agroinfiltration method enables a plethora of applications previously only possible in model species used as proxies and will serve to enable future research which will aid in advancing the future of GE crops.

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Appendix

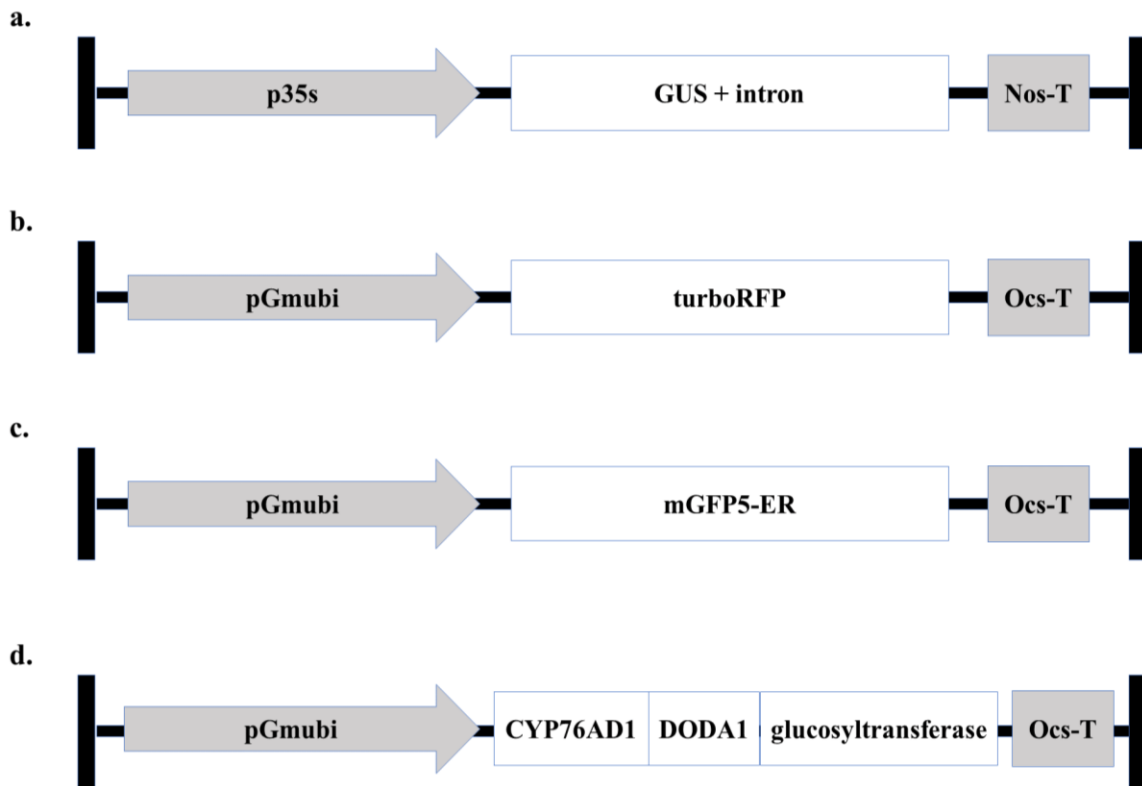


Fig. 1 Vectors used in optimization experiments. **a** Vector plasmid pTF101.1 with a CaMV35Ss (35S) promoter driving a GUS reporter with an intron and a nopaline synthase terminator (NOS) 35S:GUSplus:NOS. **b** Vector plasmid pAGM4723 with a *Glycine max* ubiquitin (Gmubi) promoter driving a red fluorescent protein (turboRFP) reporter and an *Agrobacterium* octopine synthase terminator (OCS) Gmubi:turboRFP:OCS. **c** Vector plasmid pAGM4723 with a Gmubi promoter driving a green fluorescent protein (mGFP5-ER) and an OCS terminator. **d** Vector plasmid pAGM4723 with a Gmubi promoter driving a three-gene betalain biosynthetic pathway linked by 2A peptides: CYP76A1, DODA1 and glucosyltransferase as a single open reading frame and an OCS terminator (He et al. 2020).

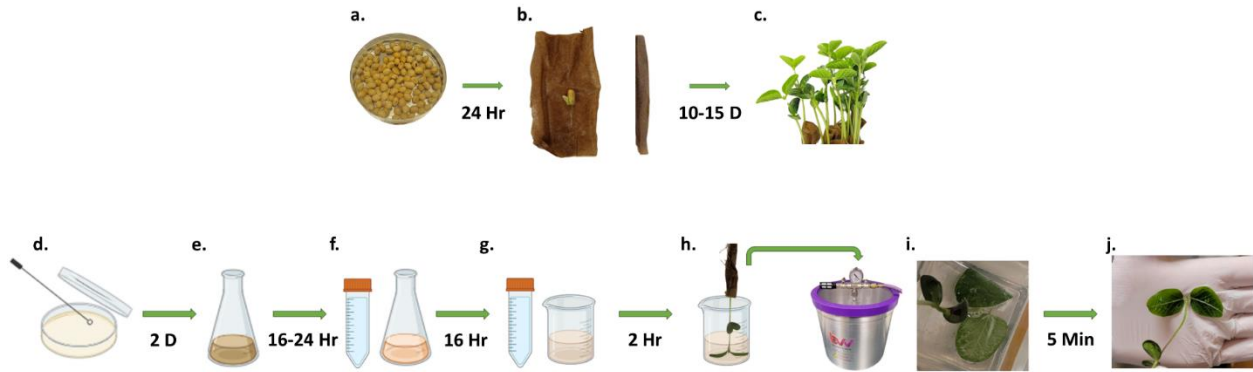


Fig. 2 Schematic of plant preparation and optimized soybean agroinfiltration process. **a** Sterile seeds were imbibed for 24 hours in water. **b** Paper towels were thoroughly moistened and cut into approximately 5 cm × 7.5 cm rectangles. Imbibed seeds were placed individually on moistened towels, rolled up, and placed upright in a beaker. **c** The paper towel-rolled seeds were placed in a growth chamber on 16-h day/8-h night cycle (~90-120 μmol intensity) at 24 °C and grown for 10-15 days to the VC or V1 stage. **d** *Agrobacterium* cultures were streaked on a YEP plate supplemented with antibiotics 4 days prior to infiltration (PTI) and grown for two days. **e** A colony from the YEP plates was used to inoculate a liquid starter culture of 50 ml YEP supplemented with antibiotics 2 days PTI, and grown for 16-24 hr on a shaker at 225 rpm at 28 °C. **f** The evening PTI, the starter culture was centrifuged at 4100 × g for 15 minutes and resuspended in AB:MES induction media to an OD₆₀₀ of 0.3, supplemented with 200 μM AS and selection antibiotics, and grown for 16 hr at 225 rpm at 28 °C. **g** The day of infiltration, the AB:MES induction media was centrifuged at 4100 × g for 15 minutes and *Agrobacterium* was resuspended in SIB to an OD₆₀₀ of 0.6, supplemented with 200 μM AS and incubated in the dark on a shaker at 50-75 rpm for 2 hr. **h** After incubation, SIB-*Agrobacterium* suspension was supplemented with 400 mg/L L-cysteine, 78 mg/L DTT, and 1% v/v Silwet L-77. SIB-*Agrobacterium* suspension beaker was placed in the vacuum chamber. Plants are then removed from the growth chamber and placed in the beaker with abaxial leaf surface facing up. **i** Plants were subjected to 5 minutes of vacuum at -84.65 kPa. The air intake valve was swiftly released to rapidly apply positive pressure and infiltrate leaf tissue. **j** Infiltrated plants were removed from the vacuum chamber, dunked in a beaker of DI water to wash off excess suspension and surfactant, patted dry with paper towel, placed back in their original growing container, lightly sprayed with water and placed under a plastic dome to maintain humidity. Finally, plants were placed in a dark cabinet for 16 hr before being placed back in the growth chamber for evaluation 72 hr post infiltration.

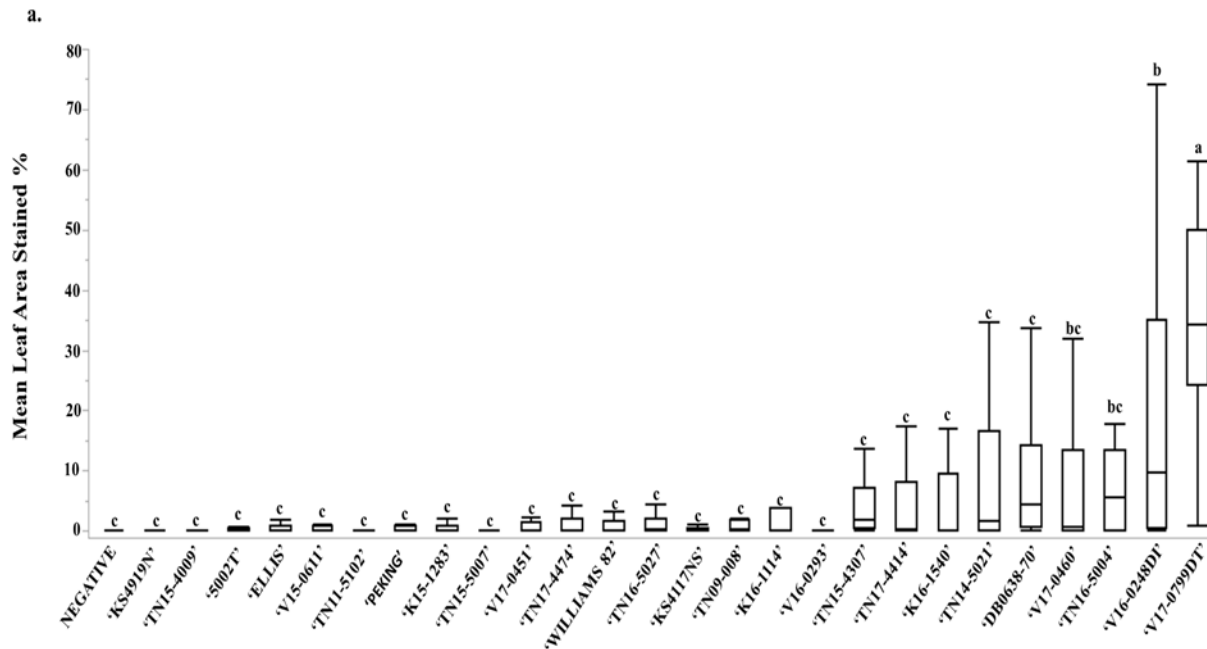


Fig. 3 Initial soybean genotype screen for amenability to agroinfiltration. Soybean lines infiltrated with *Agrobacterium* strain EHA 105 harboring 35S:GUSplus:NOS. Mixed VE and VC stage plants were subjected to 30 seconds of sonication at 42 kHz and vacuum infiltrated at - 84.65 kPa for two 30-minute intervals using SIB supplemented with 400 mg/L of L-cysteine, 78 mg/L of DTT and 1% v/v Tween-20. **a** Mean leaf area stained of infiltrated leaves. Each soybean line was infiltrated with 3 negative controls (SIB only) of the same line, but all negative controls were included as a single negative in the graph. Lines with differing letters considered significantly different. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey's HSD. **b** Representative images of all infiltrated soybean lines stained for GUS expression.

b.

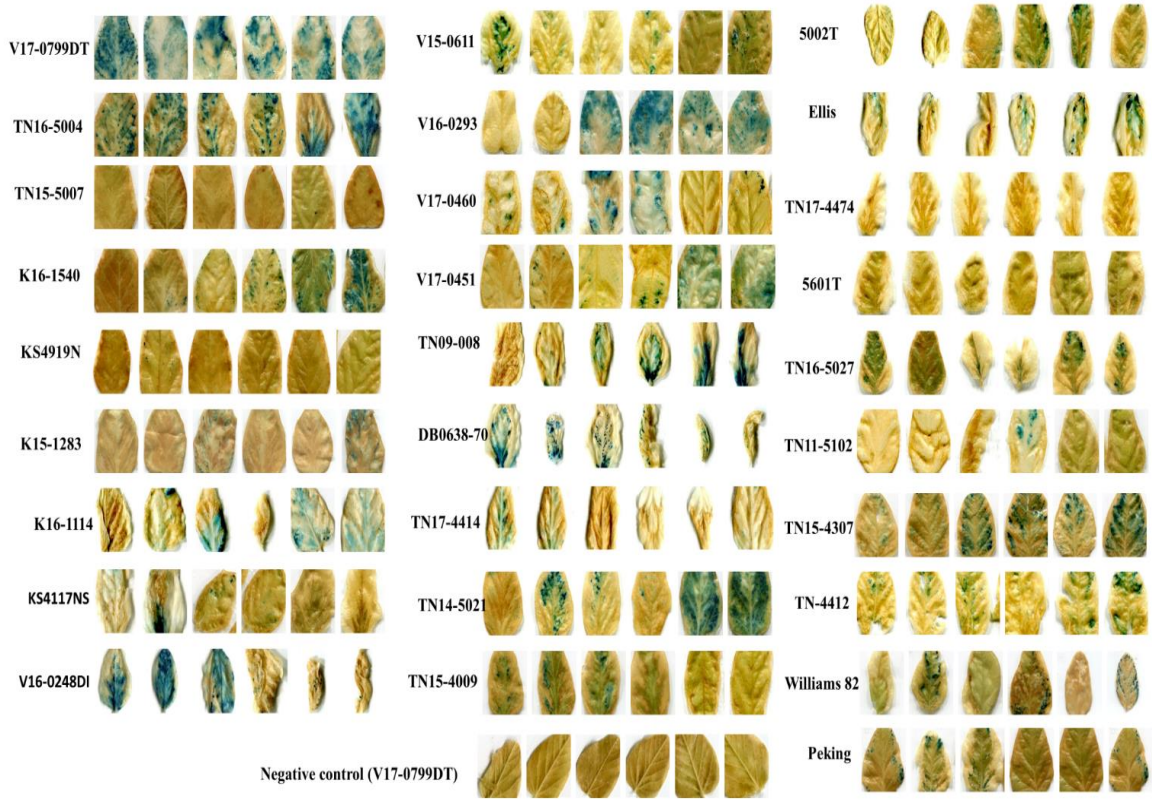


Fig. 3 continued.

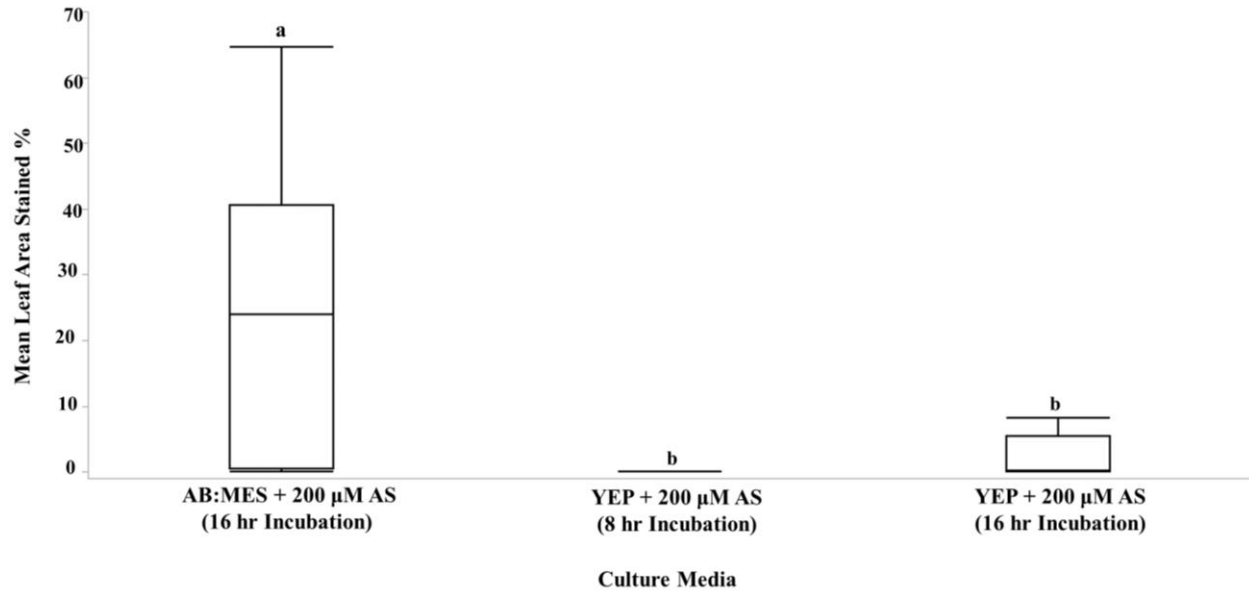


Fig. 4 Soybean line ‘V17-0799DT’ infiltrated with *Agrobacterium* strain EHA 105 harboring 35S:GUSplus:NOS incubated in AB:MES induction media + 200 μ M AS , YEP + 200 μ M AS grown the day of infiltration, or YEP + 200 μ M AS incubated overnight. Infiltrations were conducted with a single 5-minute round of vacuum at -84.65 kPa using 0.01% v/v. Silwet-L77. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey’s HSD.

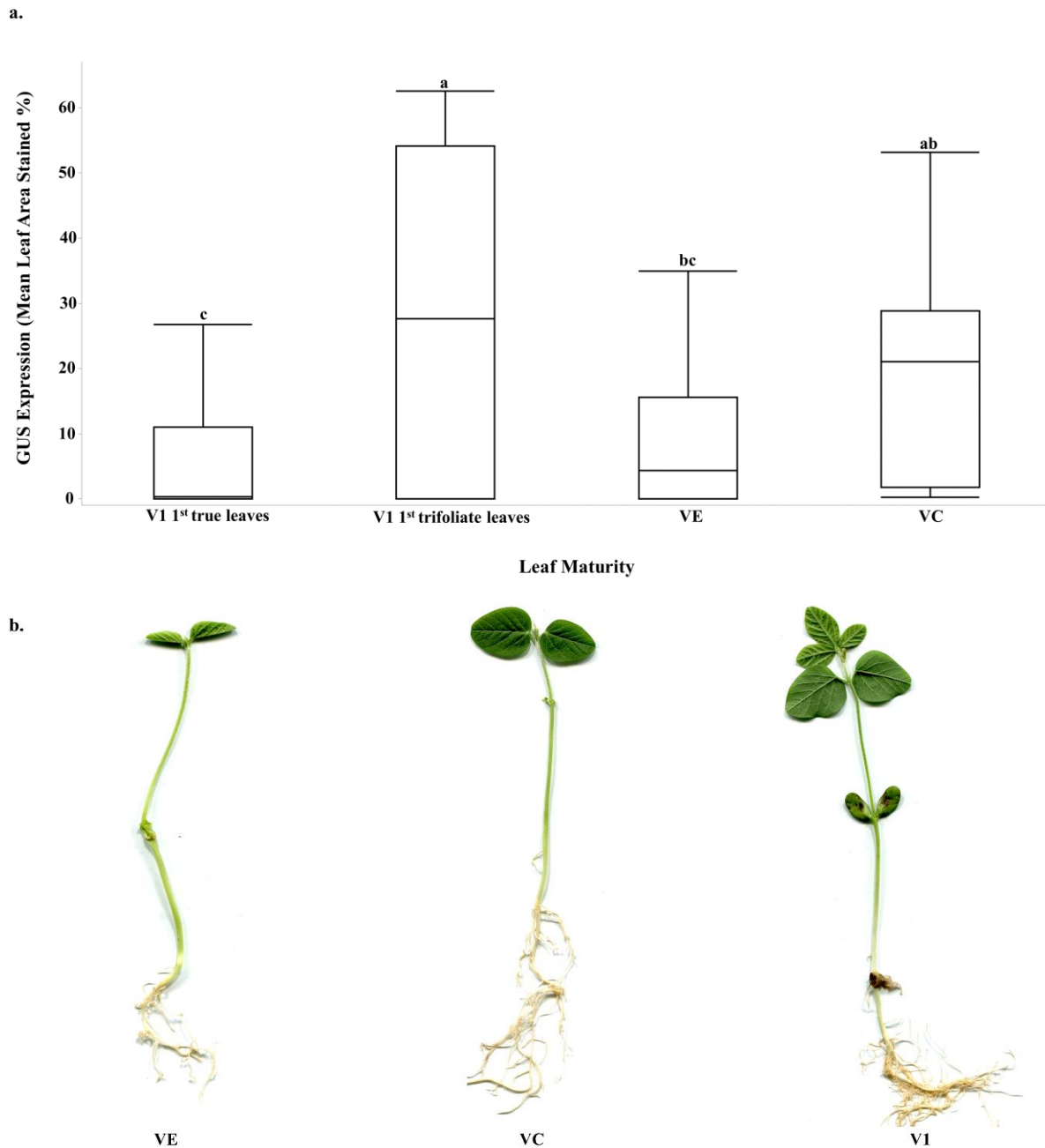


Fig. 5 Assessment of plant developmental stage. **a** Soybean line ‘V17-0799DT’ infiltrated during various developmental stages (VE-V1) with *Agrobacterium* strain EHA 105 harboring 35S:GUSplus:NOS grown in AB:MES induction media and subjected to a single 5-minute round of vacuum at -84.65 kPa using 0.01% v/v. Silwet-L77. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey’s HSD. **b** Examples of line ‘V17-0799DT’

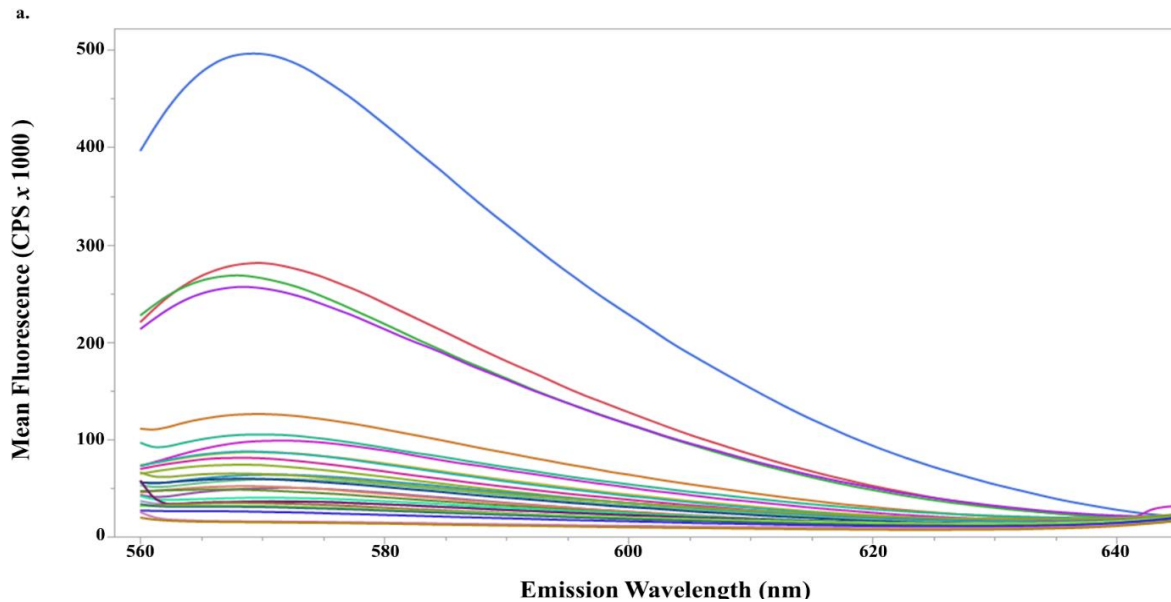
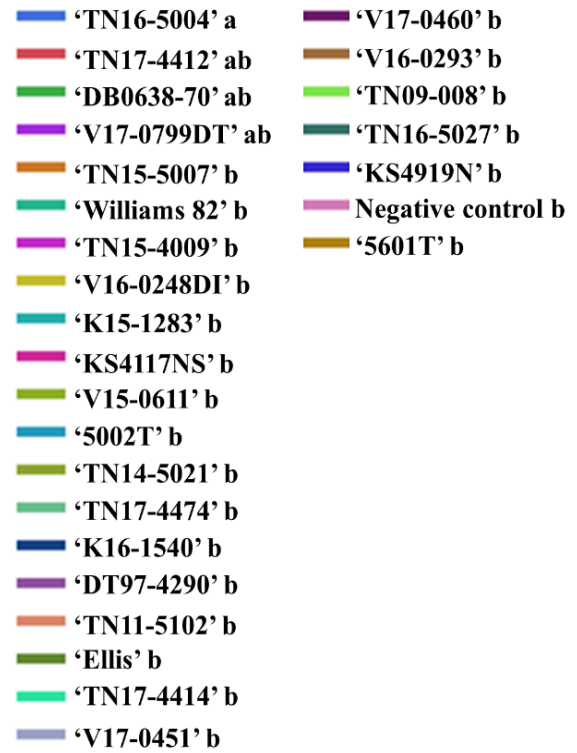


Fig. 6 Reassessment of soybean lines with a fully refined infiltration protocol using *Agrobacterium* strain EHA 105 harboring Gmubi:tRFP:OCS cultured and prepared as described in Fig. 2. VC stage plants were subjected to one 5-minute round of vacuum at -84.65 kPa in SIB supplemented 400 mg/L of L-cysteine, 78 mg/L of DTT and 1% v/v Silwet L-77. **a** Fluorescence spectroscopy of 26 infiltrated plant lines. Fluorescence emission intensity measured in counts per second (CPS). Excitation at 540 nm, emission measured from 560 nm-640 nm. Plants infiltrated with buffer and additives, but no *Agrobacterium* were used as negative controls. **b** Line ‘TN16-5004’ was the top performing genotype with a mean intensity of 476,296 cps. Peak emission (574 nm) was used for evaluation. Lines with differing letters considered significantly different. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey’s HSD. **c** qRT-PCR of lines ‘TN16-5004’ and ‘V170799DT’ for transcript abundance of tRFP relative to soybean polyubiquitin (Gmubi3). Plants infiltrated with a Gmubi:RUBY:OCS construct as well as wild type plants were used as negative controls. Line ‘TN16-5004’ showed a relative expression level of 14.16% and line ‘V17-0799DT’ showed a level of 9.5% of the reference gene, Gmubi3. Error bars represent standard error of the mean. $P < 0.05$, $n = 20$, one-way ANOVA.

b.

Soybean line	Mean Fluorescence (CPS) \pm SD
'TN16-5004'	478296 \pm 557221
'TN17-4412'	270868 \pm 413471
'DB0638-70'	251525 \pm 227833
'V17-0799DT'	243144 \pm 222479
'TN15-5007'	122175 \pm 82814
'Williams 82'	97881 \pm 73547
'TN15-4009'	97516 \pm 100269
'V16-0248DI'	84112 \pm 77009
'K15-1283'	83808 \pm 77639
'KS4117NS'	76378 \pm 93712
'V15-0611'	69443 \pm 76649
'5002T'	62666 \pm 49410
'TN14-5021'	61795 \pm 50377
'TN17-4474'	58277 \pm 59275
'K16-1540'	56843 \pm 53593
'DT97-4290'	49704 \pm 85248
'TN11-5102'	49600 \pm 52240
'Ellis'	45472 \pm 29916
'TN17-4414'	39334 \pm 39723
'V17-0451'	36359 \pm 23829
'V17-0460'	35727 \pm 18204
'V16-0293'	33121 \pm 38665
'TN09-008'	30351 \pm 16781
'TN16-5027'	29134 \pm 13235
'KS4919N'	24609 \pm 9981
Negative control	15390 \pm 195
'5601T'	14246 \pm 4049

Soybean line



c.

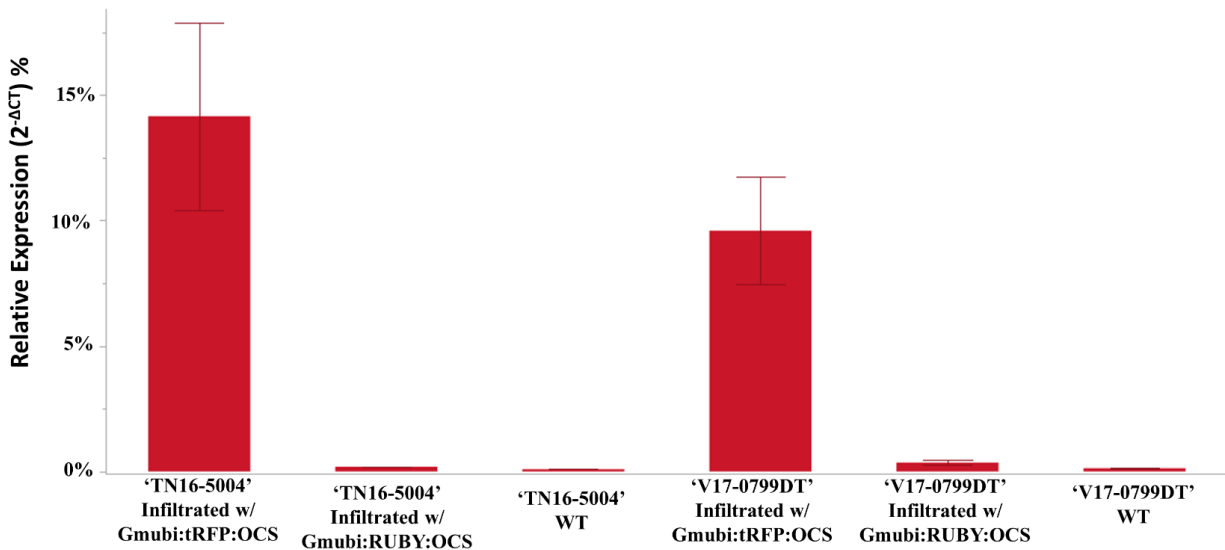


Fig. 6 continued.

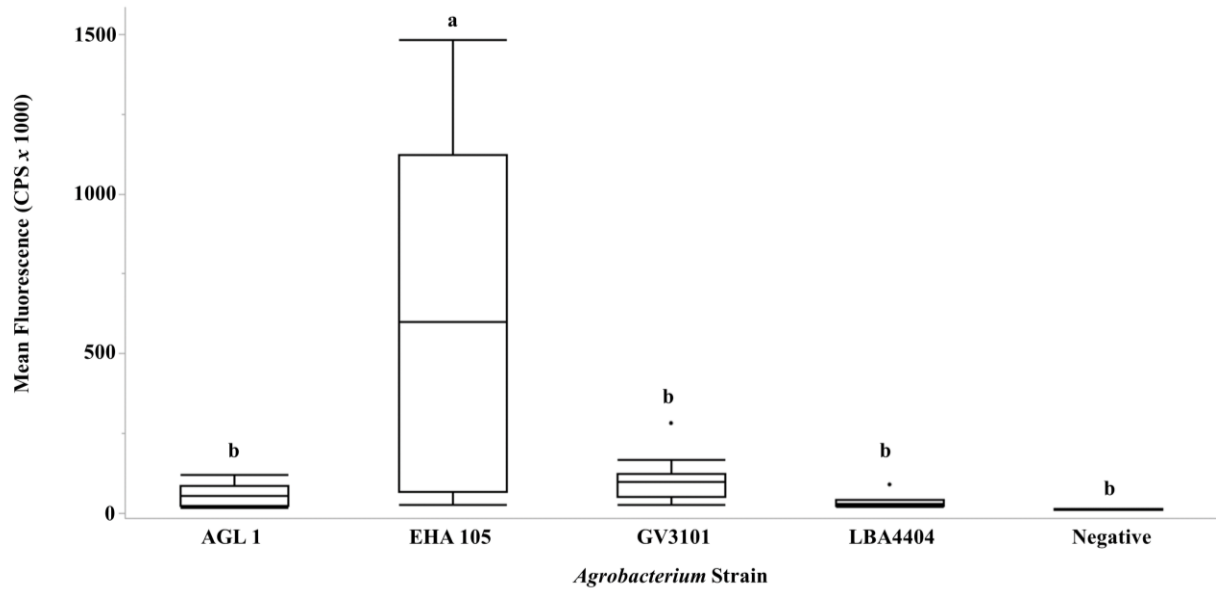


Fig. 7 *Agrobacterium* strain screen. Fluorescence spectroscopy of line ‘TN16-5004’ infiltrated as described in Fig. 2 with *Agrobacterium* strains EHA 105, LBA4404, GV3101 and AGL1 harboring Gmubi:tRFP:OCS. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, Tukey’s HSD.

Fig. 8 VC stage soybean line ‘V17-0799DT’ co-infiltrated as described in Fig. 2 using *Agrobacterium* strain harboring Gmubi:tRFP:OCS and Gmubi:mGFP5-ER:OCS at a 1:1 ratio. **a** Compound microscopy of co-infiltrated plants. Scale bar = 500 μ m. **b** Compound microscopy of negative control. Excitation for tRFP 540 nm, emission measured from 560 nm-580 nm. Excitation for mGFP5-ER 480nm, emission measured from 500 nm-520 nm **c** Confocal microscopy of co-infiltrated leaves (pseudo coloration). Scale bar =50 μ m. Excitation for tRFP 553 nm and detected at peak emission of 574 nm. Excitation for mGFP5-ER at 490 nm and detected at peak emission of 509 nm. **e** Fluorescence spectroscopy of co-infiltrated plants. Excitation for tRFP 540 nm, emission measured from 560 nm-580 nm. Excitation for mGFP5-ER 480 nm, emission measured from 500 nm-520 nm. Error bars represent standard deviation. $P < 0.05$, $n = 18$, one-way ANOVA, T-test. **f** qRT-PCR of lines ‘TN16-5004’ and ‘V170799DT’ for transcript abundance of tRFP and mGFP5-ER relative to soybean polyubiquitin (Gmubi3). Plants infiltrated with a Gmubi:RUBY:OCS construct as well as wild type plants were used as negative controls. Line ‘TN16-5004’ showed a relative expression level of tRFP and mGFP5-ER of 7.8% and 6%, respectively. Line ‘V17-0799DT’ showed a relative expression level of tRFP and mGFP5-ER of 20.8% and 17%, respectively. Error bars represent standard error of the mean. $P < 0.05$, $n = 24$, one-way ANOVA.

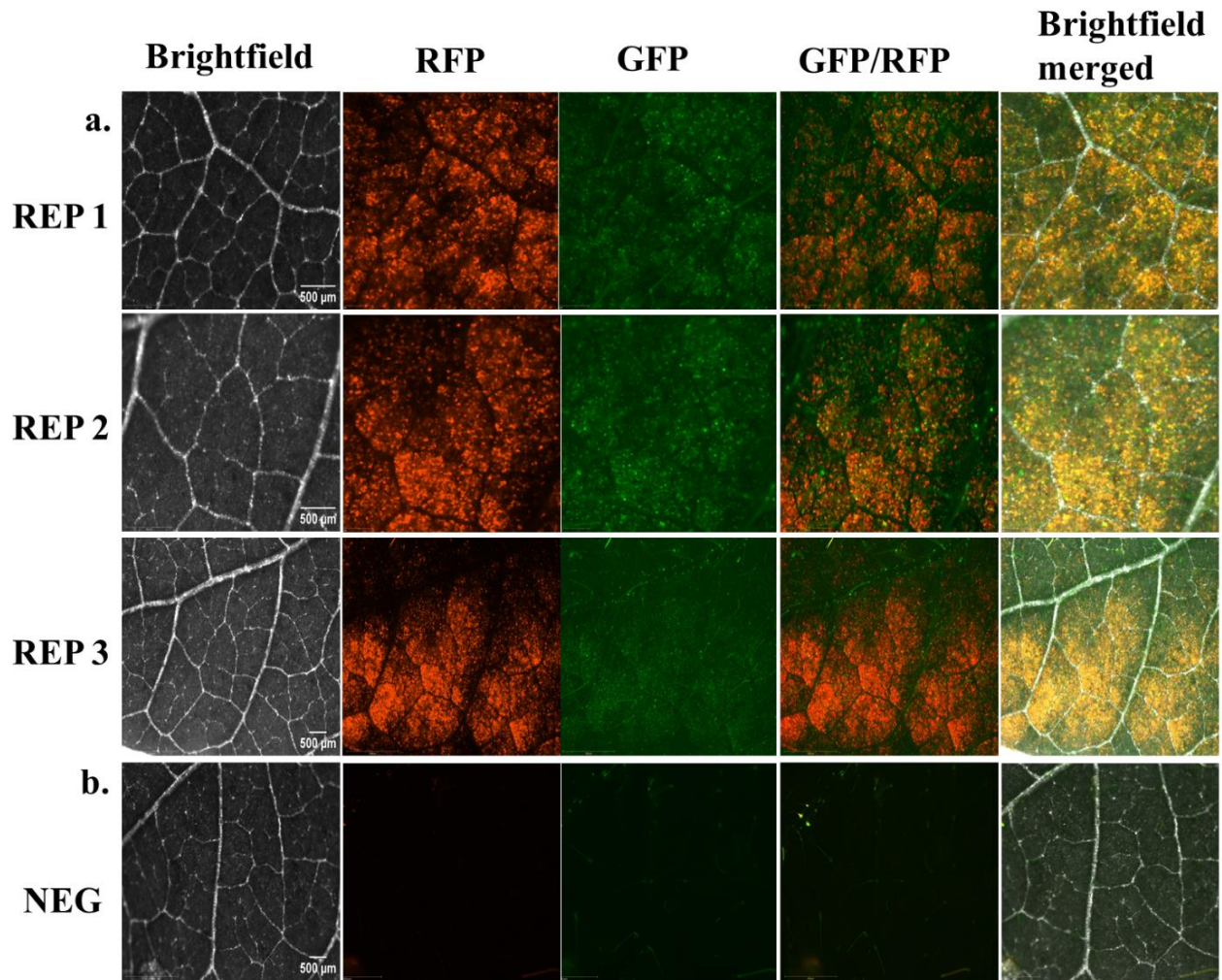


Fig. 8 continued.

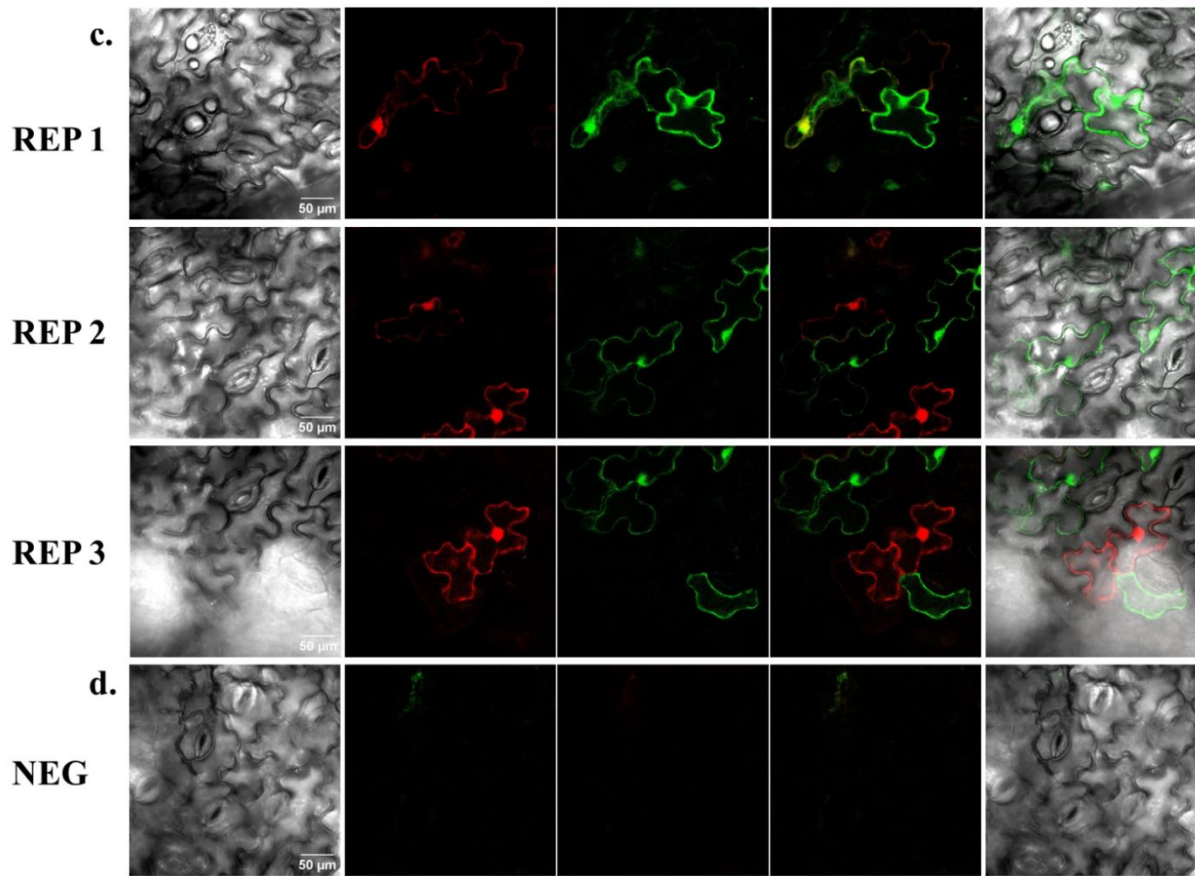


Fig. 8 continued.

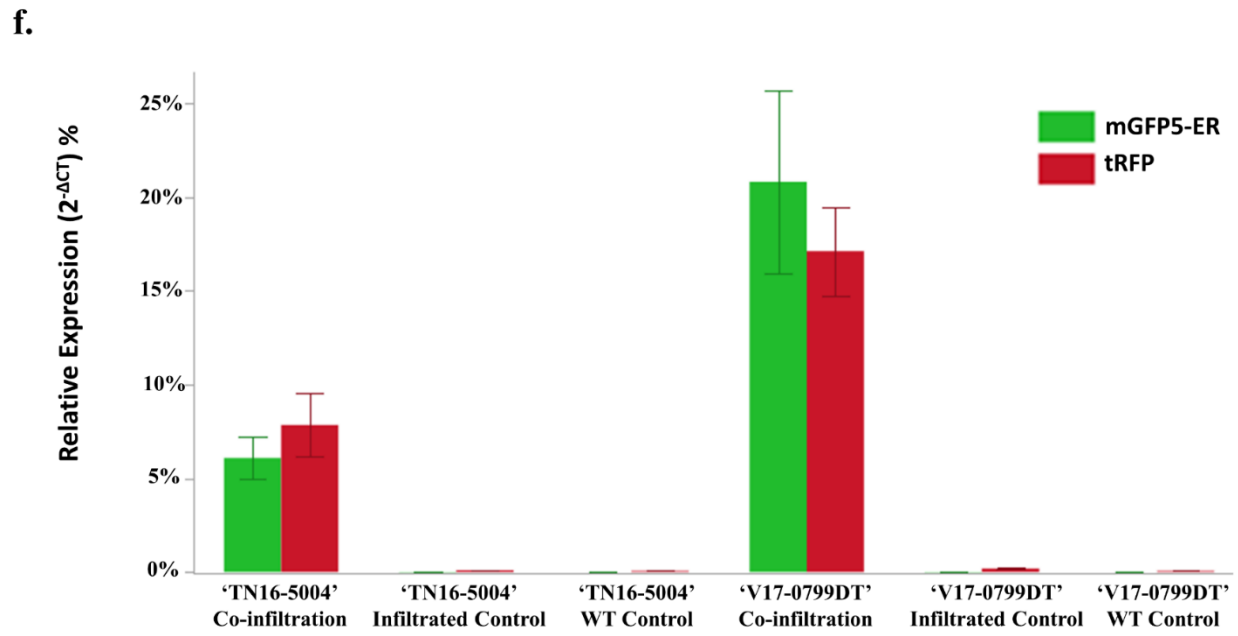
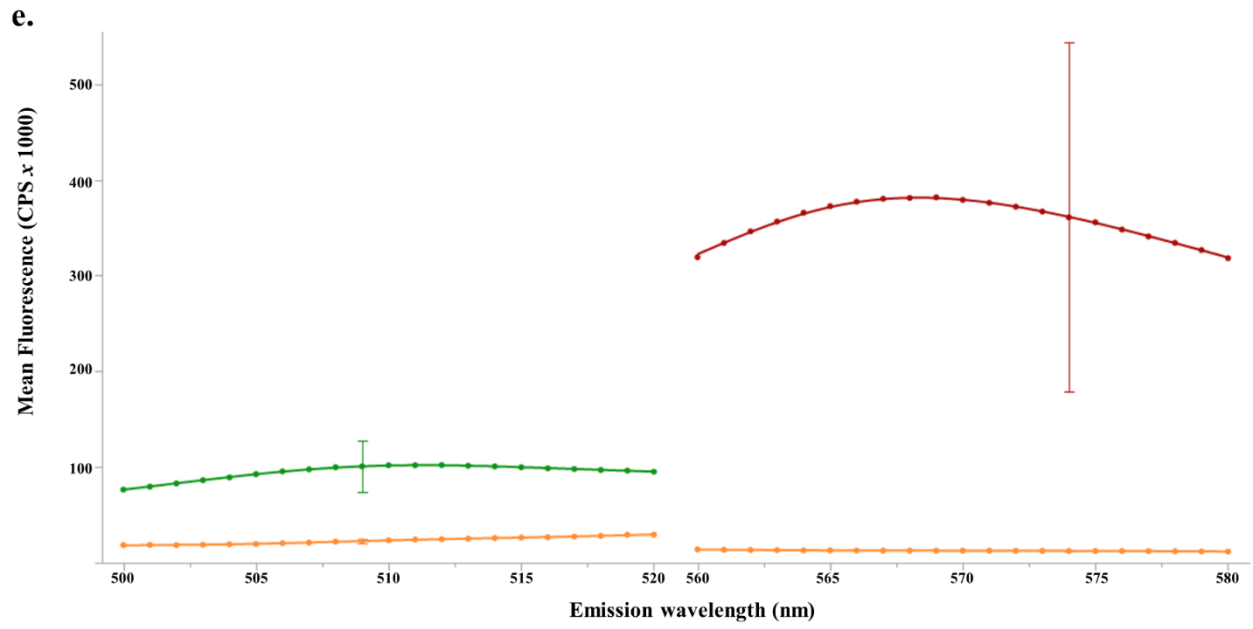


Fig. 8 continued.

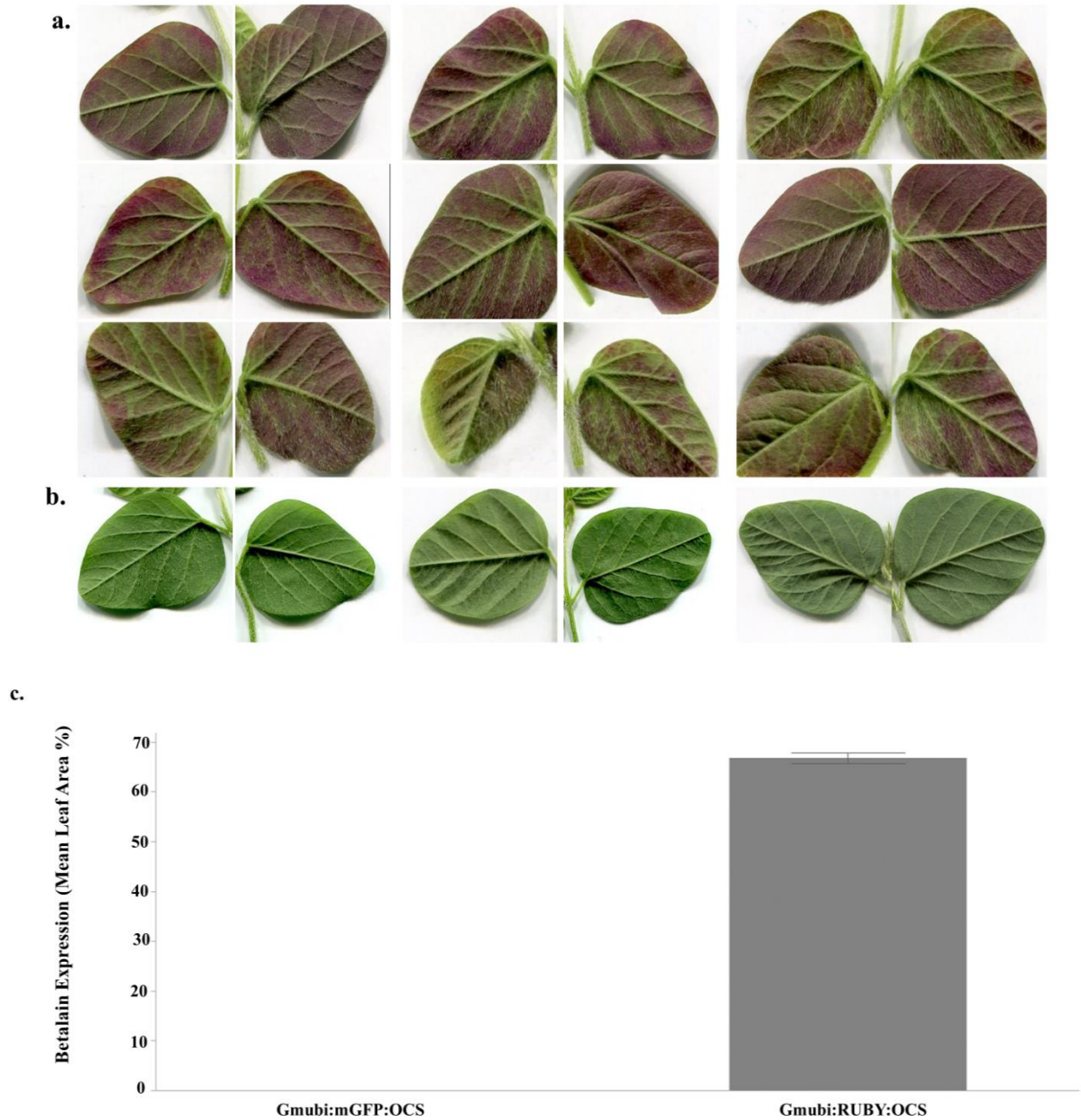


Fig. 9 VC stage soybean line ‘V17-0799DT’ infiltrated as described in Fig. 2 using *Agrobacterium* strain EHA 105 harboring Gmubi:RUBY:OCS. **a** Images showing infiltrated soybean leaves transiently expressing betalain. **b** Negative control infiltrated with *Agrobacterium* strain EHA 105 harboring Gmubi:mGFP5-ER:OCS. **c** Total leaf area showing betalain pigmentation vs. negative control. Error bars represent standard deviation. $P < 0.05$, $n = 18$, Student’s T-test.



Fig. S1 a Soybean line ‘V17-0799DT’ infiltrated after sonication 2 days post infiltration.
b Soybean line ‘V17-0799DT’ infiltrated without sonication and 0.01% v/v Silwet L-77 2 days post infiltration.

a.

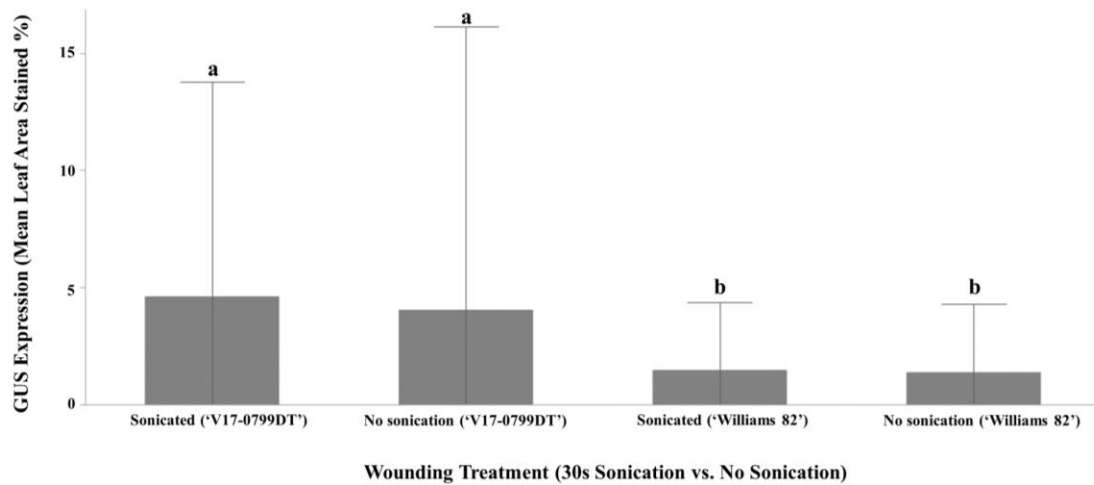
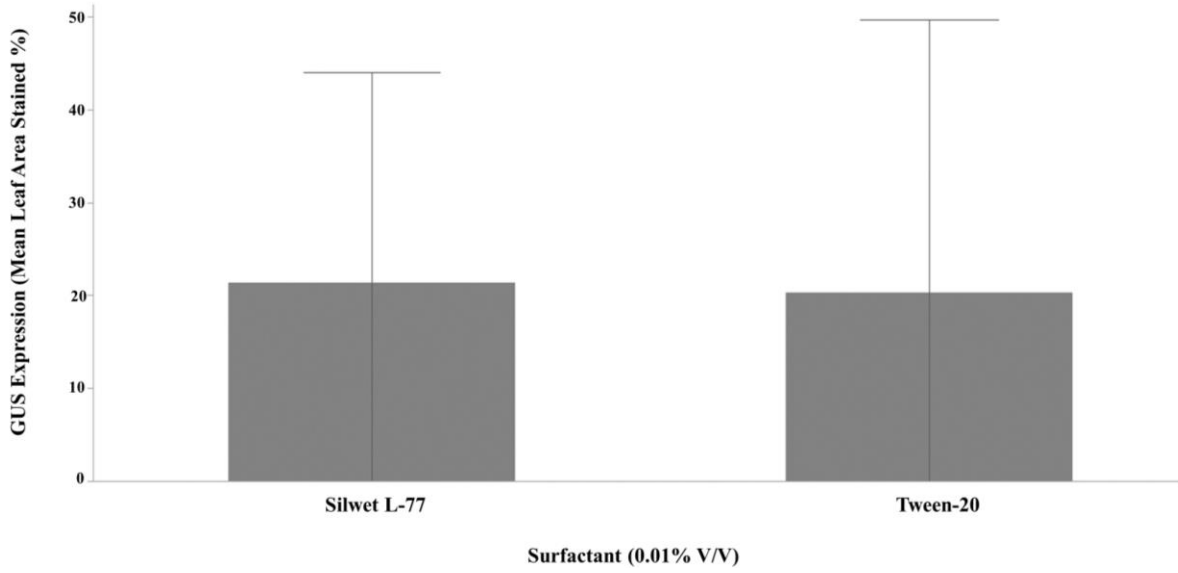


Fig. S2 Wounding treatments and surfactant test. **a** Soybean lines ‘Williams 82’ and ‘V17-0799DT’ subjected to infiltration with or without sonication using the method from Fig 2. No significant GUS expression difference between sonication and infiltration using surfactant alone was found. $P < 0.005$, $n = 18$, Student’s T-test. **b** Comparison of surfactants Silwet L-77 and Tween-20 using soybean line ‘V17-0799DT’ subjected to one five-minute round of vacuum or 2-4 rounds, respectively. $P > 0.05$, $n = 18$, Student’s T-test. **c** Comparison of differing concentrations of Silwet L-77 in SIB. A concentration of 0.01% v/v was found to be optimal. **d e f** Shows examples of soybean line ‘V17-0799DT’ after two 5-minute rounds of vacuum and release at -84.65 kPa with no surfactant, 0.01% v/v Tween-20, and one 5-minute round with Silwet L-77, respectively. $P < 0.005$, $n = 18$, one-way ANOVA.

b.



c.

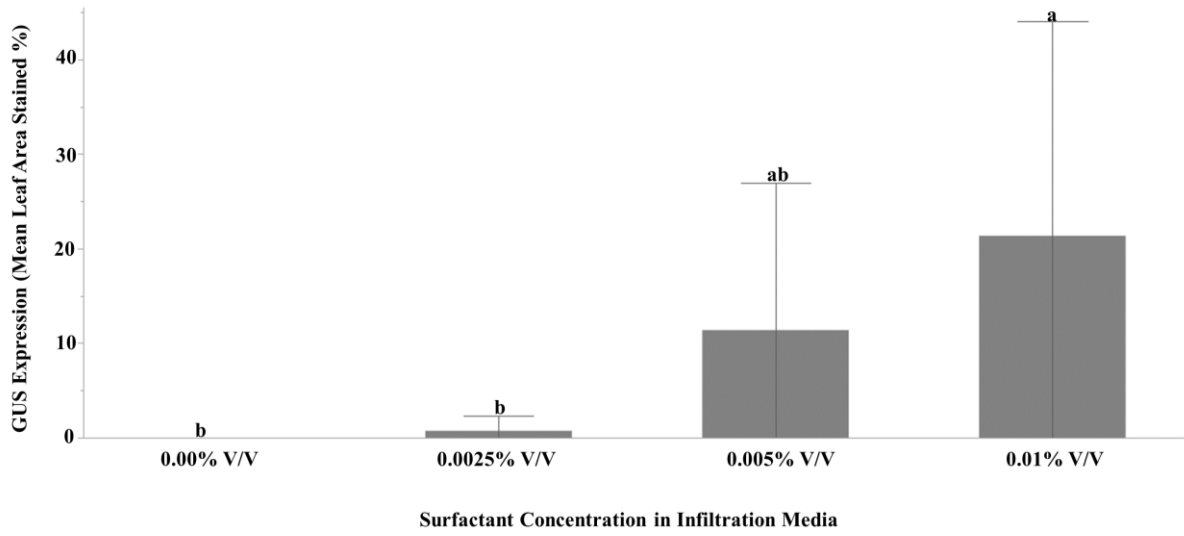


Fig. S2 continued.

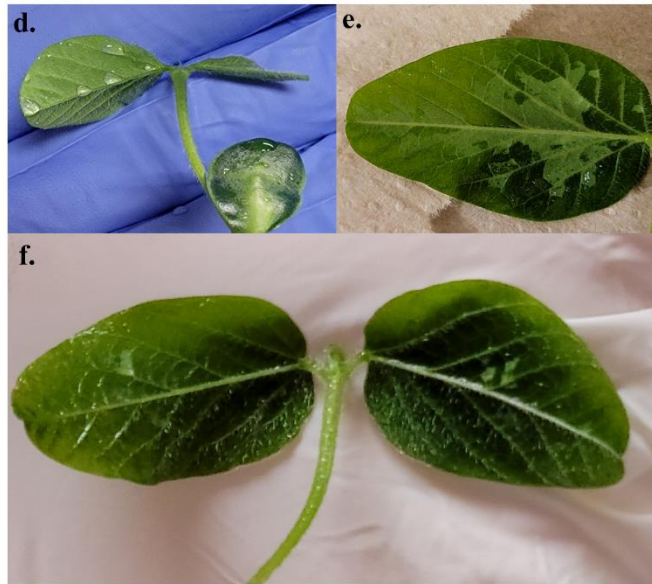


Fig. S2 continued.

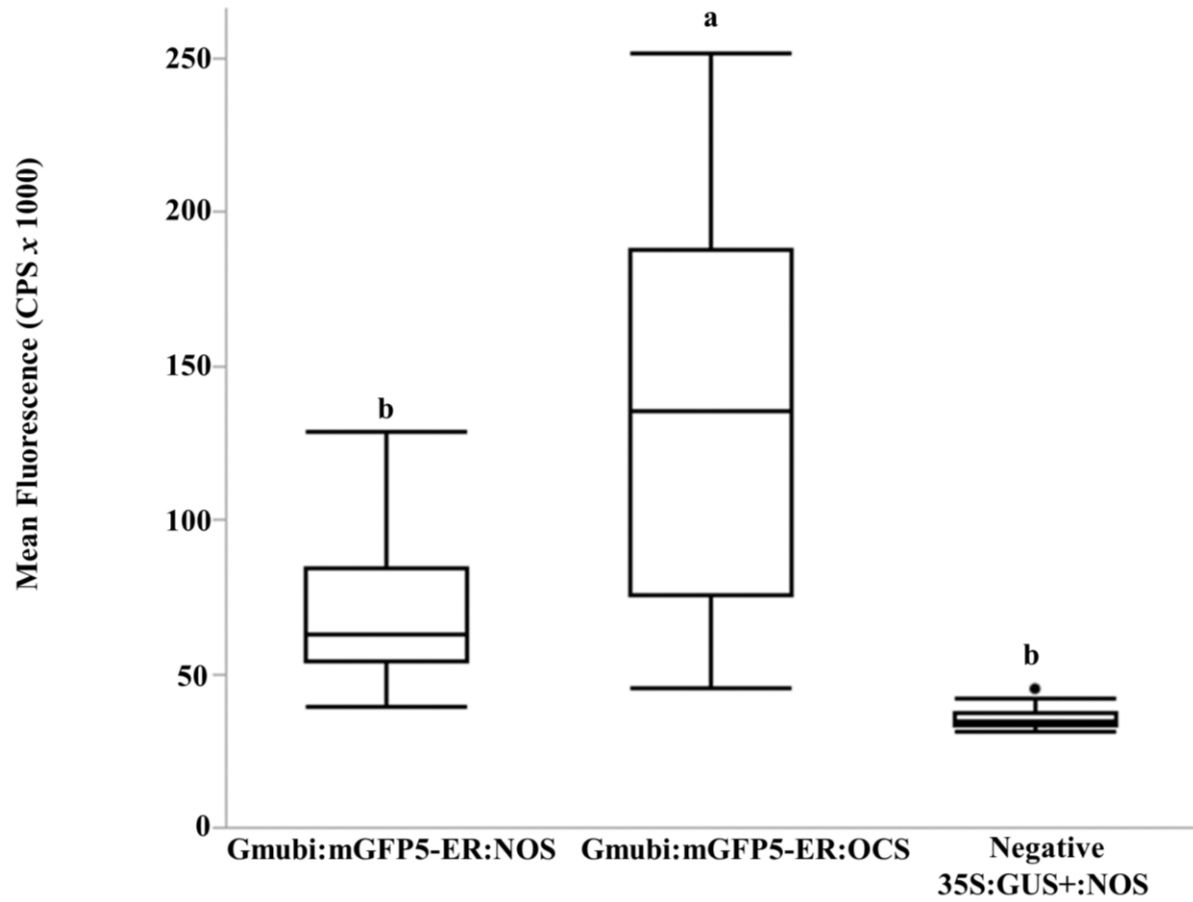


Fig. S3 Expression differences between line 'V17-0799DT' infiltrated with Gmubi:mGFP5-ER:NOS and Gmubi:mGFP5-ER:OCS

Table 1 Soybean genotypes tested. Most of germplasm tested were breeding lines developed at Virginia Tech (those beginning with “V”) and the University of Tennessee (those beginning with “T”).

Soybean line	Maturity group	Notes
5002T	4	
DB0638-70	4	
DT97-4290	4	Not tested in Fig. 3 (seed became available at later date)
‘Ellis’	4	
K15-1283	4	
K16-1114	4	Not tested in Fig. 3 (poor germination)
K16-1540	4	
KS4117NS	4	
KS4919N	4	
Peking PI 297543	Unknown	Not tested Fig. 3 (poor germination)
TN17-4412	4	
TN09-008	4	
TN11-5102	4	
TN14-5021	4	
TN15-4009	4	
TN15-4307	4	
TN15-5007	4	
TN16-5004	4	
TN16-5027	4	
TN17-4414	4	
TN17-4474	4	
V15-0611	4	
V16-0248DI	4	
V16-0293	4	
V17-0451	4	Not tested Fig. 3 (seed became available at later date)
V17-0460	4	
V17-0799DT	4	
‘Williams 82’	3	

Table 2 Description of variations of infiltration procedure as it relates to the infiltration medium suspension, surfactant, sonication, and time of vacuum exposure. Throughout the study, the infiltration medium, as well as antioxidant additives, baring variations in the *Agrobacterium* strain and/or its culturing, was consistently maintained. The initial genotype screen (Fig. 1) was conducted with sonication treatments beforehand, Tween-20 as a surfactant and two rounds of 30-minute vacuum exposure. Once we established Silwet L-77 as the surfactant of choice and ruled out sonication as a useful treatment (Fig. S1 & S2) the remainder of optimization experiments were performed without a wounding treatment, using Silwet-L77 as the surfactant and a single round of 5-minute vacuum exposure.

Agroinfiltration Medium	Composition	Additives
Soybean infiltration buffer (SIB)	10 mM MES, 10 mM MgCl ₂ ·6H ₂ O, 1L DI water at pH: 5.4	200 μM acetosyringone, 400 mg/L L-cysteine, 78 mg/L dithiothreitol (DTT), 0.01% v/v Tween-20 or 0.01% v/v Silwet L-77
AB:MES <i>Agrobacterium</i> induction media Cody et al. 2023	Per 1L 3 g (K ₂ HPO ₄), 1 g Na ₂ HPO ₄ , 1 g NH ₄ Cl, 0.31 g MgSO ₄ ·7H ₂ O, 10.66 g MES, 20 g glucose, 1 ml 2M KCl, 100 μl 1 M CaCl ₂ , 1 ml 10 mM FeSO ₄ stock pH 5.7	200 μM acetosyringone 50 mg/L kanamycin
Yeast enhanced peptone (YEP)	5 g/L NaCl, 10 g/L peptone and 10 g/L yeast extract	50 mg/L kanamycin 50 mg/L rifampicin

Table 3 Plant developmental stage/leaf ages and morphologies tested. Soybean leaves were infiltrated in the vegetative emergence (VE) stage and vegetative compound (VC) stage. Both VE and VC are the first true leaves of a germinated soybean plant with VE being the leaves when unexpanded immediately after germination and VC being defined as fully unfurled and expanded first true leaves. Soybean leaves were also infiltrated in the vegetative 1st-trifoliate leaf stage. Both the first unifoliate leaves and first trifoliate leaves were tested.

Developmental stage	Leaf type infiltrated	Description	Approximate days post imbibition
VE	First unifoliate leaves	First unifoliate leaves have emerged but are not fully unfurled or expanded.	~7-10 days
VC	First unifoliate leaves	First unifoliate leaves have emerged and fully unfurled and expanded. The meristem of the first set of trifoliate leaves is small but visible	~11-13 days
V1	First unifoliate leaves & first trifoliate leaves	First trifoliate leaves have emerged and fully unfurled	~15-20 days

Table 4 *Agrobacterium* strains tested and chromosomal backgrounds.

<i>Agrobacterium</i> strain	Chromosomal background	Reference
EHA 105	C58	(Hood et al. 1993)
LBA4404	ACH5	(Ooms et al. 1982)
GV3101	C58	(Koncz & Schell 1986)
AGL 1	C58	(Lazo et al. 1991)

Table 5 Transcript analysis primer sets. Target genes and the respective forward and reverse primers used for qRT-PCR experiments.

Gene	Primer sequence (5' to 3')
Gmubi3 (reference)	F: GTGTAATGTTGGATGTGTTCCC R: ACACAATTGAGTTCAACACAAACCG
tRFP	F: ACCACCACTTCAAGTGCACA R: CGAAAGCGAAAGGGAGAGGT
mGFP5-ER	F: CCTGTTCCATGGCCAACACT R: GCATGGCGCTCTTGAAGAAG

Supplementary Note 1. ImageJ macro code for isolation of total leaf area

Adapted from Viskol Inc. <https://visikol.com/blog/2019/02/16/building-an-imagej-macro-for-batch-processing-of-images-from-imaging-well-plates/>.

```
// Macro to measure Area, Intensity, Perimeter, and Shape of directory of images
run("Clear Results"); // clear the results table of any previous measurements
// The next line prevents ImageJ from showing the processing steps during
// processing of a large number of images, speeding up the macro
setBatchMode(true);
// Show the user a dialog to select a directory of images
inputDirectory = getDirectory("Choose a Directory of Images");
// Get the list of files from that directory
// NOTE: if there are non-image files in this directory, it may cause the macro to crash
fileList = getFileList(inputDirectory);
for (i = 0; i < fileList.length; i++)
{
    processImage(fileList[i]);
}
updateResults(); // Update the results table so it shows the filenames
setBatchMode(false); // Now disable BatchMode since we are finished
// Show a dialog to allow user to save the results file
outputFile = File.openDialog("Save results file");
// Save the results data
saveAs("results",outputFile);
function processImage(imageFile)
{
    // store the number of results before executing the commands, so we
    // can add the filename just to the new results
    prevNumResults = nResults;
```

```

open(imageFile);
// Get the filename from the title of the image that's open for adding
to the results table

// We do this instead of using the imageFile parameter so that the
directory path is not included on the table

filename = getTitle();
run("8-bit");
setAutoThreshold("Huang");
run("Set Measurements...", "area mean perimeter shape limit
redirect=None decimal=4");
// You should adjust the size input according to the sizes of nuclei in
your images

run("Analyze Particles...", "size=1-Infinity circularity=0-1.00
display");

// Now loop through each of the new results, and add the filename to
the "Filename" column

for (row = prevNumResults; row < nResults; row++)
{
    setResult("Filename", row, filename);
}

close("*"); // Closes all images
}

```

Supplementary Note 2. ImageJ macro code for isolation of leaf area stained

```
run("Clear Results"); // clear the results table of any previous measurements
setBatchMode(true);
inputDirectory = getDirectory("Choose a Directory of Images");
fileList = getFileList(inputDirectory);
for (i = 0; i < fileList.length; i++)
{
    processImage(fileList[i]);
}
updateResults();
setBatchMode(false);
outputFile = File.openDialog("Save results file");
saveAs("results",outputFile);
function processImage(imageFile)
{
    prevNumResults = nResults;
    open(imageFile);
    filename = getTitle();
    run("Color Threshold...");
    min=newArray(3);
    max=newArray(3);
    filter=newArray(3);
    a=getTitle();
    run("HSB Stack");
    run("Convert Stack to Images");
    selectWindow("Hue");
    rename("0");
```

```

selectWindow("Saturation");
rename("1");
selectWindow("Brightness");
rename("2");
min[0]=90;
max[0]=255;
filter[0]="pass";
min[1]=20;
max[1]=255;
filter[1]="pass";
min[2]=0;
max[2]=240;
filter[2]="pass";
for (i=0;i<3;i++){
selectWindow(""+i);
setThreshold(min[i], max[i]);
run("Convert to Mask");
if (filter[i]=="stop") run("Invert");
}
imageCalculator("AND create", "0","1");
imageCalculator("AND create", "Result of 0","2");
for (i=0;i<3;i++){
selectWindow(""+i);
close();
}
selectWindow("Result of 0");
close();

```



```
selectWindow("Result of Result of 0");
rename(a);
///  
run("Copy");
///  
newImage("Untitled", "RGB white", 600, 800, 1);
///  
run("Paste");
run("Make Binary");
run("Create Selection");
run("Measure");
for (row = prevNumResults; row < nResults; row++)
    {
        setResult("Filename", row, filename);
    }

close("*"); // Closes all images
}
```

Vita

Bryce N. Trull was born in Plantation, Florida. He received a B.S in Plant Science from the University of Tennessee in 2022. From 2021-present Bryce is pursuing a Master of Science degree in plant molecular genetics in the Stewart lab through a newly created 5-year MS/BS program which he helped develop for the plant science program and of which he is the first participant. His research is focused on developing an agroinfiltration method for soybean.