Isolation of Novel *Agrobacterium* and Transient Expression Assays in Soybean (*Glycine max*) and Sunflower (*Helianthus annuus*)

THESIS

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Abstract

Many laboratories routinely use *Agrobacterium* for the generation of transgenic plants; however, only a few disarmed bacterial strains are widely available and none of these strains were isolated and selected based on evaluation of transformation efficiency in target species. For this research, new *Arobacterium* strains were isolated from crown galls of various plants in Ohio and from rhizospheric soil throughout the Midwest US. These wild-type strains were isolated by plating gall and soil extracts on a semi-selective medium and screening the isolates for the presence of *virG* using PCR. The efficiency of plant transformation was evaluated by transforming hypocotyl and cotyledonary tissues of sunflower and soybean seedlings and in proliferative embryogenic tissue of soybean and then quantifying GFP expression. In sunflower, seedling cotyledonary tissue was not responsive to any of the strains tested, however hypocotyl tissues were very responsive with the highest transformation rates obtained with EHA105. With sunflower hypocotyl tissues transformed with disarmed strain EHA105, greater than 75% of the transformed cells were located in the vascular tissues. In soybean seedling tissues, tissue-specific transformation was not observed with any strain as transformed cells were evenly distributed throughout target tissue. With soybean hypocotyl, cotyledon, and embryogenic tissues, a single strain from a soybean field in North Dakota gave 5-10x higher transformation rates than EHA105, while a strain from Ohio soil gave 3-5x higher rates than EHA105.

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Chapter 1: Literature Review

Gene Introduction Overview

Plant transformation is a relatively young field, which has already revolutionized modern agriculture by contributing to many agronomic crops including soybean (Padgette et al. 1995) and maize (Koziel et al. 1993) which are produced in the developed world. For crop improvement using classical plant breeding methods, variations which are either discovered or induced, are screened and phenotypes of interest are selected for trait stabilization. While classical breeding utilizes genes from compatible species, transgenics allow for the introduction of genes from any source into the germplasm. Introduction of novel genes into plants continues to drive advancements in basic biological research and crop improvement (Finer 2010). Many of the insights derived from plant biotechnology research over the past three decades would not have been possible without the combined abilities to introduce and regulate specific transgenes. For the introduction of foreign genetic material into plants there are two main methods, biologically-mediated methods most commonly use disarmed *Agrobacterium* (Hellens et al. 2000) for introduction of genes of interest into a plant genome (Horsch et al. 1985), while the most commonly used direct DNA introduction methods utilize DNA

precipitated on microprojectiles which are accelerated into target cells, resulting in DNA integration into the chromosome (Sanford 1988; Finer and McMullen 1991). Both of these methods share a reliance on the techniques of plant tissue culture and the use of selectable markers to generate transformed fertile whole plants from single cells (Finer 2010).

Transient transformation refers to expression of the introduced DNA soon after the gene is introduced while stable transformation results from the stable introduction of DNA following integration of the DNA into the genome. For long-term evaluation of transgenes, stable gene introductions are required and this seems to be most efficiently accomplished with the use of the transformation vector, *Agrobacterium*, followed by tissue culture and selection for transformed tissues (Gelvin 2003). The effort associated with introducing a gene into many plants remains high, despite major advancements in plant transformation. For many plants, transformation rates are consistent but inefficient and more efficient methods are still needed.

Agrobacterium

Agrobacterium are near ubiquitous Gram-negative bacteria found primarily in rich moist soils in close association with plants. They are rhizospheric bacteria which may live saprophytically (Shams et al. 2012) in diverse environments yet are also pathogenic to many plant species causing both hairy root and crown gall diseases.

Host/saprophyte interactions can be commensal, having no negative impact to the plant. Because of the extreme complexity of *Agrobacterium* biology and its wide-ranging adaptations, it is often used as a model organism in microbiological research. Most useful is its capacity for gene introduction and T-DNA integration into a host genome (Zupan and Zambryski 1995). *Agrobacterium* is able to orchestrate the transfer of a single strand of DNA, chaperoned by the virulence protein complex from its own extra-chromosomal plasmid to target the host nucleus for integration (Escobar and Dandekar 2003). Naturally, *Agrobacterium* infects wounded plants in the soil or near the soil-air interface (Braun 1952), causing gall formation on many plant species (DeCleene and DeLay 1976) including most dicotyledonous and a small number of monocotyledonous species (Pitzschke and Hirt 2010). This unique case of inter-kingdom horizontal gene transfer allows for the processing and transfer to plants of bacterial DNA originating from its tumor-inducing (Ti) or root-inducing (Ri) plasmid.

Taxonomy

In the past, species taxonomy for *Agrobacterium* was based on the pathogenic characteristics of a given isolate. This type of classification was however soon abandoned once an understanding of the function, transferability, and curability of the "tumor inducing principle" was available. Crown gall disease resulted from the presence of the *Agrobacterium* Ti plasmid (Zupan and Zambryski 1995; Van Larebeke et al. 1974). Interestingly, *Agrobacterium* was one of the very earliest bacteria isolated and later

described (Smith and Townsend 1907), long before there was a real understanding of the pathogenic nature of *Agrobacterium*.

Because of a coincidental superficial similarity between plant tumors induced by *Agrobacterium* and human tumors, the disease was of early interest to the fledgling field of oncology and an isolate was given the name, *Bacterium tumefaciens* (Smith and Townsend 1907). Non-virulent strains of *Agrobacterium* were isolated from soil even earlier and given the name *Bacillus radiobacter* based upon their growth habit, producing a star-like shape under given conditions (Hofer 1941). It took thirty years before the ability to cure the bacteria of its plasmid and its ability to transfer the plasmid through conjugation to bacteria free of a plasmid was demonstrated (Hooykaas and Schilperoort 1992). The ability to cure a bacterium of its virulence plasmid made clear that strain pathogenicity was unrelated to strain classification and the trait of pathogenicity could not be used to develop a reliable taxonomy. In place of pathogenicity as a defining characteristic, biochemical assays were developed to distinguish the specialized metabolic profiles formed by the different biovars of *Agrobacterium*. The three *Agrobacterium* biovars are classified by their significant DNA and not by their plasmid based transferred DNA (T-DNA).

The biovars are extremely heterogeneous for a single bacterial species and are properly termed a species complex. Biovar 2 is relatively homogenous (Mougel et al. 2001), and is more similar to a *Rhizobium* species than to biovar 1, thus it has been

suggested that biovar 2 be reclassified as *R. rhizogenes* (Costechareyre et al. 2010). Biovar 3 contains the grape vine pathogen, *A. vitis* and has not been utilized in plant transformation.

Bacteria Isolation

Different protocols for isolation of novel *Agrobacterium* utilize semi-selective media, including 1A, 2E (Mougel et al. 2001), and Schroth's (Schroth et al. 1965; Brisbane and Kerr 1983), which contain anti-fungal (cycloheximide) and anti-bacterial compounds. Some selective media contain K_2TeO_3 at levels which is toxic for most microbes and Na_2SeO_3 as a selective agent (Mougel et al. 2001).

Biovar 1, *A. tumefaciens* and *A. rubi*, may be isolated with 1A medium, which uses arabitol as a carbon source and is amended with tellurite. Biovar 2, *A. rhizogenes* may be isolated with the medium 2E, utilizing erythritol and with four times as much tellurite compared to 1A. Biovar 3, *A. vitis* may be isolated on medium 3DG, which uses sodium L-tartrate as a carbon source (Brisbane and Kerr 1983).

Plant Transformation

Since tobacco was the first plant to be genetically transformed in 1984 (de Block et al. 1984) gene transfer via *Agrobacterium* has since been used successfully in over 100

plant species including species from most major families. For much of the history of plant transformation, the regeneration of whole transgenic plants from single cells was the limiting factor for many plants (Ellis and Croy 1993), but plant regeneration from transgenic cells is now routine for many if not most plants.

Further refinement of transformation is needed in certain plants, which remain recalcitrant to *Agrobacterium*-mediated transformation. Crops containing a transgene now dominate, making up a large amount of agricultural land available in the US today (ERS/USDA 2012). This makes for an exciting time as new techniques are refined and applied to a wide variety of crops producing an ever greater number of characteristics.

Support for research in plant molecular biology has been sustained mainly because of the promise the technology offers in improving commercial crops by both potentially producing certain traits that would be slow to stabilize using traditional breeding, and by generating unique phenotypes. The commercial success of transgenic crops was seen early with the release of the first Roundup Ready soybean line (Hinchee et al. 1993) from Monsanto.

For basic research done in the model plant species, *Arabidopsis*, exposure of gametic cells in the flowers *in planta* to *Agrobacterium* is sufficient to produce stable transgenics (Clough and Bent 1998; Chang et al. 1994; Hooykaas and Schilperoort 1992). Unfortunately techniques developed for a model plant are not always applicable to

species of agronomic interest (Collins and Shepherd 1996) and many attempts using floral dip transformation with other species have been unsuccessful (Walden and Wingender 1995). Meristematic cells, retained or induced, are of utmost interest as potential target tissues for gene introduction and whole plant regeneration.

It was once thought that difficulties with *Agrobacterium*–mediated transformation would be insurmountable in some plants and only certain dicots were responsive to transformation. With the expansion of knowledge of the T-DNA transfer process, transformation techniques have now been adapted to most plants. At one time, soybean was regarded as completely recalcitrant to transformation but it is now regularly transformed using *Agrobacterium*-mediated transformation and particle bombardment (Hadi et al. 1996; Zupan and Zambryski 1995; Birch and Franks 1991; Birch 1997).

To reduce the dependency on tissue culture, meristematic cells have been targeted using either particle bombardment (Christou 1992) or *Agrobacterium*-mediated transformation. Transformation rates for soybean for example, can also be quite low, ranging from 0.05% to 15% (Liu et al. 2004) of the target explants that produce transgenic plants. Successful gene transfer requires the development of a reliable source of target tissue and the establishment of conditions for gene transfer.

Agrobacterium-mediated transformation can be improved by manipulating bacterial growth parameters prior to inoculation, including growth phase, density, and

pre-treatment inducers used such as wounded plant extracts like acetosyringone (AS) (Stachel et al. 1985; Melchers et al. 1989) and various sugars, as well as the pH of the medium used. During post co-culture, an appropriate antibiotic necessary to control bacterial overgrowth, reducing agents and antioxidants are added to a medium in order to minimize the hypersensitive response and limit oxidation of plant tissues (Finer 2010).

There are three main selection techniques used for identifying transformed tissues. Reporter genes like the green fluorescent protein (GFP) allow for visual screening and selection of transformed cells and tissues (Chalfie et al. 1994). Herbicide resistance genes are also used (Padgette et al. 1995), where only transformed tissues survive. Finally, PCR may be used for identification of the plant cells containing the transgene. Reliable selection protocols exist for many species and tissues.

Research Objectives and Applications

With nearly $40 billion in annual US production, accounting for almost 95% of the soybean grown in the US, transgenic soybean continues to be one of our most important agricultural products (ERS/USDA 2012). The wide variation seen in *Agrobacterium* spp. transformation capabilities (Hellens et al. 2000) and the diverse reactions observed in inoculated plants suggest that transformation efficiency depends on a host pathogen interaction (Byrne et al. 1987; Hobbs et al. 1989). Many of the characterized virulent strains of *Agrobacterium* were isolated from the crown galls of

infected plants. The common laboratory strain C58 was isolated from a cherry tree in 1958 (Slater et al. 2012).

Even though *Agrobacterium*-mediated transformation has matured into a core plant biotechnology, there are still only a small number of well characterized and disarmed strains (Hellens et al. 2000) actively in use in plant transformation research (Hood et al. 1993). Transformation efficiency is often influenced by the bacterial strain used (Wroblewski et al. 2005), it would therefore be beneficial to have a more efficient strain for soybean transformation. Unfortunately, soybean does not form galls when infected with *Agrobacterium* in the field so it is not possible to harvest novel strains from soybean galls in the field.

The objectives of this work were to 1) isolate novel soybean selected *Agrobacterium* strains from soil and 2) characterize the new strains through rapid characterization of GFP expression in various soybean tissues. We have aimed to identify a new *Agrobacterium* strain that may be more useful for soybean transformation. Our approach relied on the combination of isolation of strains from field soil and then testing transformation efficiency directly with the wild-type strains on sunflower and soybean tissues.

Chapter 2: Isolation and Characterization of Novel *Agrobacterium* Strains for Soybean and Sunflower Transformation

INTRODUCTION

The genus *Agrobacterium* contains phytopathogenic bacteria that can infect many dicotyledonous species causing both hairy root and crown gall diseases (Matthysse 2006; Binns and Thomashow 1988; Nilsson and Olsson 1997). Virulent bacteria harbor the tumor-inducing (Ti) or hairy root-inducing (Ri) plasmid needed to produce the characteristic crown galls or hairy roots of the pathogens' namesake (Van Larebeke et al. 1974; Shams et al. 2012; Gelvin 2003; Chilton et al. 1977). This pathogen induced heritable change to plant cells is a unique case of inter-kingdom horizontal gene transfer, allowing bacterial DNA to be inserted and stably integrated into the host genome making *Agrobacterium* useful for gene introduction in many plant species (Gelvin 2012; Matthysse 2006; Finer 2010).

Because of its reliability and ease of use, *Agrobacterium*-mediated transformation has become the method of choice for production of transgenic plants (Jackson et al. 2013; Finer 2010). For transformation of the model plant, *Arabidopsis*, plants are simply dipped

in a bacterial suspension (floral dip), leading to the production of transgenic seed following fertilization (Clough and Bent 1998; Zhang et al. 2006). Unfortunately these techniques developed for *Arabidopsis* are not always applicable to species of commercial interest and most attempts at applying floral dip to transformation of other species have been unsuccessful (Walden and Wingender 1995). Despite the ever increasing reliance on *Agrobacterium*-mediated gene introductions for the production of transgenic plants, most of the improvements in transformation have been limited to protocol optimization and identification of tissues and plants susceptible to *Agrobacterium*, with much of this work using only a few *Agrobacterium* strains largely originating from isolates of C58 and Ach5 chromosomal backgrounds (Zhu et al. 2003; Hellens et al. 2000).

Strains derived from Ach5 and C58 chromosomal backgrounds are widely used for transformation in a wide variety of species. Unfortunately neither of these strains is especially well suited for soybean transformation. There have been only limited efforts to isolate and develop novel *Agrobacterium* strains for transformation. Hwang et al. (2013) showed no or modest increases in gall formation and percent of explant expressing a marker gene using novel wild-type bacteria that were previously isolated but not well characterized (Wroblewski et al. 2005).

The constitutively-expressed bacterial proteins, VirA and VirG are associated with virulence and are able to lead to recognition of certain plant phenolics such as acetosyringone (AS) which are produced by wounded tissues and trigger bacterial

transcription of induced virulence (*vir*) gene products (Stachel et al. 1985; Shimoda et al. 1990; Gelvin 2006; Spencer and Towers 1988). Increased expression of *vir* genes can be achieved by addition of these phenolic compounds and AS is now routinely included in co-cultivation media to enhance transformation (Godwin et al. 1991). The molecular basis for host range determination of *Agrobacterium* is still unknown, however *Agrobacterium* strains have exhibited host species specific transformation (Wroblewski et al. 2005) and Ti plasmid *vir* genes such as *virC* (Yanofsky and Nester 1986) have been shown to be instrumental in determining pathogenic range.

Agrobacterium *Strain Recovery*

A classical source for *Agrobacterium* strains has been the gall tissues of infected plants and the taxon has thus far been characterized primarily with these tumor-derived strains (Chilton et al. 1974; Bakhsh et al. 2014; Hellens et al. 2000). However, other sources that are less often utilized for isolation, including soil and fresh water, may yield an increased variety and possibly useful novel isolates of bacteria. Although *Agrobacterium* was one of the first bacteria to be studied, there was not a reliable way to isolate *Agrobacterium* from a complex environment like soil until the last decade. *Agrobacterium* can now be isolated using several semi-selective media including 1A, 2E (Mougel et al. 2001), and Schroth's (Schroth et al. 1965; Brisbane and Kerr 1983), which contain anti-fungal and anti-bacterial compounds. Some *Agrobacterium* can grow and proliferate in the presence of K_2TeO_3 at levels that are toxic to most microbes. In

addition, *Agrobacterium* has the rare ability to reduce the salt, Na_2SeO_3. K_2TeO_3 and Na_2SeO_3 are both selective agents, which are common media addenda for *Agrobacterium* selection (Mougel et al. 2001).

Plant Transformation

Reducing agents may be included in co-cultivation media in order to minimize the effects of pathogen induced hypersensitive response (HR) which may lead to premature cell death, reducing transformation rates (Olhoft et al. 2003). Both dithiothreitol (DTT) and L-cysteine are common reducing agents used to minimize oxidation of target plant tissues, thus increasing survival rates of transformed tissues (Olhoft and Somers 2001). Techniques such as sonication assisted *Agrobacterium*-mediated transformation (SAAT) were developed to increase transformation rates in different species by creating micro-wounds in the target tissue, which allows for greater access of the bacteria to the target cells (Trick and Finer 1997). Most of the approaches for improving transformation rates in plants have involved manipulation of *Agrobacterium* (Finer 2010) or modification to the transformation conditions to enhance delivery (Trick and Finer 1997; Paz et al. 2006) or minimize the HR response (Olhoft et al. 2003).

Plant cells release a range of wounding response molecules, some of which bacteria can recognize and trigger pathogen virulence (Ditt et al. 2001). Conversely, host recognition of a pathogen may trigger a downstream response for defense, generally

leading to the death of infected and nearby cells, limiting the pathogen's spread. To attenuate the effect of the hypersensitive response of plant tissue to *Agrobacterium* infection and increase the survival rate of transformed cells, the sulfhydryl reducing agents L-cysteine and dithiothreitol (DTT) (Olhoft and Somers 2001) can be added to the media used to co-culture the bacteria with the plant tissue.

Unlike past efforts in increasing transformation rates by manipulation of the transformation environment, for this research, novel strains were isolated and assayed for their ability to transform soybean and sunflower tissues. Transformation assays were performed using hypocotyl and cotyledon explants from soybean and sunflower seedlings and with embryogenic suspension cultures of soybean. In sunflower, strain EHA105 gave the highest transformation rates and showed a preference for transformation of the vascular tissue in hypocotyl-derived explants, which was not observed with the new wild-type strains. In all three soybean tissues tested, a novel strain isolated from soil gave the highest transformation rates over all *Agrobacterium* isolates tested.

MATERIALS AND METHODS

Isolation of Agrobacterium *from Soil Samples*

Soil samples were collected from various locations across Ohio and the US. Soil samples (1 g) were suspended in 5 mL sterile water and the suspension vortexed at medium speed in a Thermo Fisher Scientific Vortex Genie 2 (Model G-560; Thermo Fisher Scientific; Waltham, MA) table top unit. Suspensions were serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, and 300 µL was spread aseptically on 1A semi-selective medium (Table 2.1) (Brisbane and Kerr 1983) in 100 x 15 mm Petri plates. Plates were incubated at 28°C for a minimum of 2 d, or until colonies of at least 2 mm in diameter were present.

Putative *Agrobacterium* colonies which appeared shiny and black (Figure 2.1) were picked with sterile pipette tips and the tips were swirled in 15 µL liquid Yeast Extract Peptone (YEP) medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) and then in 15 µL H_2O, each contained in separate 96 multiwell plates. Multiwell plates containing YEP were incubated for 6-8 h at room temperature and shaken at 150 RPM. A mixture of 0.5% (*w/v*) sodium azide and 2% (*v*/v) Triton X-100 was then placed into each well in a fume hood, to reach a final percentage of 0.5% sodium azide and 1% Triton X 100. The sodium azide was included as a lysis agent and Triton X-100 as a detergent to increase lysis activity.

Lysed bacteria were heated in the 96 well PCR reaction vessel in a BioRad Laboratories PCR machine (Model # T100; BioRad Laboratories, Hercules, CA) at 95°C for 10 min and then cooled to 10°C for 5 min. Samples were centrifuged at 6,000 x *g* for 30 min in a table top Sorvall Legend RT Refrigerated Centrifuge (Model # 75004377; Thermo Fisher Scientific). A 0.5 µL sample of supernatant was removed from each well, and touchdown PCR was run with the v*irG* primer set (Table 2.2) and GoTaq Green Master Mix (Product # M3001, Promega Corporation, Fitchburg, WI) using the following conditions: 1) denaturation for 5 min at 95°C, 2) using a cycle profile of 94°C for 30 s, 3) cycle 5 times for each degree, 62°C - 57°C for 1 min, then 72°C 1 min, 72°C for 10 min and then hold at 15°C. DNA amplicons were electrophoresed on a 40 mL 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination.

Colonies which yielded amplicons following PCR were then referenced back to the YEP 96 well plate, and a sample was removed for a second round of PCR for validation. In addition, the suspension was re-streaked on 1A medium (Figure 2.2). Positive strains were re-streaked on 1A medium a minimum of three successive times and tested again for v*irG*, colonies positive after this were considered valid *Agrobacterium* and were frozen at -80°C for storage.

Isolation of Agrobacterium *from Plant Galls*

Galls were obtained from euonymus (*Euonymus obovatus*), and rose (*Rosa* sp.). Samples of tumor tissues (0.5 g) were ground with a micropestle in microfuge tube containing 1 mL sterile water and incubated overnight at 26°C. Gall extracts were serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, and 300 µL was spread aseptically on 1A medium. Plated extracts were incubated at 28°C for 48 h. In the same manner of selection of colonies from soil, *Agrobacterium* colonies, which had grown to 2 mm in diameter with a dense, shiny black morphology on 1A medium (Figure 2.1) were selected for isolation and PCR. The novel CTOHc strain was isolated from chrysanthemum (*Chrysanthemum indicum*) gall by DeeMarie Marty (Chris Taylor Department of Plant Pathology, OSU) and provided for this research.

Introduction of pCAMBIA1300-Gmubi3

The binary plasmid pCAMBIA1300 (Cambia, GenBank accession number AF234296) modified to contain GFP driven by the Gmubi3 promoter (pCAMBIA-Gmubi3, Figure 2.3) (Hernandez-Garcia et al. 2010), was introduced into each individual *Agrobacterium* strain via freeze/thaw transformation (Holsters et al. 1978). All transformed isolates were selected on a YEP medium containing 50 mg/L kanamycin, except for strain KFOH, which was insensitive to kanamycin and was selected on a medium containing 50 mg/L hygromycin. Colony PCR was performed with primer set M13 F and sGFP R (Table 2.2) to confirm successful plasmid uptake.

Single colonies were picked and re-streaked a minimum of three times on a selective medium, after which colony PCR was run again using the v*irG* and the M13/sGFP primer sets (Table 2.2).

Preparation of Agrobacterium *for Plant Transformation*

Agrobacterium strains were streaked from previously made freezer stocks onto YEP medium containing antibiotics 4 d prior to transformation. After 2 d, single colonies were picked and grown overnight in 5 mL liquid YEP containing antibiotics at 28°C. Approximately 1 mL of the overnight culture was used to inoculate 30 mL liquid YEP for an additional overnight culture.

The 30 mL liquid cultures were transferred to 50 mL plastic conical tubes and centrifuged at 3,000 *g* for 10 min at 4°C. The supernatant was discarded and the bacterial pellet was re-suspended in a liquid medium (Murashige and Skoog 1962). After the OD_{600} was adjusted to 0.5 - 0.75, AS was added to the bacterial suspensions to a final concentration of 100 μM. The bacterial suspensions were then allowed to incubate at room temperature for about 30 min.

Plant materials

Agrobacterium strains were evaluated for transformation efficiency using seedling materials of sunflower (cv. 'RHA280') and soybean (cv. 'Thorne'). Seeds were surface sterilized with a 4% (*v/v*) bleach solution for 10 min with shaking at 150 rpm, and rinsed five times with sterile water, until the odor of bleach was no longer present. Seeds were germinated in between water-saturated sterile paper towels in Magenta vessel (GA-7 containers, Magenta Corp., Chicago, IL) for 5 d at 25°C before use for transformation.

Soybean embryogenic suspension cultures were initiated from *Glycine max* cv. 'Thorne', on D40 medium and maintained in 30 mL FN medium (Finer and Nagasawa 1988) in 125 mL baffled flasks, shaken at 150 RPM at 25°C. Suspension culture tissue was subcultured every 2-3 wk, using 5-10 pieces of rapidly proliferating embryogenic tissue, taken about 1 wk prior to transformation

Plant Transformation

Seedlings were placed into a sterile Petri dish containing 5 mL of bacterial suspension in MS medium with AS and dissected directly in the solution. For preparation of cotyledons, both ends of cotyledons were excised and discarded, and the remaining cotyledon segments were then cut into 2-3 mm cross sections. For preparation of stem segments, the roots were removed and hypocotyls were cut into 2-3 mm sections. After dissection, explants were blotted on dry filter paper, and immediately placed onto a solid co-culture medium. For soybean tissues, the solid co-culture medium was modified to

contain 500 mg/L DTT. Tissues were co-cultured for 2 d at 25°C, and then transferred to medium containing MS salts (Murashige and Skoog 1962), Gamborg's B5 vitamins (Gamborg et al. 1968), 3% sucrose and 400 mg/L Timentin for 3 d. GFP expression was measured 5 d after initial inoculation or 3 d after transfer of the tissue to the medium containing Timentin.

For soybean suspension cultures, 10 clumps of embryogenic tissue were placed in 13 x 100 mm borosilicate thin walled glass test tubes containing 5 mL bacterial suspension. Tissues in the tubes were sonicated for 10 s, while keeping tissue at least 3 mm below the water surface in the water bath. The bacterial solution was then discarded and the embryogenic tissue was blotted on sterile filter paper to remove excess bacteria.

Embryogenic tissues were co-cultured in liquid FN medium containing 100 μM AS and 500 mg/L cysteine for 2 d, and then transferred to fresh FN medium containing 400 mg/L Timentin and 500 mg/L-cysteine for 3 d. GFP expression was quantified by counting the total number of GFP foci per tissue clump using a MZFLIII stereomicroscope (Leica, Heerbrugg, Switzerland) and averaged per treatment.

Calculations and Data Analysis

Each experiment was performed as three separate biological replicates for each treatment. Mean GFP expressing foci counts were calculated per cut side of hypocotyl or

cotyledon explants and embryogenic tissue clumps 2-3 mm in size. Data was analyzed in R (Version # 3.0.3, R Core Team, Vienna, Austria) and Minitab (Version # 16.0, Minitab Inc., State College, PA) using ANOVA procedure and PROC GLM. Means separation was carried out using Fisher's test.

RESULTS AND DISCUSSION

Isolation of Novel Strains

For this research, five novel *Agrobacterium* strains were isolated from soil collected from soybean fields in the US, one strain was collected from creek bed soil and three additional strains were collected from galls of *Chrysanthemum indicum*, *Euonymus obovatus*, and *Rosa* sp. (Table 2.3, 2.4). An additional 4 previously-identified strains were added to this group of novel strains to generate the set of thirteen different *Agrobacterium* strains that were used in this work (Table 2.3). All 13 strains were modified to contain the pCAMBIA vector so that GFP expression could be quantified following transformation of sunflower and soybean seedlings and soybean embryogenic cultures.

Transient Expression in Sunflower

In sunflower seedling explants transformed with the pCAMBIA1300 Gmubi3 binary plasmid (Hernandez-Garcia et al. 2010) some tissues were more responsive to transformation using EHA105, a previously identified and well-studied laboratory strains (Figure 2.4). EHA105 produced the highest overall transformation efficiency (Figure 2.5). Use of EHA105 gave higher transformation rates in vascular tissues compared to the soil-derived strains, which showed no such preference for tissue-specific transformation.

With the novel soil-derived strains obtained in this study, GFP foci were evenly distributed throughout the tissues (Figure 2.6). Further analysis of tissue-specific transformation in sunflower hypocotyls revealed that the C58 strain also showed a preference for transformation of vascular tissue. As C58 is the wild-type progenitor strain of EHA105 (Lee and Gelvin 2008) this similarity in transformation targeting was not unexpected. For all other known and novel strains, no tissue-specific transformation was observed with sunflower hypocotyl tissues. Contrary to previous studies, the cotyledonary tissue of sunflower was surprisingly unresponsive to transformation using all strains (Rao and Rohini 1999), significant GFP expression was not observed in treated sunflower cotyledons with any strain. The unresponsive nature of sunflower cotyledonary tissues in this study may have resulted from using tissue, which was not precultured or at a very responsive state of development. Sunflower and soybean seedlings were treated similarly so that comparisons between these different plants could be made.

Transient Expression in Soybean

In soybean hypocotyl explants, cotyledon explants and embryogenic tissues, use of strain EHA105 resulted in a very low efficiency of transformation (Figure 2.7). Although EHA105 gave the highest efficiency of transformation of sunflower hypocotyls, this same strain worked very inefficiently for transformation of all soybean tissues based on GFP foci counts.

Of the novel strains that were obtained, three soil derived strains showed improved transformation rates in all soybean tissues tested. Interestingly, strains produced a consistent transformation profile across all soybean tissues; the more efficient strains gave high transformation rates with soybean hypocotyls (Figure 2.8), cotyledons (Figure 2.9) and embryogenic cultures (Figure 2.10, 2.11). The least effective strains showed low transformation rates in all soybean tissues. In addition, tissue-specific transformation of vascular versus ground tissue in hypocotyls and cotyledons of soybean was not observed (data not shown). This is in contrast to our results obtained with sunflower cotyledons, which were unresponsive to transformation with all strains and EHA105 seemed to preferentially transform vascular tissues in hypocotyl explants. *Agrobacterium* strains JTND and KFOH gave the highest transformation efficiency in all soybean tissues tested.

Strain KFOH was recovered from soil from a non-cultivated field while strain JTND was obtained from a field that had been under cultivation for soybean production. None of the wild-type strains were disarmed prior to evaluation for soybean transform. The effects of disarming on transformation efficiency or tissue-specificity of transformation in soybean and sunflower are not known although the C58 strain is the wild-type progenitor to EHA105 (Lee and Gelvin 2008; Kiyokawa et al. 2009) and gave lower transformation rates compared to its disarmed version.

Transformation Overview

In general, the percent of tissue transformed, was significantly affected by the bacterial strain used (Figure 2.6, 2.8, 2.9, 2.11). For many studies on transformation, the percent explants that contained some transformed cells is typically reported. In this research, the number of cells expressing GFP (Sheen et al. 1995) within each explant was reported. This seems to be an accurate measure of transformation efficiency and these numbers were reflective of the higher transformation rates obtained in this study.

In this study using wild-type *Agrobacterium*, short term analyses of GFP expression were used to estimate transformation efficiency. More long-term studies of tumor formation caused by the introduced bacterial genes were not performed, as some previous studies have done (Hwang et al. 2013; Lewis and Bliss 1994). While these types of assays do test for transformation ability, there is more involved in the process than just DNA transfer. For inoculation of embryogenic cultures, sonication was used to micro-wound the embryos, allowing for more access points for the bacterium to infect host cells (Gaba et al. 2006). Wounded tissues produce phenolics, which trigger the transfer T-DNA (Melchers et al. 1989). Embryo sonication and explant excising activate this phenolic compound production and has been shown to increase transient expression in soybean seedlings (Trick and Finer 1997). Because of the ability to inhibit oxidation, reducing agents including DTT and L-cysteine have been used to minimize the appearance of necrosis, leading to increased transient expression (Finer 2010).

Despite an apparent wide variety of *Agrobacterium* strains currently in use in plant transformation, many of the strains come from limited chromosomal backgrounds, mainly C58 (Hood et al. 1986; Lazo et al. 1991; Hood et al. 1993) and Ach5 (Hoekema et al. 1983; Hellens et al. 2000). One of the most commonly used *Agrobacterium* strains is EHA105, a derivative of C58 and originally obtained from a cherry gall (Slater et al. 2012). Since crown gall has never been reported in soybean fields, isolation of *Agrobacterium* from soybean galls could not be undertaken. To our knowledge there has not been an effort to isolate a host specific transformation vector for *Agrobacterium*-mediated transient expression in soybean or sunflower until now.

Future Direction

Factors required for T-DNA transfer from *Agrobacterium* are found on the Ti or Ri plasmid and molecular analysis has led to a basic understanding of the role of this extrachromosomal element in *Agrobacterium*-host interactions. *Agrobacterium*-mediated transformation may be described as five sequential steps, or factors, consisting of (1) recognition of the presence of a susceptible host, (2) *vir* gene induction and expression, (3) bacterial attachment to a host cell, (4) transfer of T-DNA to the host cell, and (5) T-DNA translocation and integration into the chromosome.

In theory any of these bacterial factors may be manipulated to increase transformation rates, and there is likely much work to be done. In contrast, it has been the

approach of this research to attempt to select a bacterial strain for a specific host rather than matching a host to a pre-selected bacterium. The basis for the enhanced transformation rates of soybean tissue with strains JTND and KFOH demonstrated in this study is not known. As there are many factors, which influence *Agrobacterium*-mediated transformation, we have not yet determined which steps in the transformation process have been made more efficient and if transformation may be further improved with disarming.

TABLE AND FIGURES

Table 2.1 1A recipe, semi-selective medium for *Agrobacterium* spp., *w/v*, per 1000 mL.

L (-) arabitol	3.04 g
NH_4NO_3	0.16 g
KH_2PO_4	0.54 g
K_2HPO_4	1.04 g
Sodium taurocholate	0.29 g
$MgSO_4 \cdot 7\ H_2O$	0.25 g
Agar	15.0 g
Crystal violet, 0.1% (*w/v*)	2.0 mL
Cycloheximide, 2%	1.0 mL
Na_2SeO_3, 1%	6.6 mL
K_2TeO_3	80 mg

Table 2.2 Oligonucleotide primers used in this study.

Name	Primer sequence (5'-3')	Length (bp)	Target
VirG	ATcTYAATTTRggKcgYgAAgA	539	Virulent strain of *A. tumefaciens*
	cAcRTcMgcgTcRAAgAAATA		
sGFPF / M13R	ccgTAggTggcATcgc	1569	pCAMBIA insert
	cAggAAAcAgcTATgAc		

Table 2.3 Strains of *Agrobacterium* used in transformation assays. The first nine listed strains were isolated in the course of this work, soybean field isolates for the purpose of comparing to the current top preforming strains available, and gall isolates for a comparison to other novel wild-type isolates.

Strain	Sample name	Sample location	Substrate isolated from
SDOH	Sandusky	Sandusky, OH	Soybean field
DSOH	Dan Schwartz	Central OH	Soybean field
EROH	Erie	Erie, OH	Soybean field
JTND	Joel Thorsrud	Hillsboro, ND	Soybean field
CTOHb	Blue3	Ottawa, OH	Soybean field
KFOH	Kim Finer	Wooster, OH	Creek bed soil
BGOH	Brad Goodner	Hiram, OH	Euonymus gall
CTOHr	Rose	Wooster, OH	Rose gall
CTOHc	Chrysanthemum	Wooster, OH	Chrysanthemum gall
EHA105	EHA105	Lab strain	Cherry gall
C58	C58	Lab strain	Cherry gall
J2	J2	Lab strain	Cherry gall
K599	K599	Lab strain	Cucumber

Table 2.4 Samples obtained and tested from soil and crown galls. Columns are; the name of the sample location, any potential isolate's code, city or county and state of origin, ± *virG* PCR results, ± successfully isolation, ± pCAMBIA plasmid uptake and expression of kanamycin resistance, ± ability to transform sunflower and soybean seedlings, and ± ability to transform soybean embryos.

Sample name	Code	Location	*virG* PCR	Stain isolated	pCAMBIA introduced	Viable on kan 50	Sunflower seedling	Soybean seedling	Soybean embryo
Dan Schwartz	DSOH	Cortland, OH	+	+	+	+	+	+	+
Joel Thorsrud	JTND	Hillsboro, ND	+	+	+	+	+	+	+
Sandusky	SDOH	Sandusky, OH	+	+	+	+	+	+	+
Kim Finer	KFOH	Wooster, OH	+	+	+	+	+	+	+
Erie	EROH	Erie, OH	+	+	+	+	+	+	+
Field Blue3	CTOHb	Ottawa, OH	+	+	+	+	+	+	-
Brad Goodner	BGOH	Hiram, OH	+	+	+	+	+	+	-

Continued

Table 2.4 continued

Chris Taylor	CTOHr	Wooster, OH	+	+	+	+	+	+	-
Chris Taylor	CTOHc	Wooster, OH	+	+	+	+	+	-	-
John Finer	JHOHe	Wooster, OH	+	+	+	+	+	-	-
Dale Profit	DPOH	Van Wert, OH	+	+	+	+	-	-	-
98	CTOH98	Ottawa, OH	+	+	-	-	-	-	-
Harold Gloe	HGMO	Hermann, MO	+	-	-	-	-	-	-
Jay Myers	JMND	Colfax, ND	+	-	-	-	-	-	-
Trudi Soil	Trud	Moorland, Ohio	+	-	-	-	-	-	-
Brent Kohls	BKND	Clifford, ND	+	-	-	-	-	-	-
Colfax	Colx	Colfax, OH	+	-	-	-	-	-	-
Hillsborough	Hils	Hillsborough, OH	+	-	-	-	-	-	-

Continued

Table 2.4 continued

Fayetteville	Fayet	Fayetteville, OH	+	-	-	-	-	-	-
Chuck Parker	CPOH	Canton, OH	+	-	-	-	-	-	-
Rodney Bacon	RBOH	New Springfield, OH	-	-	-	-	-	-	-
Jeff Bentley	JBOH	Deerfield, OH	-	-	-	-	-	-	-
Ed Burtch	EBOH	Celina, OH	-	-	-	-	-	-	-
Jeff Clever	JCOH	Croton, OH	-	-	-	-	-	-	-
Daniel Corcoran	DCOH	Waverly, OH	-	-	-	-	-	-	-
Dennis Denlinger	DDOH	Brookville, OH	-	-	-	-	-	-	-
Don Grimes	DGOH	Orrville, OH	-	-	-	-	-	-	-
Jeff Magyar	JMOH	Orwell, OH	-	-	-	-	-	-	-

Continued

Table 2.4 continued

Jeff Roehm	JROH	Hillsboro, OH	-	-	-	-	-	-	-
John Steritz	JSOH	Lynchburg, OH	-	-	-	-	-	-	-
Doug Utz	DUOH	New Washington, OH	-	-	-	-	-	-	-
Jerry Ward	JWOH	Waldo, OH	-	-	-	-	-	-	-
Mike Yeagle	MYOH	Lindsey, OH	-	-	-	-	-	-	-
Todd Hesterman	THOH	Napoleon, OH	-	-	-	-	-	-	-
Keith Kemp	KKOH	West Manchester, OH	-	-	-	-	-	-	-
Patrick Knouff	PKOH	Minster, OH	-	-	-	-	-	-	-
Carl Rhodes	CROH	Lisbon, OH	-	-	-	-	-	-	-
Mike Bellar	MBKS	Howard, KS	-	-	-	-	-	-	-
Harvey Pyle	HPND	Fargo ND	-	-	-	-	-	-	-

Continued

Table 2.4 continued

Harvey Morken	HMND	Casselton, ND	-	-	-	-	-	-	-
Keith Phillips	KPMO	Kahoka, MO	-	-	-	-	-	-	-
Mike O'Leary	MOMN	Danvers MN	-	-	-	-	-	-	-
Vernon Pooch	VPMN	Farwell MN	-	-	-	-	-	-	-
Greg LeBlanc	GLMN	Crookston MN	-	-	-	-	-	-	-
Monte Peterson	MPND	Valley City, ND	-	-	-	-	-	-	-
Cecil Deschene	CDMN	Argyle MN	-	-	-	-	-	-	-
Vanessa Kummer	VKND	Colfax, ND	-	-	-	-	-	-	-
Field 1 Piketon	F1Pike		-	-	-	-	-	-	-

Continued

Table 2.4 continued

Field 2 Piketon	F2Pike		-	-	-	-	-	-	-
Field 1	Corcor		-	-	-	-	-	-	-
Wood	WOOD		-	-	-	-	-	-	-
Western	West.		-	-	-	-	-	-	-
Rohr1	Rohr1		-	-	-	-	-	-	-
Rohr2	Rohr2		-	-	-	-	-	-	-
Rohr3	Rohr3		-	-	-	-	-	-	-
Rohr4	Rohr4		-	-	-	-	-	-	-
Rohrx	Rohrx		-	-	-	-	-	-	-
Blue7	Blue7		-	-	-	-	-	-	-
Blue11	Blue11		-	-	-	-	-	-	-

Continued

Table 2.4 continued

Blue15	Blue15		-	-	-	-	-	-	-
Field2	Field2		-	-	-	-	-	-	-
Field3	Field3		-	-	-	-	-	-	-
91	91		-	-	-	-	-	-	-
92	92		-	-	-	-	-	-	-
93	93		-	-	-	-	-	-	-
96	96		-	-	-	-	-	-	-
97	97		-	-	-	-	-	-	-

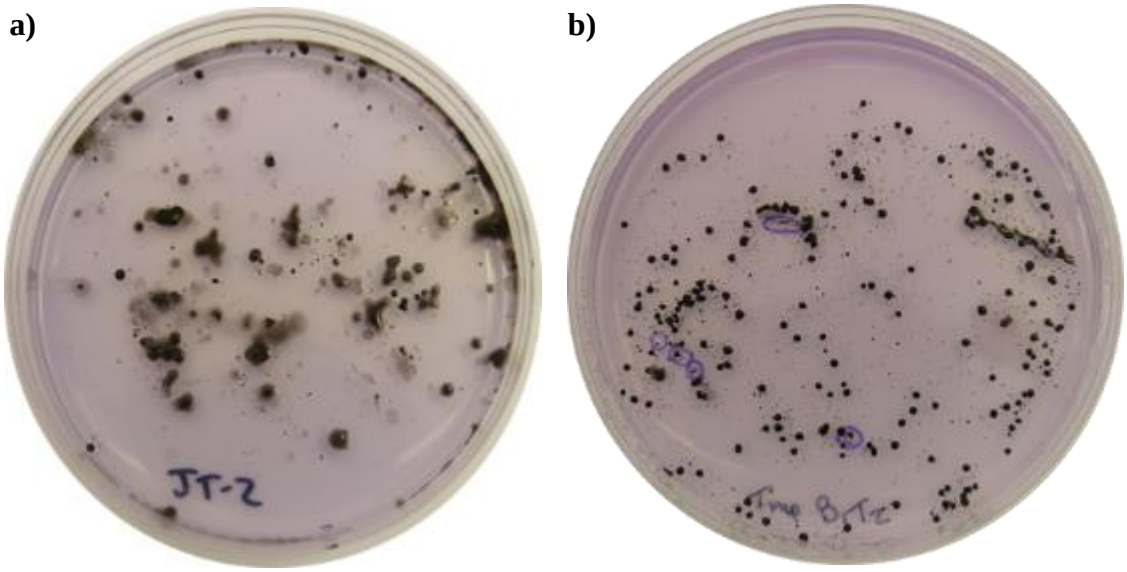


Figure 2.1 Typical growth seen on 1A semi-selective medium with tellurite added. a) A soil solution at 10^{-2} dilution. b) A gall suspension at 10^{-2} dilution.

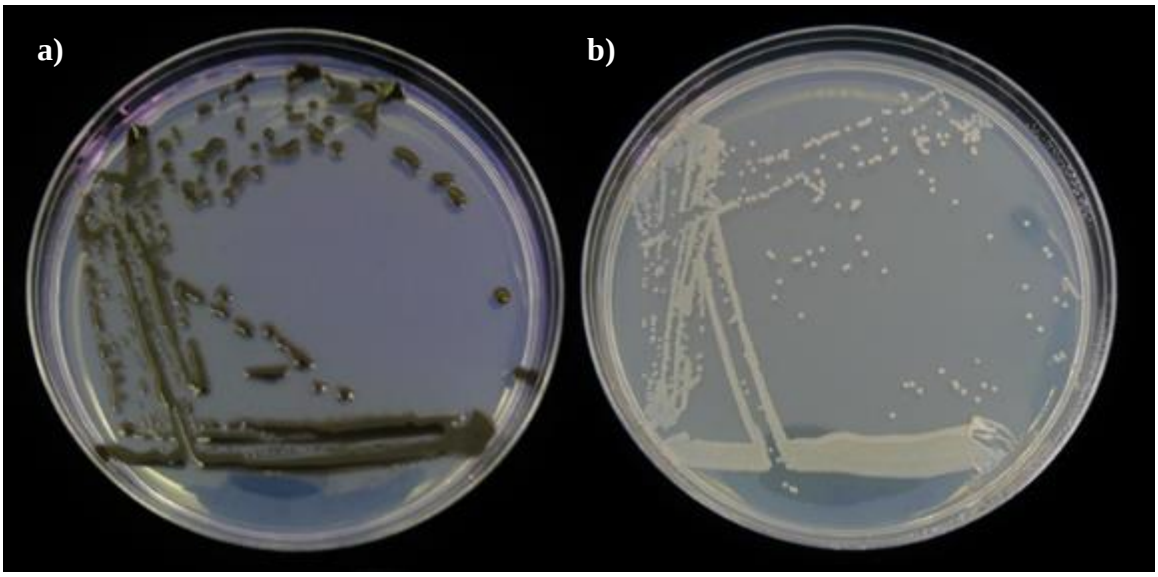


Figure 2.2 Positive *virG* colonies streaked on a) 1A medium, then b) YEP medium.

a)

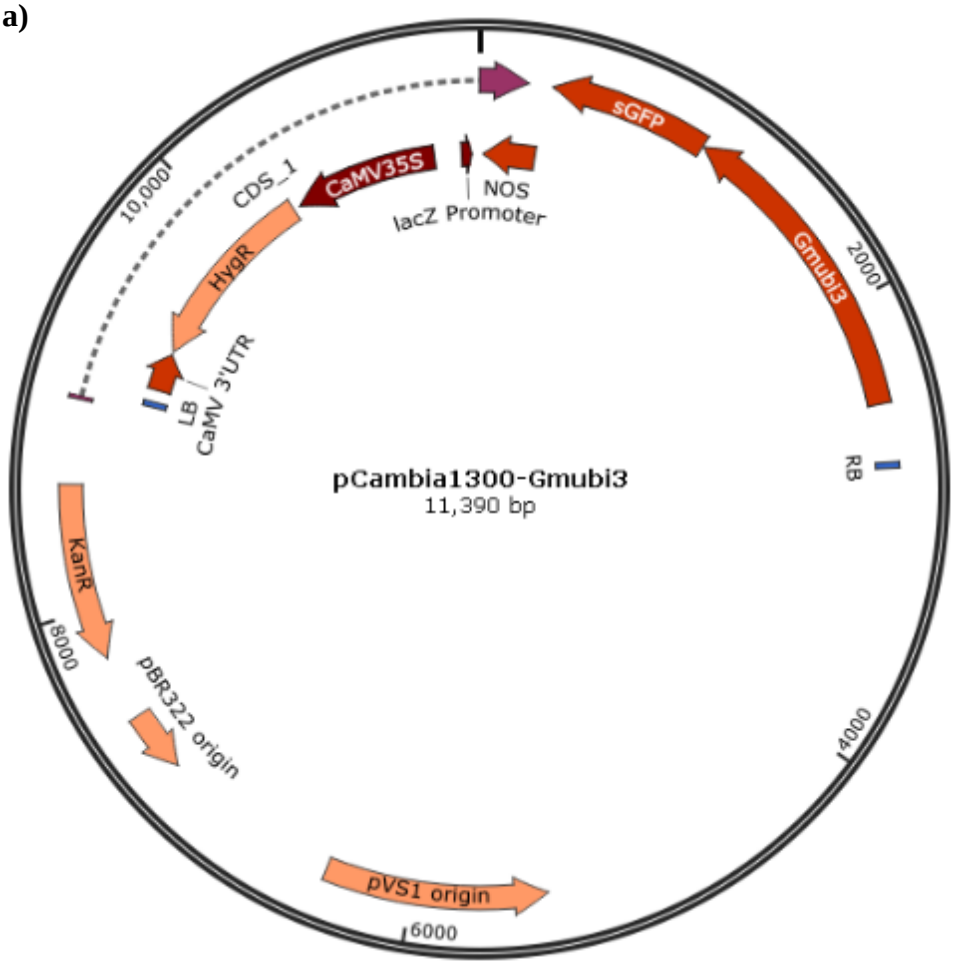
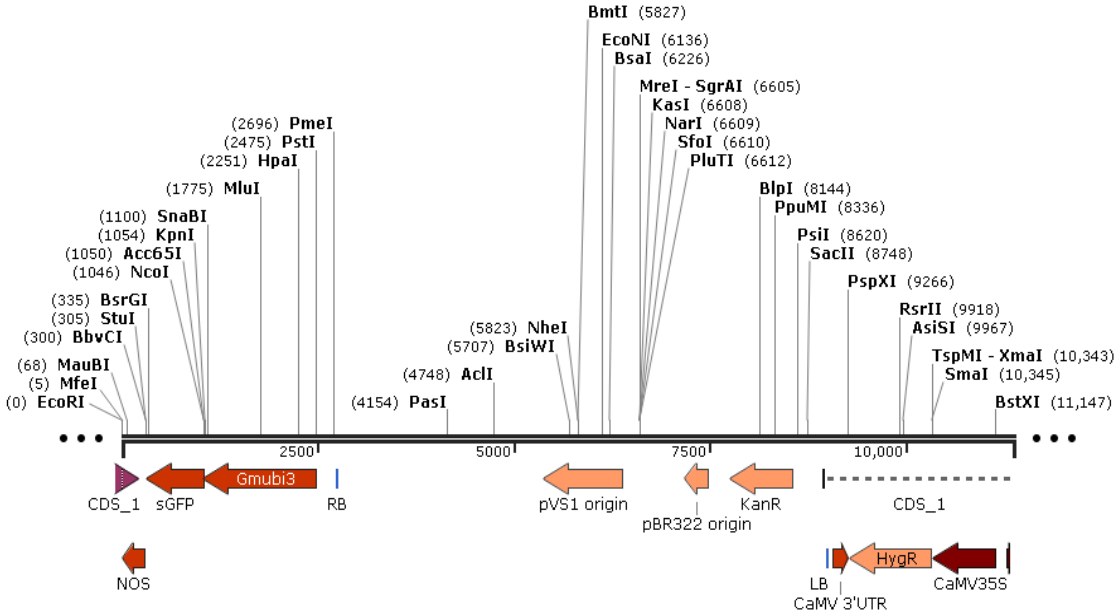


Figure 2.3 pCAMBIA1300 GmUbi3 plasmid map a) with and b) without splice sites.

Continued

Figure 2.3 continued

b)



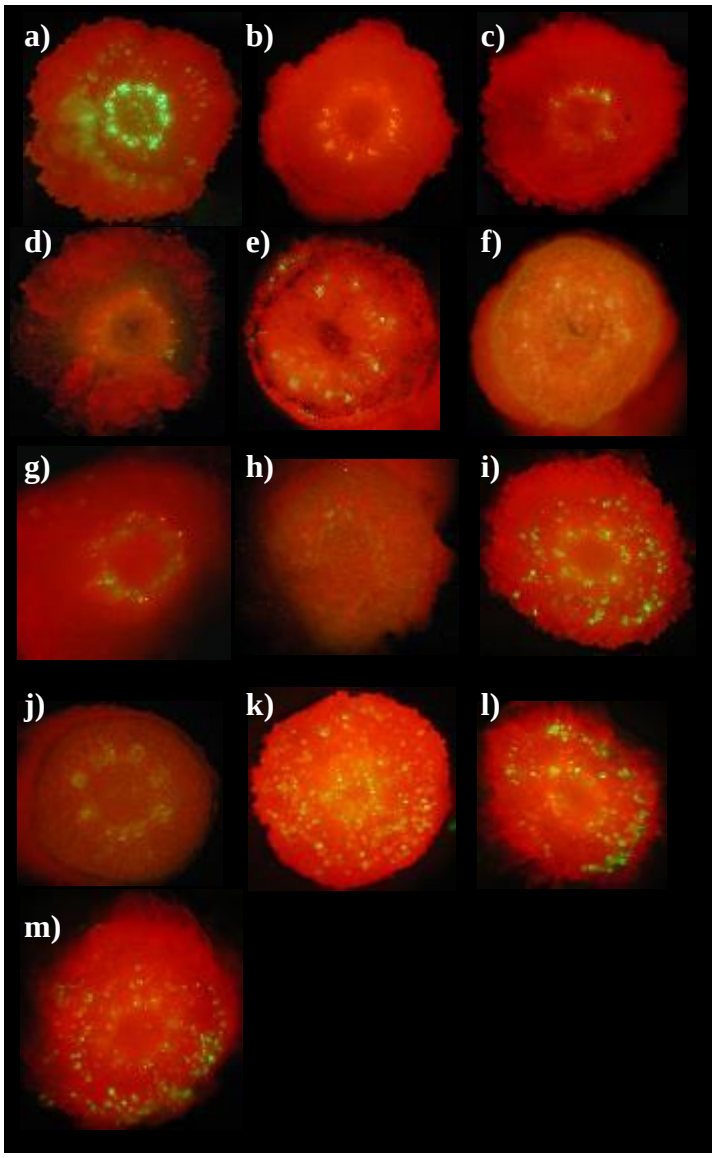


Figure 2.4 Sunflower hypocotyl tissues expressing GFP. Tissue-specific transformation is seen in C58 derived strains, contrary to expression patterns seen in most novel strain. a) EHA105, b) C58, c) J2, d) K599, e) BGOH, f) CTOHc, g) CTOHr, h) CTOHb, i) DSOH, j) EROH, k) JTND, l) KFOH, m) SDOH.

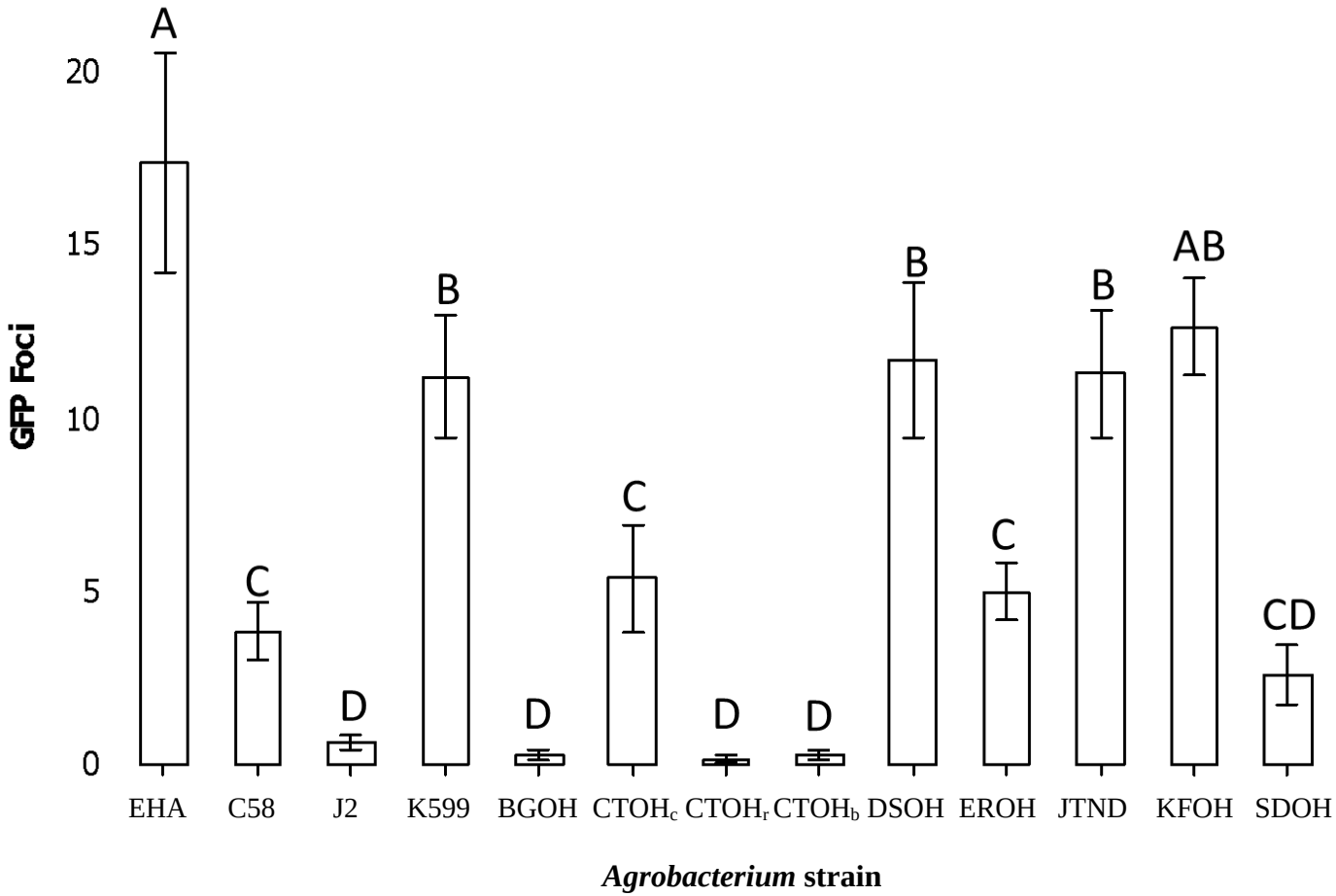


Figure 2.5 Mean GFP foci in 5 d sunflower seedling hypocotyl explants. Data are mean ± standard error (SE) from at least three independent experiments. Target material was inoculated, co-cultured for 48 h and then transferred to an antibiotic-containing medium for control of *Agrobacterium* growth. After an additional 72 h, transformation efficiency was quantified by counting expressing foci per explant. Sunflower hypocotyl explants showed that strain EHA105 had the highest expression.

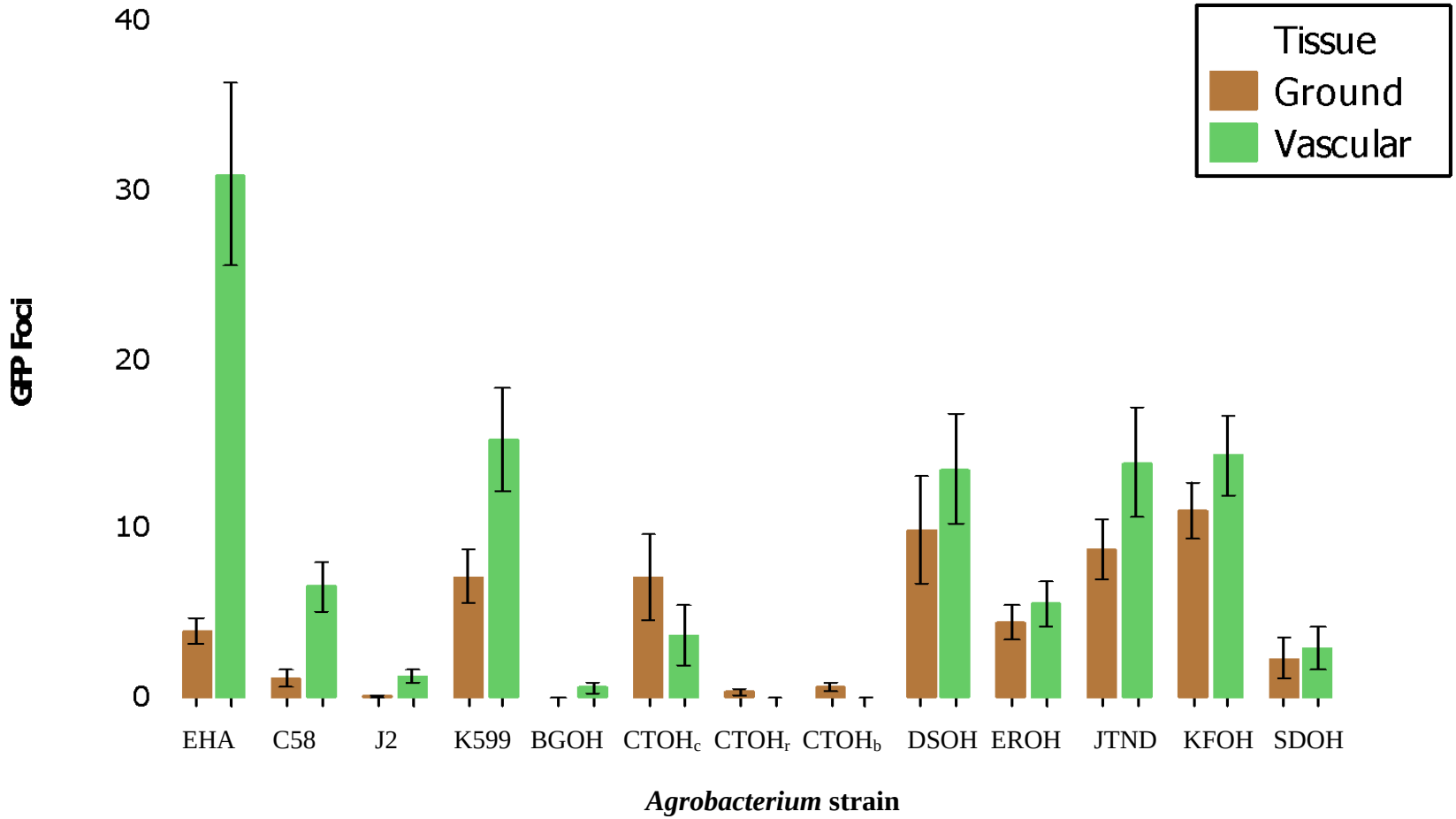


Figure 2.6 Mean GFP foci in 5 d sunflower seedling by tissue type. Three known strains containing the C58 chromosomal background, C58, EHA105 and J2, all showed preferential transformation of vascular tissues. Novel isolates did not show a significant difference in type of tissue transformed. Data are mean ± standard error (SE) from at least three independent experiments.

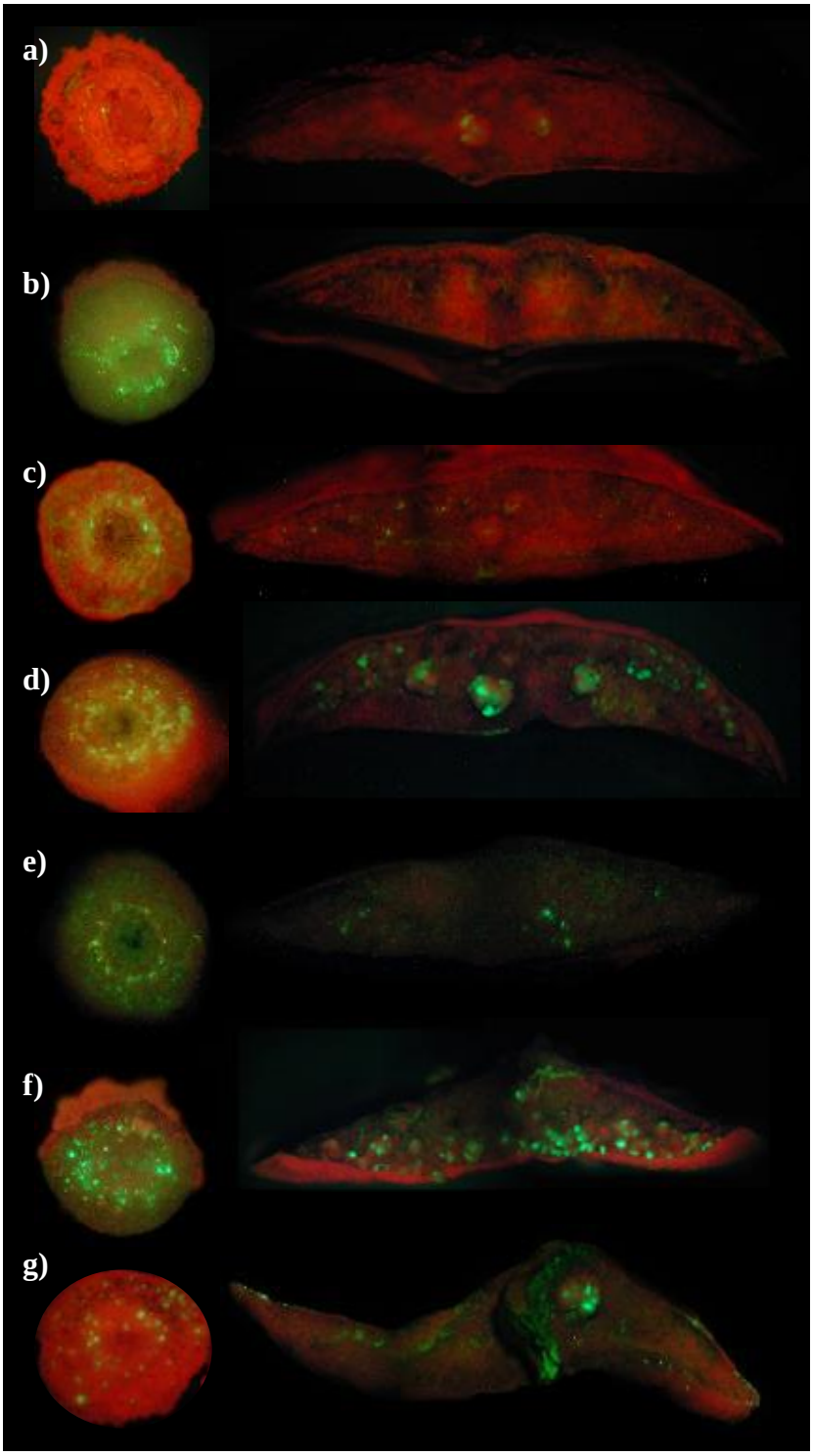


Figure 2.7 Soybean hypocotyl and cotyledon tissues expressing GFP: a) EHA105, b) C58, c) K599, d) DSOH, e) EROH, f) JTND, g) KFOH.

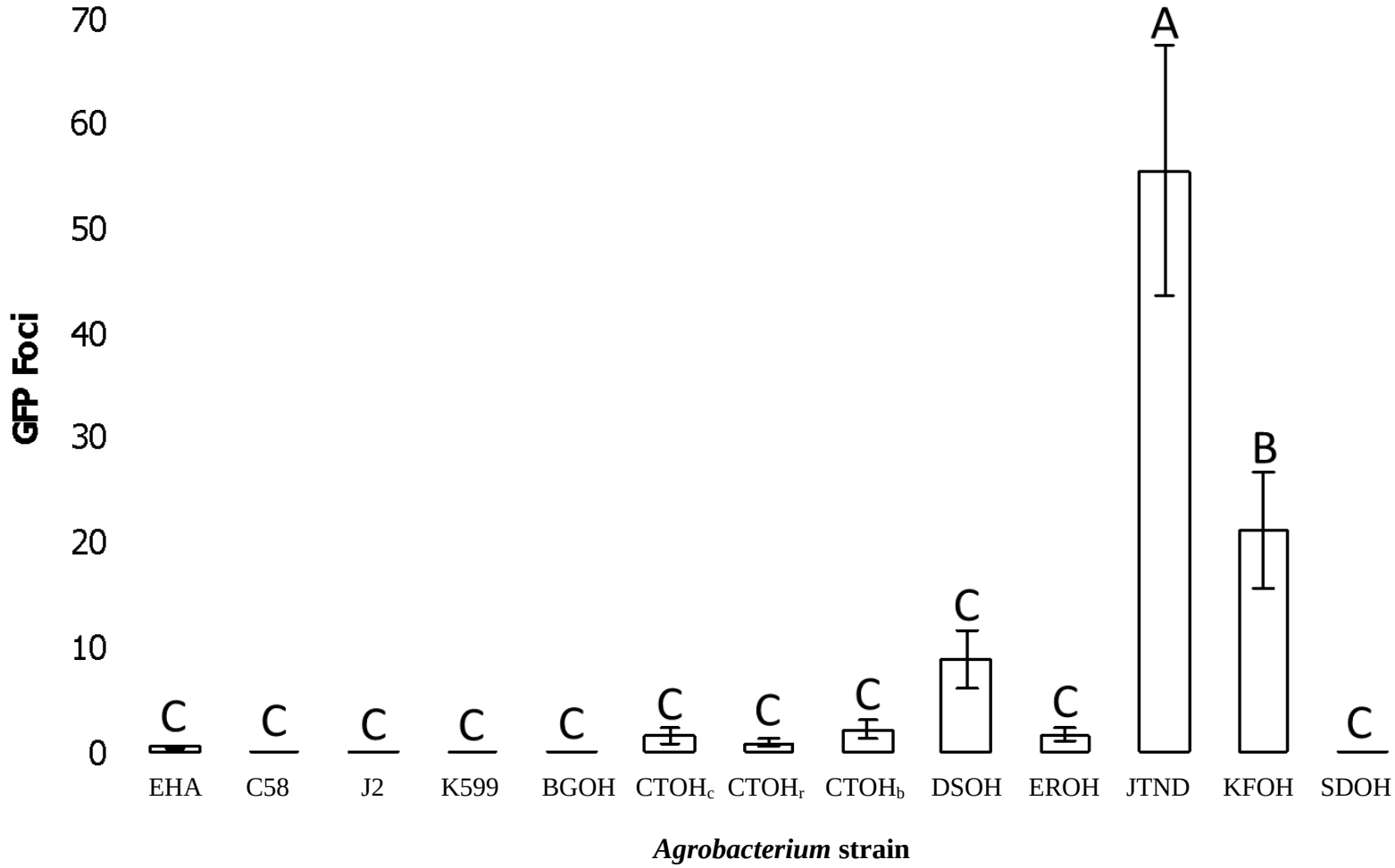


Figure 2.8 Mean GFP foci in 5 d soybean seedling hypocotyl explants. Data are mean ± standard error (SE) from at least three independent experiments.

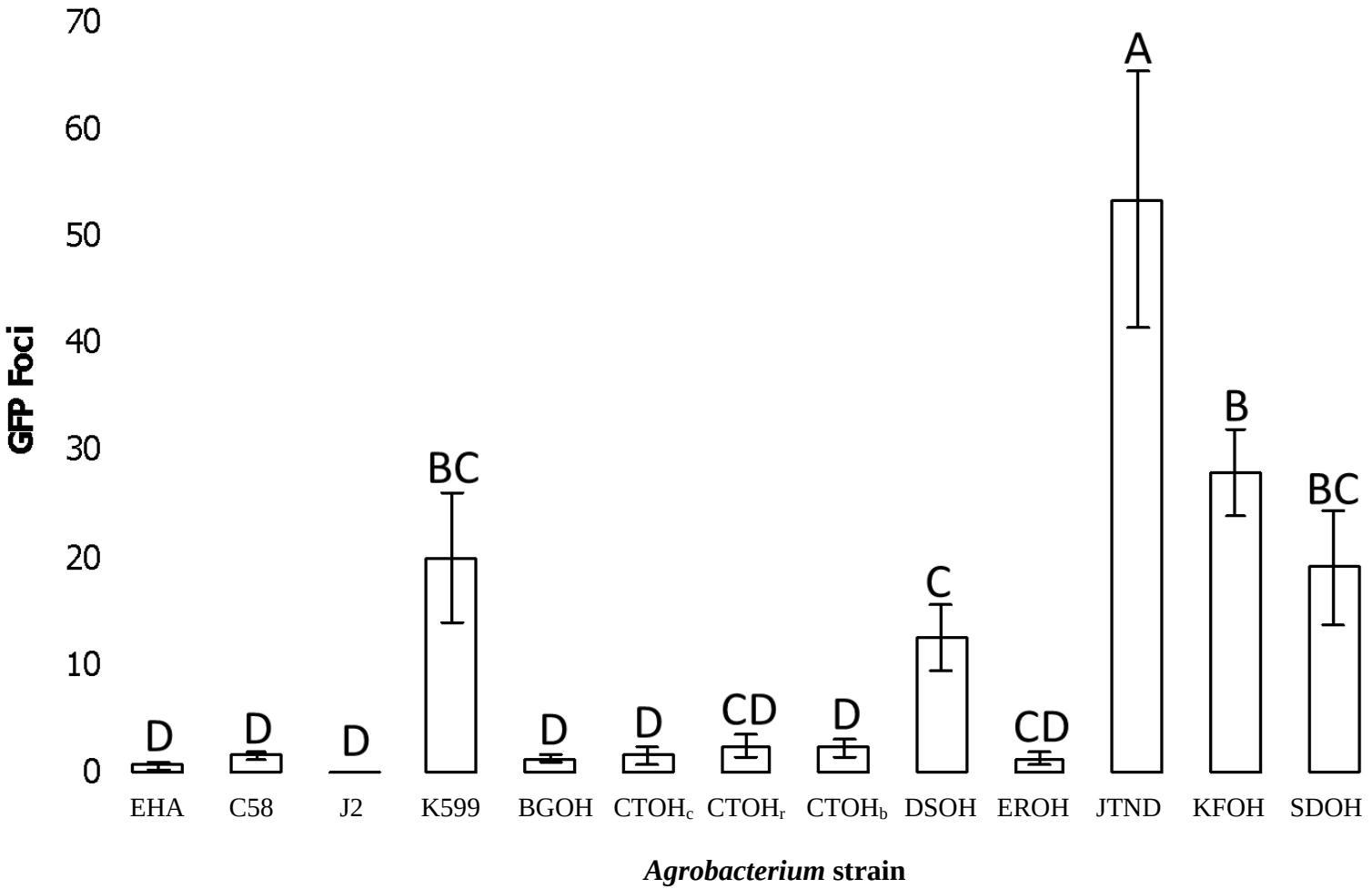


Figure 2.9 Mean GFP foci in 5 d soybean seedling cotyledon explants. Data are mean ± standard error (SE) from at least three independent experiments.

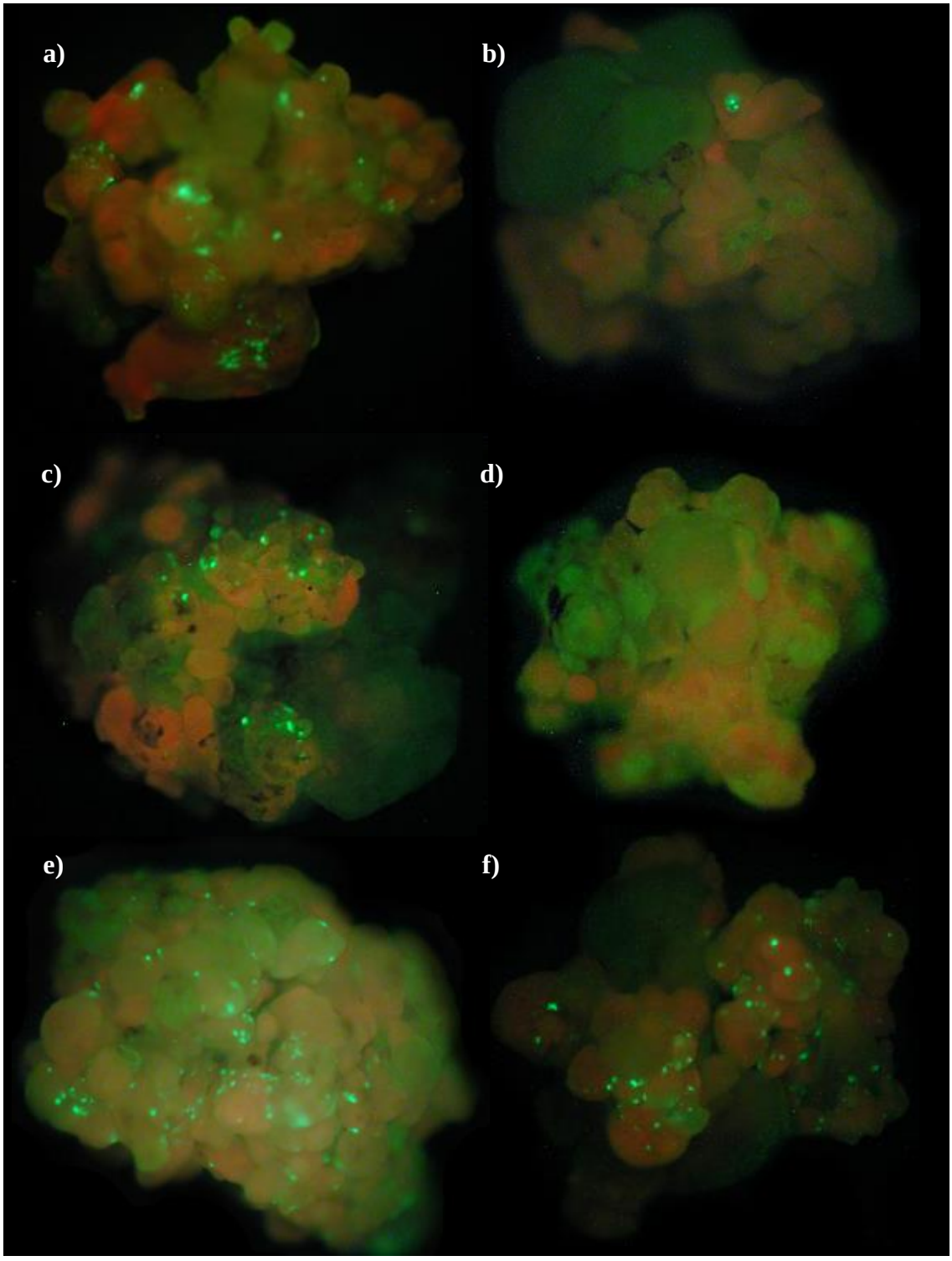


Figure 2.10 Soybean embryogenic suspension cultures expressing GPF. Shown after 5 d transformation assay, 2 d co-culture and 3 d recovery a) EHA105, b) C58, c) DSOH, d) EROH, e) JTND, f) KFOH

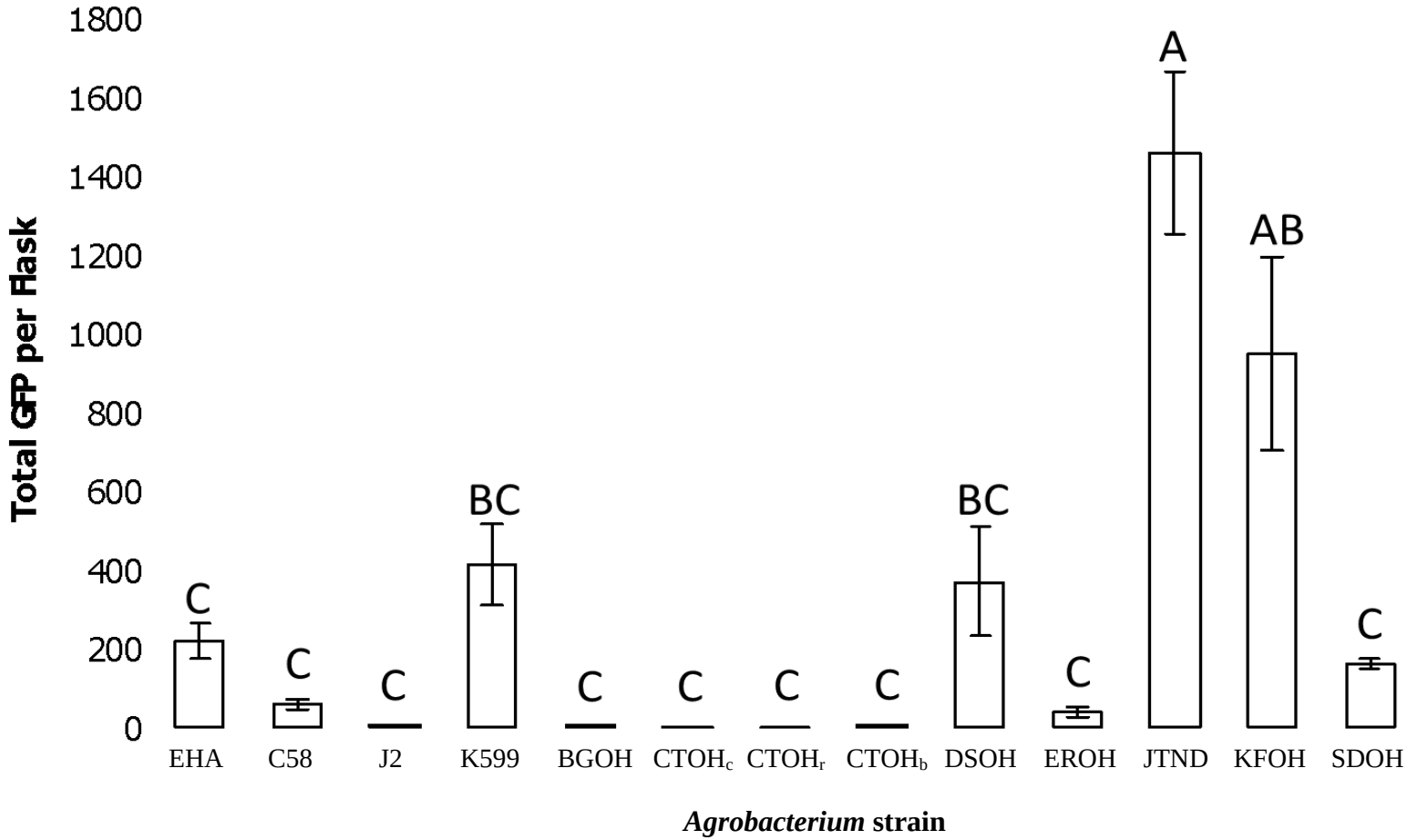


Figure 2.11 Mean GFP foci in 5 d soybean embryogenic suspension cultures. Data are mean ± standard error (SE) from at least three independent experiments.

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