REGENERATION AND *AGROBACTERIUM*- MEDIATED TRANSIENT EXPRESSION OF TWO CHILI PEPPER (*CAPSICUM SPP*. L.) CULTIVARS

A Thesis

by

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Submitted to the Graduate and Professional School of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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December 2021

Major Subject: Horticulture

ABSTRACT

Chili pepper is a staple crop for humans. We tried to develop an efficient tissue-culture and transient gene expression system in two pepper genotypes, A108 and Hab51p2-1. Using MS agar supplemented with 2mg/L of Zeatin, 0.5mg/L of BA, Kinetin, and IAA, we succeeded in regenerating these two chili pepper cultivars. When we used cotyledons, the induction percentages of callus for A108 and Hab51p2-1 were 96.6 and 100, respectively. The simultaneous induction percentage for both shoot and root for A108 was 10.3 and that for Hab51p2-1 was 5.8. When we used hypocotyls, the induction percentages of callus for A108 and Hab51p2-1 were 95.8 and 96.2, respectively. In addition, we developed a transient gene expression system for A108 and Hab51p2-1 peppers using two strains of *Agrobacterium tumefaciens*, AGL-1 and GV3101 transformed with the CGEL-21 vector harboring the EGFP and GUS genes to establish a pepper transformation system by agroinfiltration. Expression of both reporter genes was detected using fluorescence microscopy, PCR, histochemical GUS assay, and quantitative real-time PCR. Using quantitative real-time PCR, we demonstrated that the AGL-1 strain showed better performance in the transformation system for A108 pepper with a normalized transient GUS gene expression level of 2.03359±0.54354, compared to the GV3101 strain with a transient expression level of 0.79618±0.28541. For transformed Hab51p2-1 pepper, we detected transient expression of GUS gene in all transformed groups, although the expression level was relatively lower than that of the A108 transformed groups, suggesting that more studies are needed to validate the reason for the low transient expression level in the Hab51p2-1 pepper genotype.

DEDICATION

This thesis is dedicated to my father, Sangheui Seo and my mother, Sunju Lee for their love, unconditional support, and for their patience and encouragement during times of stress.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Kevin Crosby for supporting the project and giving me the opportunity to conduct this research for my Master's program. I appreciate the time and patience made for me throughout the project.

Also, I would like to thank my committee member, Dr. Qingyi Yu for giving guidance for establishing the tissue culture system in pepper and to Dr. Michael Thomson for letting me conduct the research in his lab and supporting tools of transient expression in pepper.

Thanks to Dr. Nikolaos Tsakirpaloglou for giving me advice and guidance for transient expression experiment on peppers.

Last but not the least, I would like to thank my mother and father giving support and encouragement during the degree program.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This research was supervised by a thesis committee consisting of Dr. Kevin M. Crosby and Dr. Patricia Klein of the Department of Horticultural Sciences, Dr. Qingyi Yu of the Department of Plant Pathology and Microbiology, and Dr. Michael Thomson of the Department of Soil and Crop Sciences.

All other work conducted for the thesis was completed by the student independently.

Funding Sources

This work was funded by TDA Specialty Crop Block Grant SC-1819-16- Enhancing Pepper Crop Health and Value by Integrating Novel Genetics and Mineral Nutrition Management, and TDA Specialty Block Grant SC-2021-37- Improving Quality and Profitability of Texas Pepper Production with Cropping Strategies and Novel Texas A&M Genetics.

NOMENCLATURE

BAP	Benzyl-aminopurine
cwt	Hunderedweight
EGFP	Enhanced green fluorescence protein
GA_3	Gibberellic acid
GUS	ß-glucuronidase
$HgCl_2$	Mercury (II) chloride
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
MS	Murashige & Skoog
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Pepper Production

World production of pepper in dry and fresh weight

Pepper (*Capsicum spp*.) is an important vegetable and essential spice used as a basic ingredient in various cuisines around the world. It is widely spread all over the world and is rich in vitamins and minerals such as vitamin A, B-complex, C, E, manganese, and potassium [**7,41**].

World total production of chilies and peppers in fresh weight was 12,976,927 million tons in 1994 and 38,027,164 million tons in 2019 [**19**]. Fig 1. and Fig 2. show that Asian countries produced more peppers than any other region [**19**].

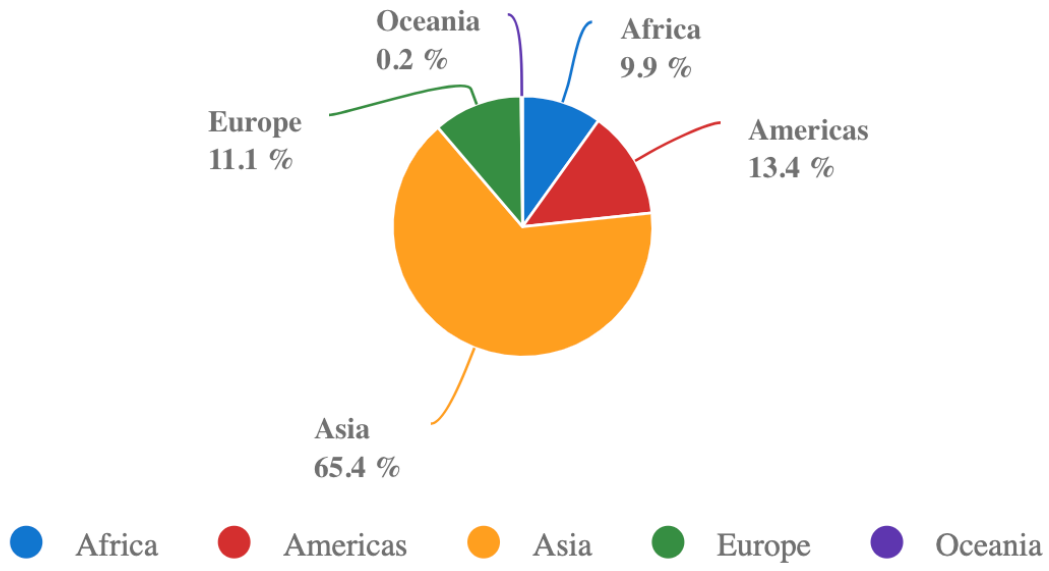

Figure 1. Total production share of peppers (fresh) by region; sum 1994-2019.

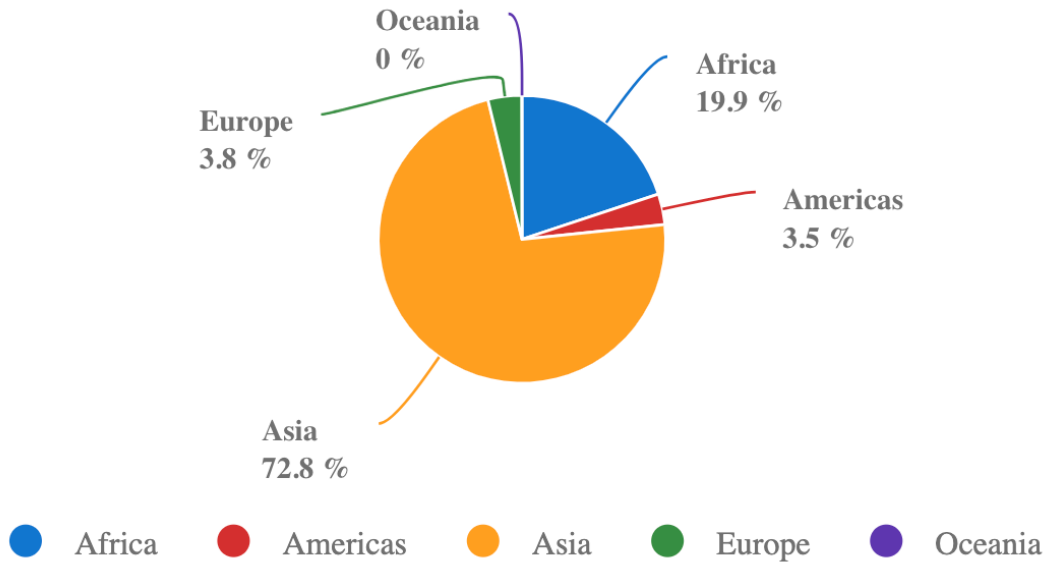

Figure 2. Total production share of peppers (dry) by region; sum 1994-2019.

Pepper production in the USA

Most bell peppers in the United States are produced in the field using drip irrigation and mulch. Besides field-grown peppers, greenhouse-grown bell peppers are available throughout the year. Fresh-market peppers are harvested by hand every week or so for about four to six weeks. In 2017, total US bell pepper and chili pepper production that was measured in dollars was $625,577,000 and $113,562,000, respectively [**16**]. However, the yield for US bell pepper and chili pepper was 341 cwt/acre, and 208.5 cwt/acre, respectively [**17**].

According to the USDA Economic Research Service, in 2016, only 5.3 percent of bell peppers produced in the USA were exported to other countries, while 60.2 percent of peppers consumed in the USA were imported [**17**].

Brief pepper botany and genetics

Origins and distribution

Pepper (*Capsicum spp*.) belongs to the family of Solanaceae which also includes tomato, potato, tobacco, and petunia. There are 38 reported species of *Capsicum* [**31**]. Five pepper species, *C. annuum, C. frutescens, C. baccatum, C. chinense,* and *C. pubescens*, are cultivated and consumed crops around the world. The origin of the pepper is thought to be from the tropical South American region from Mexico to Bolivia [**11, 32**]. *C. annuum, C. frutescens* and *C. chinense* originated from wild progenitors [**11, 35**]. *C. annuum* is a commonly cultivated species, which originated from wild bird pepper. It was domesticated in Mexico and was first introduced in Europe by Columbus and other explorers [**3, 12**].

Genetic diversity of pepper

The analysis of genetic diversity in peppers has been performed by random polymorphic DNA, inter-simple sequence repeats, or simple sequence repeats (SSR) [**10, 29, 52**]. Genus *Capsicum* was analyzed by means of isozyme, nuclear, and chloroplast DNA markers in the early 1990s [**43, 46**]. A recent study analyzed 28 SSR loci in 1,352 non-redundant accessions of 11 *Capsicum* species from 89 countries [**29**]. This study showed the interspecific structure of the genus *Capsicum*, and the origin of the wild cultivated species of *C. annuum*. The genetic structure of the domestic *C. annuum* species are closely related to cultivar types and human selection.

Conservation importance of pepper wild relatives

Wild plants have evolved through natural selection in diverse environments and subjected to abiotic stresses such as poor soil, or climate, as well as biotic stresses, such as pests and diseases. The wild relatives provide us the tools to use when we breed crops to adapt to difficult environments. Khoury and colleagues [**18**] have found that more than 95 percent of wild chili peppers are not well protected in gene banks. Conservation of wild chili peppers in gene banks allows pepper scientists to study the complex genetic traits and to continue pepper propagation by storing pepper seeds.

Plant breeding in wild species

Crossing with wild species has been recognized as a promising crop improvement tool to increase genetic diversity and incorporate useful traits into cultivated species. However, crossing wild and domesticated species can result in cross incompatibility between the wild species and the cultivated crop [**51**]; F_1 hybrid sterility [**51**]; infertility of the segregating generations [**51**]; reduction of recombination between the chromosomes between the two species [**51**]; and linkage drag, having negative effect for the genes that are tightly linked to the trait of interest [**51**]. In species lacking these drawbacks wild introgression breeding has been used to develop new crop varieties having disease resistance [**51**] and other useful traits.

Genetic engineering of pepper

Agrobacterium tumefaciens

Pepper's breeding and production is being challenged by a variety of pests, diseases, and abiotic stresses [**44**]. New trait development in pepper is hindered by interspecies crossing barriers and by the lack of an efficient regeneration system, which is critical for new-trait introduction by genetic transformation. The major hurdles in *Capsicum* regeneration are the low frequency of shoot formation and the development of poorly formed shoot buds and leafy shoots, which cannot elongate, because of the lack of a shoot apical meristem [**6, 15, 29, 48**].

Agrobacterium tumefaciens as a vector to create transgenic plants is being used for many agronomically and horticulturally important species [**47**]. Currently, a high proportion of economically important crops such as corn, soybeans, cotton, and canola are transgenic, and more crop species are to be generated by *Agrobacterium*-mediated transformation [**38**]. The genus *Agrobacterium* has a variety of species. *A. tumefaciens* causes crown gall disease, *A. rhizogenes* causes hairy root disease, *A. rubicauses* is responsible for cane gall disease, and *A. vitis* causes galls on grape and a few other plant species [**33**]. The critical aspect for the purposes of plant genetic engineering is the host range of the *Agrobacterium* strains. *Agrobacterium* can transfer DNA to a remarkably broad group of organisms including numerous dicot and monocot species [**26, 27**] as well as gymnosperms [**42, 50**].

The molecular principle of genetic transformation of plant cells by *Agrobacterium* is to transfer a region of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid resident in *Agrobacterium* into the plant nuclear genome. Ti plasmids are on the order of 200 to 800 kbp in size [**11, 24, 34**]. The transferred DNA (T-DNA) is called the T-region on the Ti or Ri plasmid. The size of T-regions on native Ti and Ri plasmids are about 10 to 30 kbp [**5**]. The

processing of the T DNA from the Ti plasmid and its transfer from the bacterium to the plant cell is mediated by virulence (vir) genes which are carried by the Ti plasmid [**14, 23**].

There are several studies on the transformation of pepper using *Agrobacterium tumefaciens*. Six different red pepper cultivars (ACA-10, Kashi Anmol, LCA-235, PBC-535, Pusa and Jawala) were regenerated using hypocotyl explants and an *Agrobacterium*-mediated transformation protocol [**27**]. The explants (hypocotyls, cotyledonary leaves, and leaf discs) from axenic seedlings of six red pepper cultivars were cultured on either Murashige and Skoog medium (MS) without hormone or MS medium supplemented with BAP (6-benzylaminopurine) or in combination with IAA (indole-3-acetic acid). Inclusion of IAA led to callus formation at the cut ends of explants, formation of rosette leaves and unclear shoot buds. Addition of gibberellic acid (GA3) in the shoot elongation medium at 0.5 mg/l concentration augmented the elongation in two cultivars, LCA-235 and Supper. Chili cultivar, Pusa Jawala was transformed with *Agrobacterium tumefaciens* containing the βC1 ORF of satellite DNA β molecule associated with Chili leaf curl Joydebpur virus. Transformants were identified by PCR and Southern hybridization analysis [**27**]. Heidmann et al. [**13**] improved pepper transformation by the ectopic expression of the *Brassica napus* BABY BOOM AP2/ERF transcription factor. Transient activation of BABY BOOM in the progeny plants triggered prolific cell regeneration, producing many somatic embryos which could be regenerated into seedlings.

A new and efficient system for *A. rhizogenes* transformation of the cultivated species *Capsicum annuum* was also reported [**1**]. Hypocotyls and foliar organs of Yolo Wonder (YW) and Criollo de Morelos 334 (CM334) pepper cultivars were injected with the two constructs, pBIN-gus and pHKN29-gfp of *A. rhizogenes* strain A4RS. Foliar explants of both pepper genotypes transformed by A4RS-pBIN-gus or A4RS-pHKN29-gfp induced transformed roots.

To construct an efficient method for genetic transformation of pepper, Zhou et al. [**52**]

used a monitoring system expressing GFP (green fluorescent protein) as a reporter marker incorporated into *Agrobacterium*-mediated transformation. A callus-induced transformation system was used to transform the GFP gene. GFP expression was confirmed in all tissues of T0, T1 and T2 peppers.

Lee et al. [**48**] used two genes, TMV-coat protein (CP) and PPI1 (pepper-PMMV interaction 1 transcription factor), to transform chili pepper inbred lines P915 and P409 using *Agrobacterium* co-culture. Eighteen transformed T0 plants were produced. The key to the pepper transformation protocol was to select calli having shoots since selection of calli with no shoots will result in non-transformants.

A stable transformation method for hot pepper using the hygromycin phosphotransferase (*hpt*)/hygromycin selection strategy was studied [**25**]. Explants from aseptic pepper seedlings were inoculated with *Agrobacterium tumefaciens* containing pCAMBIA1301. Several calli were developed on the medium containing hygromycin and then shoots were successfully reproduced from the hygromycin-resistant calli. The *hpt* gene integration, its expression in the transgenic pepper plants (T0), and its transmission to the progeny (T1) were confirmed by Southern and Northern hybridization analysis [**25**].

The study to standardize an efficient system for *Agrobacterium*-mediated genetic transformation of *Capsicum annuum* L. cv. California Wonder was performed [**45**]. *Agrobacterium tumefaciens* LBA 4404 strain carrying a binary vector pBI121, *npt-II* and *gus* genes was applied for co-cultivation with cotyledon and hypocotyl explants. Regeneration of possible transgenic shoots was obtained on MS basal medium supplemented with 6.0 mg/L of BAP, 0.3 mg/L of IAA, 500 mg/L of cefotaxime and 50 mg/L of kanamycin. The elongation of malformed rosettes was obtained on MS basal medium containing 2.25 mg/L of BAP, 2.0 mg/L of GA3, 500 mg/L of cefotaxime and 50 mg /L of kanamycin.

A wide range of pepper genotypes for regeneration and transformation with an *Agrobacterium tumefaciens* shooter strain were evaluated [**4**]. The genetic transformation of 107 doubled haploid (DH) pepper genotypes in 12 cultivar groups was performed with *Agrobacterium tumefaciens* harboring the cucumber mosaic virus (CMV) coat protein (CP) gene. The regenerated plants were selected and confirmed by PCR detection of the CP gene. Eighteen PCR-positive DH plants were identified.

An efficient transformation method of chili pepper cultivars (*Capsicum annuum* L.), Pusa Sadabahar and Pusa Jwala using *Agrobacterium tumefaciens* was reported [**30**]. The transformation of peppers was done with *Agrobacterium tumefaciens* strain LBA4404 harboring pCAMBIA2301 construct with GUS reporter and NPT-II marker genes. Co-cultivation of the hypocotyl explants with *Agrobacterium* cells for 72 h was optimal for obtaining high transformation efficiency of 30% in both cultivars.

Biolistic transformation

Plant transformation can also be accomplished using biolistics or microprojectile bombardment which can be used for transferring exogenous DNA to plant nuclear and chloroplast genomes. This method contains both transgene and coating of gold or tungsten particles (~ 2 microns in diameter) which are blasted by a small explosion to go into plant cells directly [**37**]. Theoretically, it can be used on any plant species and their subcellular organelles to transform them [**36**]. Since it does not require specific vectors, it simplifies the cloning process. Both biolistics and *Agrobacterium* have been used for generating stable transgenic plants and transient gene expression on various plant species successfully [**36**]. Compared with the biolistics gene delivery method, studies have shown that *Agrobacterium* performs significantly better in transformation efficiency, transgenic expression, and inheritance [**36**]. Also, it is feasible in most dicotyledonous and in a limited number of monocotyledonous plants

[**23**]. The biolistics approach may produce higher copy numbers of a transgene, and total randomness of integration of genes into the plant genome. For the application of producing pharmaceutical proteins, using *Agrobacterium* as a gene delivery method is more desirable than biolistics. In the last 20 years, various methods have been established for *Agrobacterium*- based gene delivery methods into plant genomes [**23**]. Recently, a method called agroinfiltration has been the most promising technology for transformation applications [**44**].

Syringe agroinfiltration

Syringe agroinfiltration is a useful tool for transient gene expression experiments as it allows for delivery of viral genes into the intercellular space of the plant genome [**38**]. This method is established in several plant species [**38**] and several advantages were demonstrated. Without the need for specialized equipment, it is a simple procedure having flexibility on infiltrating the whole leaf with one target DNA or infiltrating multiple DNAs into different areas of one leaf which allows multiple assays to be performed on a single leaf [**38**]. In addition, it is applicable in a broad range of studies including those examining plant pathogens, abiotic stresses, transgene silencing, localization and function of proteins, and interactions between different proteins [**44**]. Infiltration of an entire leaf can be performed for obtaining characterization of biochemical activity for recombinant proteins, purification, and preclinical functional studies [**20, 21**]. The use of a vacuum chamber was first established for plant species that do not respond well to syringe agroinfiltration [**38**]. First, the plant leaves were submerged into an infiltration media which contains the *Agrobacterium* strain that harbors the target recombinant DNA. Then the submerged plants are exposed to a negative atmospheric pressure in a vacuum chamber. The vacuum draws the air out of the interstitial spaces which are filled by *Agrobacterium*-containing media when the vacuum is released. Compared with syringe

infiltration, it is more complex and requires investments in vacuum pumps, vacuum chambers, and large volumes of *Agrobacterium* culture. Also, it's hard to conduct multiple assays on a single leaf. However, it gives a significant ability for expression of transgenes and analysis of protein function for plant species that do not work with syringe infiltration. As vacuum infiltration is more robust and allows for infiltration of many plants in a short period of time, it facilitates development of large amounts of proteins, which allows rapid production of human pharmaceuticals in plants.

Expression of transgenic proteins using Agroinfiltration

Agroinfiltration was known for having a higher level of transgene expression than that of stable transgenic plants [**38**]. Mostly this is because the transgene does not integrate into the plant nuclear genome randomly with uncertain transcriptional activities, which leads to an elimination of the "position effect" [**9,40**]. Since the amount of recombinant protein produced is limited, searching for an optimized expression of vectors can also help to achieve an even higher level of protein production.

Selection of vector on Agroinfiltration

In Agroinfiltration various vectors, including non-viral or plant virus-based vectors can be used [**44**]. While non-viral based vectors like 35S cauliflower mosaic virus (CaMV) promoter give a higher level of accumulation of proteins in the transient experiment rather than in stable transgenic plants, the yield of the target protein is still relatively low [**3**]. Since plant virus-based vectors have an efficient replication ability or transcription in plant cells they accumulate a higher level of recombinant proteins [**9, 28**]. The first successful expression of a transgene in plants was the use of CaMV replacement vector, in which the bacterial

dihydrofolate reductase gene replaced the insect transmission gene [**8**]. However, these viruses have limitations on their packaging capacity and easily lose their functions of essential genome when only a small amount of their genomes are removed or substituted. As a result, these vectors have a limitation for the size of the transgene and impact on the improvement of the transient expression system. The next generation was the use of viral vectors that are based on single-stranded RNA viruses. The abundance of these viruses has allowed the identification of viral vectors with a large packaging capacity and which are more tolerable for gene substitution and insertion. A study has shown the expression of various transgenes using replacement vectors that were based on tomato bushy stunt virus (TBSV) and tobacco mosaic virus (TMV) [**28**]. The viral coat protein (CP), which is essential for the cell-to-cell movement of many viruses, was used for the replacement of the target gene. If infection of the entire plant and the use of insertion vectors that contain the complete functional viral genome with the addition of the target gene is needed it gives a possibility of a loss of systemic infectivity [**28**]. Vectors derived from tobacco mosaic virus (TMV) and potato virus X (PVX), also allow the expression of the inserted transgene while retaining systemic movement and infection [**28**]. Introducing viral vectors into plant tissues by mechanical inoculation of infectious viral particles or viral nucleic acids presents several challenges to its application. For example- the virus which requires transmission by specialized insects cannot be used and using an RNA-virus-based vector requires a laborious and hard to achieve, *in vitro* process of generating RNA-based vectors. Limitations on the host range of viruses also present another barrier for its broad application, however, the use of agroinfiltration has effectively resolved problems associated with viral vector delivery. It delivers directly and efficiently the DNA or cDNA form to the nucleus of plant cells without an *in vitro* transcription process. Using the transcription and the processing activities of the host cell, infectious and autonomously replicating nucleic acid constructs are produced from the delivered DNA [**28**]. Since Agroinfiltration allows

eliminating the need for the viral systemic spreading function, the coat protein (CP) can be deleted, thus providing various options for selection of type and size of the transgene, and the concern about transgene loss during systemic spreading is also eliminated. In addition, Agroinfiltration also provides a tool for introducing viral vectors to a broad range of plant species beyond the natural virus hosts and to those that are not mechanically transmissible in nature.

CRISPR-Cas9

Microorganisms have developed a unique adaptive immune system known as CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat sequences with the protein Cas9), which is used by bacteria and archaea for defense against invading foreign DNA [**14**]. It is composed of a Cas9 nuclease, a target recognizing CRISPR RNA (crRNA), and auxiliary non-coding trans-activating crRNAs (tracrRNAs) [**22**]. The nuclease Cas12a was found in the CRISPR/Cpf1 system of the bacterium *Francisella novicida* [**49**]. Cas12a showed several differences from Cas9. It causes a 'staggered' cut in double stranded DNA as opposed to the 'blunt' cut produced by Cas9 and requires only a CRISPR RNA (crRNA) for successful targeting. In contrast, Cas9 requires both crRNA and a transactivating crRNA (tracrRNA). Cas12a's small crRNAs are ideal for multiplexed genome editing. The nuclease 13 is characterized in the bacterium *Leptotrichia shahii*. Cas13 is an RNA-guided RNA endonuclease, which does not cleave DNA, but only single-stranded RNA. Cas13 is guided by its crRNA to a ssRNA target and binds and cleaves the target. Like Cas12a, the Cas13 remains bound to the target and then cleaves other ssRNA molecules non-discriminately [**2**].

CRISPR allows us to generate genomic diversity for plant breeding. Shen et al. [**39**] edited eight yield and quality relevant genes in rice simultaneously. The mutation rates in transgenic rice were from 50 to 100%. They could isolate mutants having homozygous mutated

alleles of all eight targeted genes and homozygous octuple, septuple and sixtuple mutants as well as heterozygous mutants for all targeted genes. Another recent study showed that two QTLs regulating grain size (GRAIN SIZE3, *GS3*) and grain number (Grain number 1a, *Gn1a*) were edited in five different widely cultivated rice varieties [**40**]. Zhou et al. [**52**] also succeeded in simultaneous editing of three yield related QTLs in elite rice backgrounds by targeting the same two QTLs, *GS3* and *Gn1a*, and GRAIN WIDTH and WEIGHT 2 (*GW2*).

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CHAPTER II

REGENERATION OF TWO CHILI PEPPER (*CAPSICUM SPP.* L.) CULTIVARS

Introduction

Pepper (*Capsicum* spp.) belongs to the family Solanaceae which includes tomato, potato, tobacco, and petunia. The genus *Capsicum* consists of 33 wild plant species and 5 domesticated species: *C. annuum, C. baccatum, C. chinense, C. frutescens,* and *C. pubescens* [**9**]. Pepper is an essential spice used as a basic ingredient in various cuisines around the world. It is widely spread all over the world and is rich in vitamins and minerals such as vitamin A, B-complex, C, E, manganese, and potassium [**6, 42**].

Pepper has both agronomic and economic value, so it is necessary to have an efficient system to generate various pepper cultivars for human consumption. Although agronomic traits in peppers have been introduced by conventional breeding, it still has a limitation of genetic improvement due to sexual incompatibility between the various domesticated species [**26**]. Pepper is well known to be a recalcitrant species for *in vitro* manipulation and regeneration. Some studies reported the regeneration of pepper using different types of explant sources such as shoot tip [**10**], rooted hypocotyls [**44**], cotyledon, hypocotyl, embryo [**2**] and somatic embryogenesis [**25**]. As regeneration is highly dependent on genotype, explant, and the condition of medium [**18, 28, 35, 36**], the establishment of an efficient regeneration system by *in vitro* tissue culture is critical for the creation of genetically diverse peppers.

Developing an established tissue culture system for multiple pepper genotypes will be useful for genetic manipulation in peppers using micropropagation, plant transformation, and CRISPR. Micropropagation is the technology of multiplying stock plant material rapidly in order to produce multiple progeny plants by plant tissue culture methods. It can be used to supply many plantlets from a stock plant which produces few seeds and does not respond well

to vegetative reproduction for planting [**39**]. Plant transformation is the method to insert DNA from one plant into the genome of another plant and can be used to research the effect of certain genes in crops, and to improve crop traits including nutrient, yield, disease resistance, and stress tolerance [**23**]. *Agrobacterium* can be used for transforming the plants. *Agrobacterium* is a soil bacterium, which has the capability of transferring its own DNA into plant cells. *Agrobacterium* containing genes of interest are inoculated onto wounded plant tissue explants, and *Agrobacterium* then transfers the gene of interest into the DNA of the plant tissue [**16**]. CRISPR (clustered regularly interspaced short palindromic repeats) uses a pair of molecular scissors which snip DNA in certain places to remove unwanted parts or edit existing sequences [**4**]. The CRISPR-Cas (CRISPR-associated) system is a prokaryotic immune system which provides resistance to foreign genetic elements in plasmids and phages [**5**]. RNA containing the spacer sequence makes Cas proteins recognize and cut out foreign pathogenic DNA [**31**]. The CRISPR gene editing system has a variety of applications such as crop breeding, basic biological research, and disease treatment and generally utilizes the cas9 gene [**47**].

In this study, we sought to establish an efficient tissue culture system using two unique pepper genotypes; A108 and Hab51p2-1, which are genetically very distinct from *C. annuum* lines reported in the previous literature. We hope that our method will provide a tool for genetic manipulations and biotechnology for these breeding lines since none of the tissue culture systems previously reported used *Capsicum annuum x C. baccatum* species.

Materials and methods

Seeds

Seeds of two different genotypes of chili Pepper (*Capsicum spp.*), A108 and Hab51p2-1, were used. A108 is an interspecific hybrid between *Capsicum annuum* x *C. baccatum*, a BC_2F_3 line, and Hab51p2-1 is an Elite, F_{10} inbred line of *C. chinense* were used in this experiment. All the seeds were obtained from the Texas A&M University Horticulture Teaching, Research and Extension Center (HortTREC) (College Station, TX).

Preparation of MS (Murashige and Skoog) medium

MS medium (PhytoTechnology Laboratories, Shawnee Mission, KS, USA) containing agar and sucrose was dissolved in distilled water in a flask and was adjusted to pH 5.8 with 1N NaOH (Sigma-Aldrich, St. Louis, Missouri, USA) before being autoclaved at 121℃ for 20 minutes. The autoclaved MS medium was dispensed into a petri dish.

Germination

Seeds (A108 & Hab51p2-1) were surface sterilized with 70% ethyl alcohol for 5 minutes and then the seeds were soaked in 0.2 % Mercury (II) chloride ($HgCl_2$) (Sigma-Aldrich, St. Louis, Missouri, USA) for 5 minutes with continuous stirring. The seeds were rinsed with double distilled water for 5 minutes 3 times and were incubated in MS media containing 0.8% agar and 1% sucrose at 24±2℃ in the dark for 14 days. The germinated seeds were used as explant sources for regeneration.

Callus induction

The cotyledonal leaves and hypocotyls from the germinated seedlings were excised into 2 mm pieces and incubated in solid MS media supplemented with 2mg/L Zeatin (Sigma-Aldrich, St. Louis, Missouri, USA), 0.5mg/L BAP(Sigma-Aldrich, St. Louis, Missouri, USA), 0.5mg/L Kinetin (Sigma-Aldrich, St. Louis, Missouri, USA), and 0.5 mg/L IAA (Sigma-Aldrich**,** St. Louis, Missouri, USA) in a petri dish at 24±2℃ in the dark.

Shoot and root induction

White fragile calli were selected after 6 weeks and were incubated in MS basal salt supplemented with 2mg/L Zeatin, 0.5mg/L BAP, 0.5mg/L Kinetin, and 0.5mg/L IAA in a petri dish at 24±2℃ under 24h light with intensity of 200μmol $m^{-2}s^{-1}$. Calli with shoots were used for the induction of roots in MS basal media containing 3% sucrose under 24±2℃, 24h under light, intensity of 200μmol $m^{-2}s^{-1}$ for 5 weeks.

Acclimatization

Regenerated plants with successful establishment of roots were incubated in 1:1 ratio of MS basal salt and vermiculite (Palmetto, U.S) in the culture room under 16/8h light/ dark conditions.

Statistics analysis

Statistical analysis was performed on % data between A108 and Hab51p2-1 cultivars using student's T test of JASP 0.14 (https://jasp-stats.org/). A P-value less than 0.05 was considered statistically significant.

Results

In vitro regeneration of two chili pepper cultivars

It is known that pepper is harder to be regenerated than other solanaceous crops such as tobacco, tomato, and potato [**27**]. We tried to establish efficient regeneration systems for two pepper genotypes, A108 and Hab51p2-1, which can be used for genetic manipulation of pepper genes using genetic engineering technologies such as *Agrobacterium*-mediated transformation and CRISPR gene editing.

Seeds of A108 (**Fig. 3a & 3b**) and Hab51p2-1 (**Fig. 3c & 3d**) cultivars were germinated for 14 days.

Cotyledons (**Fig. 4a**) and hypocotyls (**Fig. 4c**) from germinated seedlings of A108 were induced for calli for 7 weeks. The calli of cotyledons and hypocotyls were then treated for shoot and root induction. Shoots and roots were induced in calli of cotyledons (**Fig. 4b**) and hypocotyls (**Fig. 4d**) 5 weeks after incubation.

Cotyledons (**Fig. 5a**) and hypocotyls (**Fig. 5c**) from germinated seedlings of Hab51p2-1 were induced for calli for 7 weeks. The induced calli of cotyledons and hypocotyls were induced for shoots and roots. Shoots and roots were induced in calli of cotyledons (**Fig. 5b**) and hypocotyls (**Fig. 5d**) 5 weeks after incubation.

We calculated the success rate for induction of cotyledons and hypocotyls of both cultivars, A108 and Hab51p2-1 (**Table 1 & 2**). When we planted cotyledons of A108 and Hab51p2-1 on MS agar (**Table 1**), calli were formed in 58 (96.6%) out of 60 explants from A108, and 103 (100%) out of 103 explants from Hab51p2-1. When roots and shoots were induced on MS agar, roots were induced in 9 (15.5%) out of 58 calli of

A108 and in 11 (10.6%) out of 103 calli of Hab51p2-1, shoots were induced in 7 (12.06%) out of 58 calli of A108 and in 23 (22.3%) out of 103 calli of Hab51p2-1, and both roots and shoots were induced in 6 (10.3%) out of 58 calli of A108 and in 6 (5.8%) out of 103 calli of Hab51p2-1. When we planted hypocotyls of A108 and Hab51p2-1 on MS agar (**Table 2**), calli were formed in 69 (95.8%) out of 72 explants from A108, and 78 (96.2%) out of 81 explants from Hab51p2-1. When roots and shoots were induced on MS agar, roots were induced in 25 (36.2%) out of 69 calli of A108 and in 19 (24.3%) out of 78 calli of Hab51p2-1, shoots were induced in 24 (34.7%) out of 69 calli of A108 and in 18 (23.0%) out of 78 calli of Hab51p2-1, and both roots and shoots were induced in 6 (8.6%) out of 69 calli of A108 and in 10 (12.8%) out of 78 calli of Hab51p2-1.

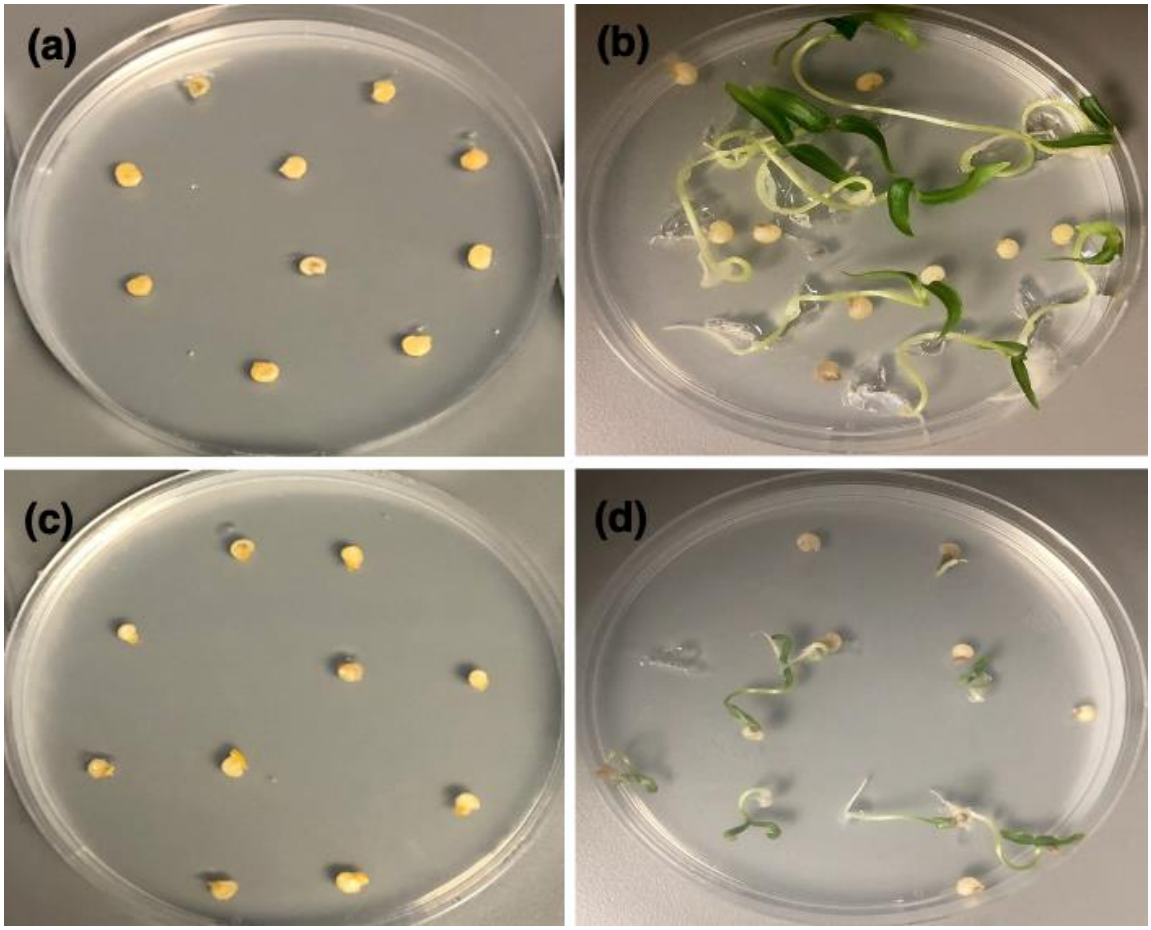

Figure 3. Germination of two pepper cultivars, A108 and Hab51p2-1.

Seeds (A108 & Hab51p2-1) were surface sterilized and were incubated in MS media with 0.8% agar and 1% sucrose at 24±2℃ in the dark for 14 days.

(a) Germination of A108 0 days after incubation; **(b)** Germination of A108 14 days after incubation; **(c)** Germination of Hab51p2-1 0 days after incubation; **(d)** Germination of Hab51p2-1 14 days after incubation

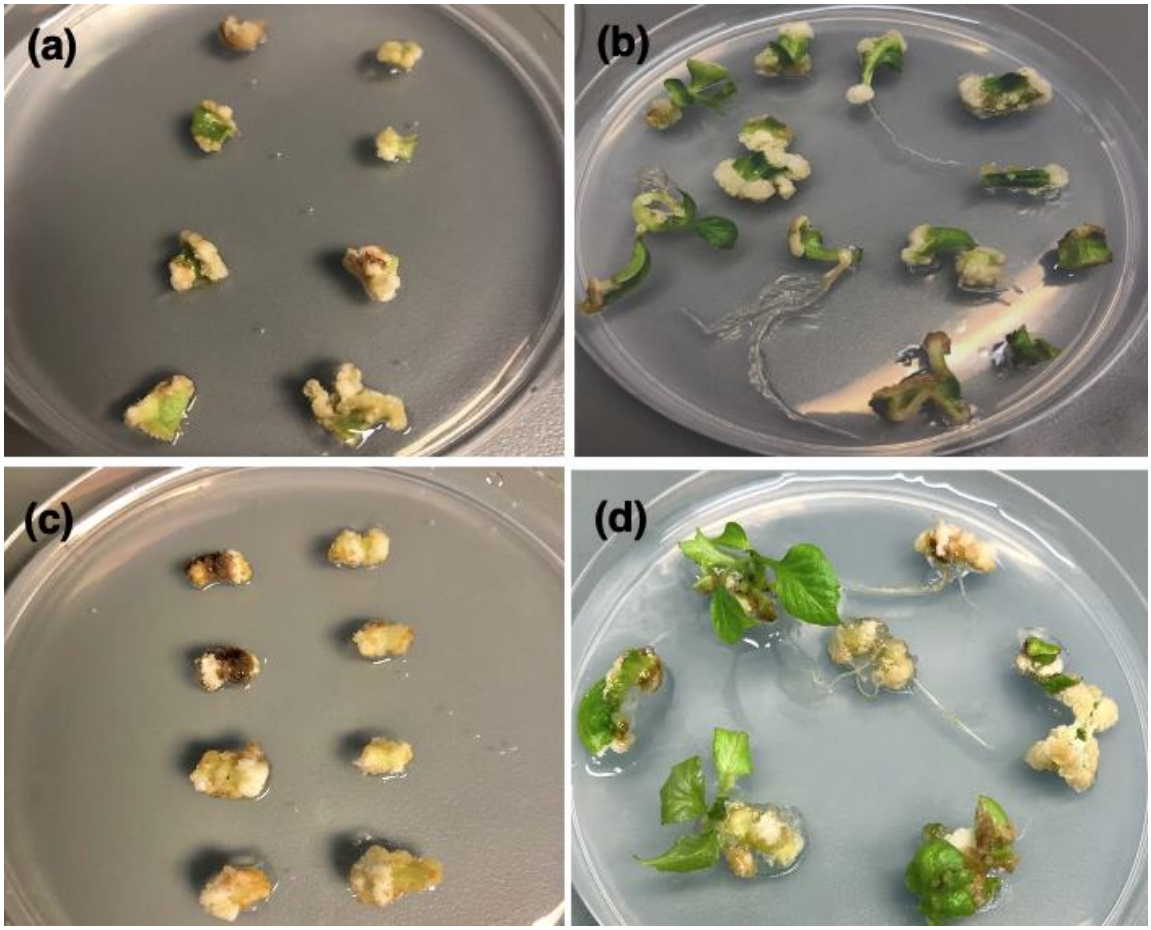

Figure 4. Callus, shoot, and root induction in A108 explants. The callus was induced in the excised cotyledonal leaves and hypocotyls from the germinated seedlings of A108 cultivar on solid MS media supplemented with 2mg/L Zeatin, and 0.5mg/L each of BA, Kinetin, and IAA in a petri dish, and calli were further incubated on MS basal salt supplemented with 2mg/L Zeatin, and 0.5mg/L each of BA, Kinetin, and IAA to produce shoots. Roots were induced in calli with shoots on MS basal media containing 3% sucrose.

(a) Callus induction of A108 cotyledons 7 weeks after incubation; **(b)** Shoot and root induction of A108 from cotyledon callus 5 weeks after incubation; **(c)** Callus induction

of A108 hypocotyls 7 weeks after incubation; **(d)** Shoot and root induction of A108 from hypocotyl callus 5 weeks after incubation

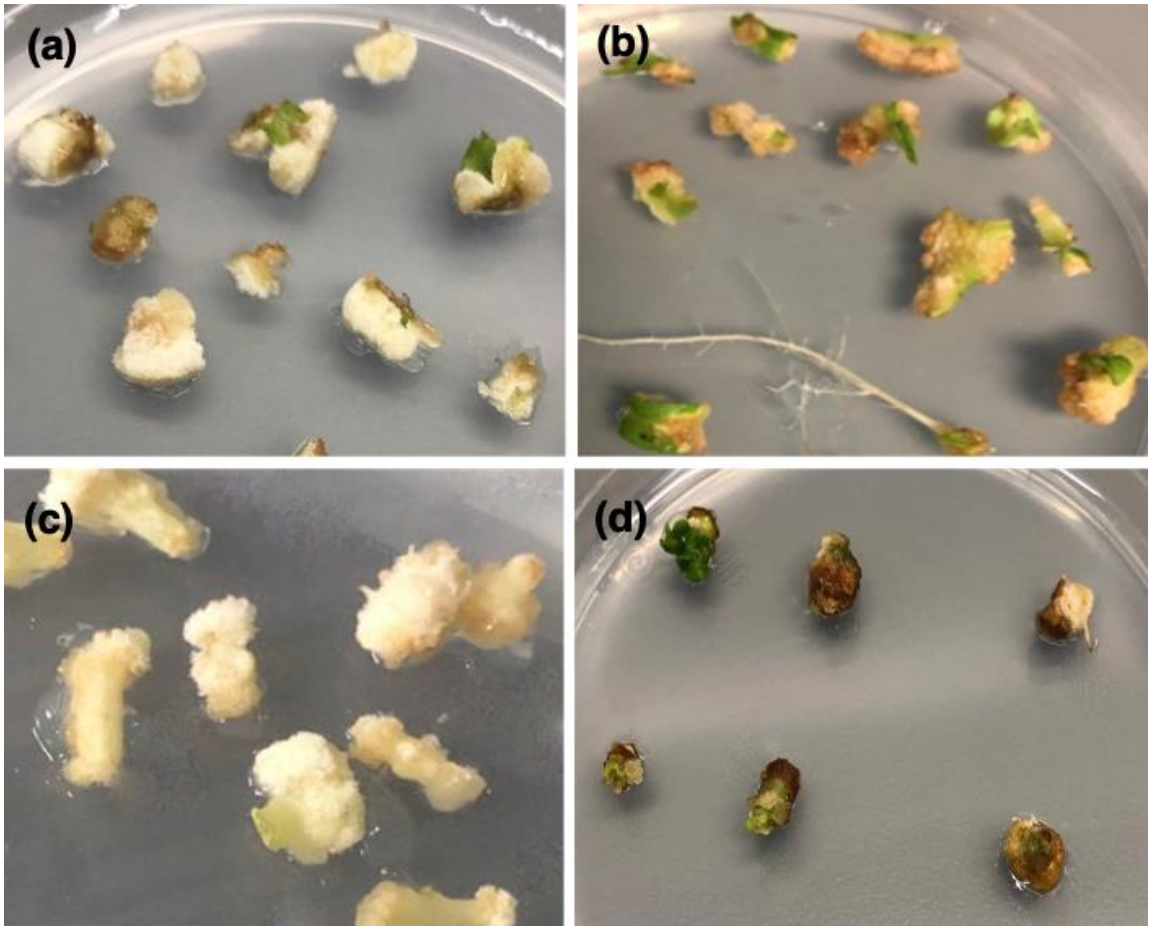

Figure 5. Callus, shoot, and root induction in Hab51p2-1 explants. The callus was induced in the excised cotyledonal leaves and hypocotyls from the germinated seedlings of Hab51p2-1 cultivar on solid MS media supplemented with 2mg/L Zeatin, and 0.5mg/L each of BA, Kinetin, and IAA in a petri dish, and calli were further incubated on MS basal salt supplemented with 2mg/L Zeatin, and 0.5mg/L each of BA, Kinetin,

and IAA to produce shoots. Roots were induced in calli with shoots on MS basal media containing 3% sucrose.

(a) Cotyledon callus induction of Hab51p2-1 7 weeks after incubation; **(b)** Shoot and root induction from cotyledon callus of Hab51p2-1 5 weeks after incubation; **(c)** Hypocotyl callus induction of Hab51p2-1 7 weeks after incubation; **(d)** Shoot and root induction of Hab51p2-1 from hypocotyl callus 5 weeks after incubation

Summary of callus and regeneration hormone concentrations is described in Table 3 and Table 4, respectively.

Table 1. Induction rate of callus, roots, and shoots in cotyledons of two chili cultivars

Pepper seed	Callus		Regeneration: Formation of roots and shoots					
			Root		Shoot		Root & shoot	
	success no./total no.	%	success no./total no.	%	success no./total no.	%	success no./total no.	%
A108	58/60	96.6	9/58	15.5	7/58	12.06	6/58	10.3
Hab51p2-1	103/ 103	100	11/103	10.6	23/103	22.3	6/103	5.8
P-value	<0.001		0.013		0.031		0.019	

Table 2. Induction of callus, roots, and shoots in the hypocotyls of two chili cultivars

Pepper seed	Callus		Regeneration: Formation of roots and shoots					
			Root		Shoot		Root & shoot	
	success no./total no.	%	success no./total no.	%	success no./total no.	%	success no./total no.	%
A108	69/72	95.8	25/69	36.2	24/69	34.7	6/69	8.6
Hab51p2-1	78 / 81	96.2	19/78	24.3	18/78	23.0	10/78	12.8
P-value	<0.001		0.051		0.035		0.015	

Table 3. Callus Induction hormone combination

	Kaaby, E. *et al.* 2015 [**22**]	Prakash, A. H. *et al.* 1997 [**37**]	Ko, M.K. *et al.* 2007 [**26**]	Current study
Zeatin	-	-	2mg/L	2mg/L
BAP	-	0.5 mg/L	-	0.5mg/L
Kinetin	2mg/L	-	-	0.5mg/L
NAA	-	2mg/L	-	-
IAA	2mg/L	-	0.2mg/L	0.5mg/L

Table 4. Regeneration hormone combination

	Valera-Montero *et al.* 1992 [**44**]	Sanatombi *et al.* 2008 [**40**]	Ko, M.K. *et al.* 2007 [**26**]	Current study
Zeatin	-	91.2µM	2mg/L	2mg/L
BAP	5mg/L	31.1 µM	-	0.5mg/L
Kinetin	-	4.7 µM	-	0.5mg/L
NAA	-	-	-	-
IAA	0.3mg/L	-	0.05mg/L	0.5mg/L

Acclimatization of regenerated plantlets

Elongated and wholly regenerated plantlets from cotyledons of A108 (**Fig. 6a**) were acclimatized in MS basal salt containing vermiculite (**Fig. 6b**). Elongated and wholly regenerated plantlets from cotyledons of Hab51p2-1 (**Fig. 6c**) were acclimatized in MS basal salt containing vermiculite (**Fig. 6d**). The acclimatized plantlets survived for only 3-4 weeks.

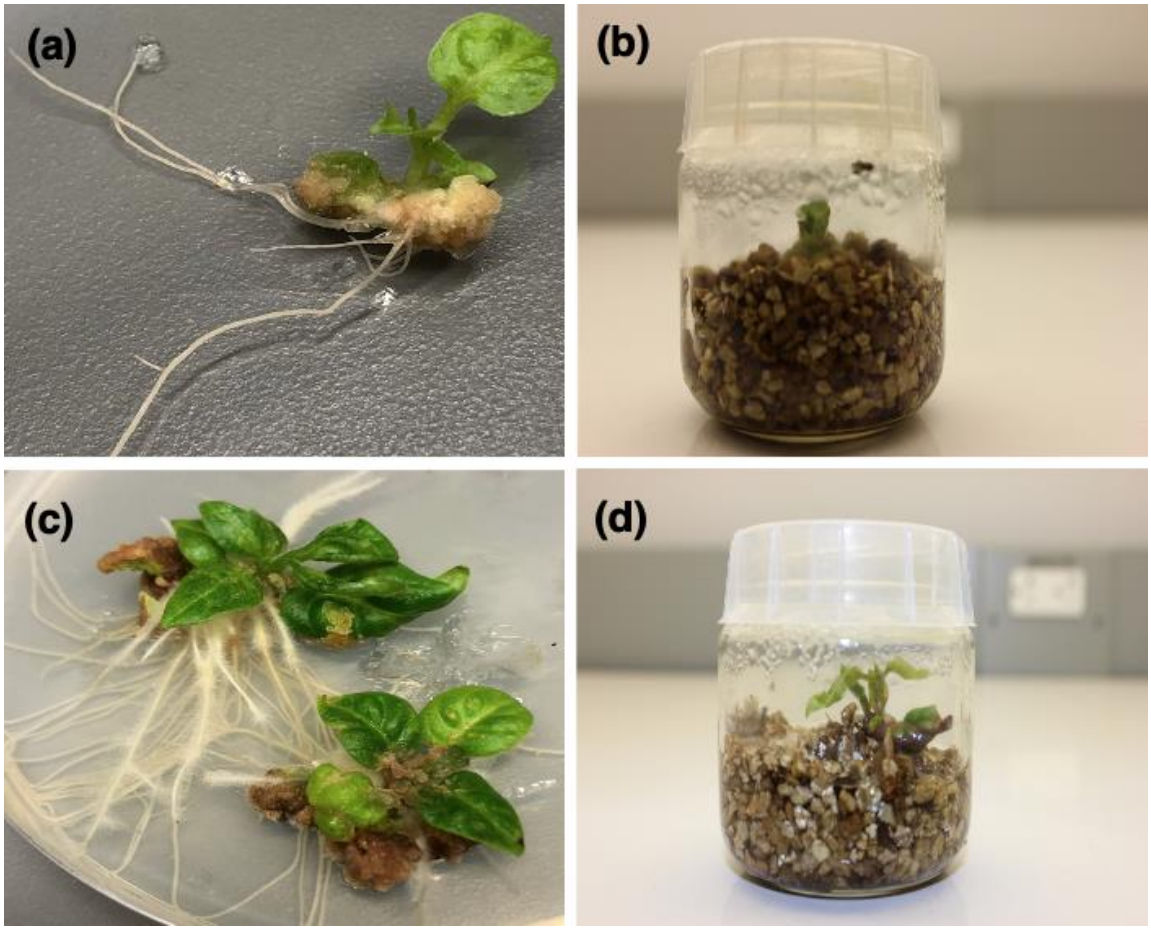

Figure 6. Acclimatization of regenerated pepper plantlets. The regenerated plants with shoots and roots were acclimatized in MS basal salt with vermiculite. **(a)** Whole

regenerated pepper of A108 cotyledon 13 weeks after incubation; **(b)** Acclimatized A108; **(c)** Whole regenerated pepper of Hab51p2-1 cotyledon 13 weeks after incubation; **(d)** Acclimatized Hab51p2-1

Discussion

In this study, we successfully established a regeneration system for two chili peppers. This is the first report of a tissue culture system of *Capsicum annuum* x *C. baccatum* pepper.

Since pepper is one of the essential ingredients in human diets, and is being consumed by humans in many countries around the world, the developed efficient regeneration systems for peppers can facilitate the creation of a variety of peppers containing diverse traits, which are useful to growers and beneficial to human health.

Previous studies faced fungal contamination while studying the regeneration of peppers [**33**]. Various studies treated seeds with commercial bleach (sodium hypoclorite) to eliminate contamination at the sterilization process [**3, 7, 22, 26, 41, 45**].

A previous study reported the steilization process for tissue culture for leaf, nodal, and seed explants while transferring field sources into tissue culture on *Aquilaria malaccensis* using Clorox® bleach, $HgCl_2$, and Benomyl. Highest 'clean and alive' percentage for seed sterilization of 90.0 ± 1.9^a was achieved when using 0.2% $HgCl_2$ for 12 minutes [**14**].

We successfully induced calli from the explants of cotyledons and hypocotyls of seedlings that were germinated from seeds on MS medium containing 1% sucrose and 0.8% agar. When we used 3% sucrose, the germination was also sucessful, but the seeds started to germinate 4 to 5weeks after germination. It took 9 to 10 weeks to obtain fully germinated seedlings, while it only took 2 weeks to get full germination using a 1%

sucrose concentration from our optimized protocol. It seems that the higher sucrose concentration is responsible for the delay in germination.

Organogenesis in *Capsicum* was reported using explants from cotyledon, hypocotyl, leaf, shoot tip, embryo, root, and seed explants [**8, 15, 17, 27**]. When we used shoot tips and leaf discs no response for regeneration was detected.

Studies reported that the use of cotyledonary leaves and hypocotyls as an explant served as the best sources for regeneration [**19, 30, 38**]. When we tested using these explants, we obtained a similar result and demonstrated that the hypocotyl provides better performance for regeneration.

For induction of callus, we combined the plant hormones auxin and cytokinin, using 2mg/L Zeatin, and 0.5mg/L BAP, 0.5mg/L Kinetin, and 0.5mg/L IAA and incubating in the dark. This combination of hormones is different than those reported in previous studies which used 2mg/L IAA and Kinetin [**22**], 2mg/L NAA and 0.5mg/L BAP [**37**], and 2mg/L Zeatin, 0.2 mg/L IAA in [**26**].

For induction of shoots, we added 2,4-D hormone with our hormone combinations such as 2mg/L Zeatin, 0.5mg/L 2,4-D, 0.5mg/L BAP, 0.5mg/L Kinetin, as described [**2, 22**]. We deteced watery calli in two different types of explants which were not able to induce shoots. Also, previous study reported $AgNO_3$ promotes the highest induction rate of shoots in peppers [**18, 20**]. However, other papers reported the drawbacks for $AgNO_3$ compound which acts as an inhibitor of ethylene activity, and which causes chlorosis, primordial leaf abscission and loss of plant vigor [**32, 34**].

Since previous studies reported that BAP hormone is the most effective on shoot bud induction compared to any other cytokinin-related hormones [**19, 21, 40**], we included BAP for inducing shoots. Agarwal et al. [**1**] reported that high levels of BAP (25.0 µM) or combinations with IAA or IBA induce shoots for explants of shoot tips in *C. annuum* L. var. 'Mathania'.

Some papers have reported the formation of rosette-like shoots that were unable to elongate, as a phenomenon frequently observed during *in vitro* culture of chili using explants of cotyledons [**2, 13**]. However, based on our hormone compositions no rosette-like leaf structure was detected or formed and the shoots that were induced were able to elongate.

The acclimatized two genotypes of peppers did not survive over 4 weeks. Suggesting that the development of the optimal root system and soil conditions is a critical step for increasing survival rate of regenerated pepper plantlets. The study of callus induction from explants of fruit and seedlings from chili pepper showed that callus was induced from all explants cultured on MS medium supplemented with IAA and Kinetin [**22**]. However, the acclimatization was not successful.

We found that some previous studies were not successful in developing a tissue culture system for peppers. Calli alone were induced on mature leaf explants of pepper cultivars, Edino and Brujo on MS agar supplemented with BAP, indole-3-butyric acid (IBA) or 2,4-dichlorophenoxyacetic acid (2,4-D) [**24**]. Hypocotyl explants from *Capsicum annuum* and *Capsicum frutescens* were planted on MS medium supplemented with 2.5mg/L NAA and 2.5 mg/L BAP and only calli were induced well [**29**].

The developed novel regeneration system in two distinct genotypes of peppers can be applied to embryo rescue [**11, 43**], genetic manipulation [**46**], and to biotechnology [**12**]. In conclusion, we successfully regenerated two pepper inbred lines, A108 and Hab51p2-1 and we hope that our work contribute to the development of pepper regeneration system.

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CHAPTER III

AGROBACTERIUM-MEDIATED TRANSIENT EXPRESSION OF TWO CHILI PEPPER (*CAPSICUM SPP.* L.) CULTIVARS

Introduction

Pepper (*Capsicum spp.*) is an essential spice and condiment for various cuisines in the World [**11**]. It has been used as a seasoning, and medicinal property, fresh and processed vegetables, spice, dried forms, food dye, and plants for pharmaceutical and cosmetic properties [**6,14**]. Peppers contain healthy minerals, vitamins, amino acids, and phytochemicals such as phenolics and flavonoids, which function as antioxidants lessening deteriorative diseases [**13,16,22**].

Agrobacterium tumefaciens has been used for plant genetic transformation to produce proteins [**21,22,26**]. Genetic transformation can be a valuable method for the assessment of gene function. *Agrobacterium* integrates the virulent DNA into the plant genome [**29**]. *Agrobacterium*-mediated stable genetic transformation has been successful on several species in the Solanaceae family [**11,13**]. The genus *Capsicum* is hard for stable genetic transformation with a low frequency of regeneration rate (0.5~1%) [**4,9,10,29**].

The transient expression methods like protoplast transformation [**20**], biolistics [**19**], and agroinfiltration [**8**] are simple techniques, which make broad leaf parts transformed [**25**]. Microinjection agroinfiltration by infiltrating *Agrobacterium tumefaciens* cultures through plant leaves allows transient expression. Agroinfiltration

transformation has been reported in many plant species such as *Arabidopsis thaliana* [**15,25**], tomato [**17,26,30**], lettuce [**29**], pepper [**9**] potato [**26**], common bean (*Phaseolus vulgaris*) [**22,25**] soybean (*Glycine max*) [**17,22**], and pea (*Pisum sativum*) [**18,25,30**].

In this study, we used the agroinfiltration method to perform transient expression of the enhanced green fluorescent protein (EGFP) and β-glucuronidase (GUS) genes with two *Agrobacterium* strains in two pepper genotypes to establish a pepper transient transformation system.

Material and Methods

Plant material

Two different genotypes of chili Pepper (*Capsicum spp.*), A108 and Hab51p2-1, were used. A108 is an interspecific hybrid between *Capsicum annuum* x *C. baccatum*, a BC_2F_3 line, and Hab51p2-1 is an Elite, F_{10} inbred line of *C. chinense*. Both peppers were grown in the greenhouse using soil mixtures of Potting mix (Promix, USA), Vermiculite (Palmetto, USA), and Perlite (Aero-soil, USA) at a ratio of 2:0.5:1. Fully established leaves of two peppers were used for agroinfiltration.

Vector

Vector CGEL-21 harboring the CaMV 35S promoter, enhanced green fluorescent protein (EGFP) gene, and the β-glucuronidase (GUS) gene was kindly provided by the Crop

Genome Editing Laboratory (CGEL), Texas A&M AgriLife Research Center, College Station, TX.

Transformation of *Agrobacterium tumefaciens*

Two different types of *Agrobacterium* strains; AGL-1 and GV3101 were used in this experiment. All strains were transformed with vector CGEL-21 using the freeze-thaw method [**28**]. *A. tumefaciens* strains AGL-1 and GV3101 harboring CGEL-21 vector were streaked on an LB (Luria-Bertani) media plate containing the antibiotics rifampicin (10ug/ml), kanamycin (50ug/ml), carbenicillin (50ug/ml), and gentamycin (100ug/ml). Colonies of AGL-1 containing CGEL-21 encoding the EGFP and GUS genes were inoculated into liquid LB media (10g/L tryptone, 10g/L NaCl, pH 7, 5g/L yeast extract) containing rifampicin (10ug/ml), carbenicillin (50ug/ml), and kanamycin (50ug/ml). GV3101 containing the CGEL-21 vector encoding the EGFP and GUS genes was inoculated in LB medium containing gentamicin (100ug/ml) instead of carbenicillin. The cultures were then grown in a shaking incubator at 29°C, 250rpm, overnight. After the culture became cloudy, the culture was pelleted at 25°C, 4,000 rpm for 20 minutes.

Infiltration Media

After centrifugation of the culture, the pellet was washed 2 times in infiltration medium (IFM) (10mM MES pH 5.6, 10mM $MgCl_2$, 150µM Acetosyringone) and then it was resuspended in 10ml of IFM. The final concentration was matched to 0.4~0.5 at OD_{600} by diluting it in IFM. The final cultures were then incubated for 2 hours at room temperature in a shaker at 80 rpm before use.

Delivery of transformed bacteria into pepper

The activated transformed bacteria in IFM were delivered using a needle-less syringe by directly applying it to the abaxial side of 6~7 week-old leaves. After infiltration, the plants were then incubated for 5 days at room temperature. This process was repeated at least 3 times.

EGFP microscopy

EGFP signals were detected with an Olympus SZX10 fluorescence microscope under a blue light equipped with GFP and GFPA filter having a light exposure time of 1 second. Only the GFPA filter blocks the green autofluorescence of chlorophyll.

Histochemical GUS assay

Detection of the activity of GUS was performed as described in [**5**]. We used formaldehyde instead of glutaraldehyde. The chlorophyll was completely removed using 200-proof ethanol for 3 days.

EGFP and GUS gene confirmation of transformed peppers by PCR

Leaf tissues were collected where the EGFP and GUS signal had been detected by fluorescence microscopy. Total genomic DNAs were extracted using a CTAB protocol [**1**]. The forward primer sequence and reverse primer sequences for EGFP and GUS are shown in **Table 5**. The PCR reactions were carried out in a total volume of 25 μL containing 12.5 μL of 2x KAPA Plant PCR Buffer (Sigma-Aldrich, St. Louis, Missouri, USA), 0.2 μL 2.5U/μL KAPA 3G Plant DNA Polymerase (Sigma-Aldrich, St. Louis, Missouri, USA), 0.75 μL 10μM Forward Primer, 0.75 μL 10μM Reverse Primer, 9.8 μL Nuclease free H_2O, and 1 μL genomic DNA (100ng). The reactions were amplified in a thermal cycler; 1 cycle of 3 minute at 95℃, followed by 25 cycles of 20 seconds at 95℃, 15 seconds at 58℃, and 15 seconds at 72℃, with a final 1 cycle of 1 minute at 72℃ before soaking at 4℃. The reaction products were then separated in a 1.0% agarose gel containing SYBR™ safe DNA gel stain (10,000x) (100V, 40 minutes). 1 kb plus DNA ladder (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used for sizing the PCR products which were recorded using a ChemiDoc system.

Table 5. Primer sequences

Primer name	Forward	Reverse
Actin	tct cct gaa gag cac cct gt	tac atg gca ggg aca ttg aa
β-tubulin	gga gat gtt cag gag ggt ga	atg ttg ctc tcg gct tca gt
EF1α	gcc tca aac tcc aag gat ga	ggc tcc ttc tcg agt tcc tt
EGFP (NT-143, NT-144)	ctt gac cat ggt aga tct gag g	cgg ctt tgc ctt gaa agt cc
GUS (NT-39, NT- 40)	gca cca tca aga cgt tct cc	ctt ctg tgg gtc gag ttc ct

Confirmation of housekeeping gene amplification

Total genomic DNAs of wild-type pepper leaves were extracted using the CTAB protocol [**1**]. PCR reactions were composed of 12.5 μL 2x KAPA Plant PCR Buffer (Sigma-Aldrich, St. Louis, Missouri, USA), 0.2 μL 2.5U/μL KAPA 3G Plant DNA Polymerase (Sigma-Aldrich, St. Louis, Missouri, USA), 0.75 μL 10μM Forward Primer, 0.75 μL 10μM Reverse Primer, 9.8 μL Nuclease free H_2O, and 1 μL genomic DNA (100ng). The housekeeping gene primers are shown in **Table 5**. The reactions were amplified in a thermal cycler; 1 cycle of 3 minute at 95℃, followed by 28 cycles of 20 seconds at 95℃, 15 seconds at 58℃, 15 seconds at 72℃, and a final 1 cycle of 1 minute at 72℃ before soaking at 4℃. The reaction products were then separated on a 1.0% agarose gel containing SYBR™ safe DNA gel stain (10,000x) (100V, 35 minutes). 1 kb plus DNA ladder (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used for sizing the PCR products which were recorded using a ChemiDoc system.

Real-Time qPCR

Total RNA was isolated using the GeneJET Plant RNA Purification kit (Thermo Scientific™) using 100 mg of frozen (-80℃) powdered leaf tissues according to the manufacturer's protocol. All RNA samples were treated with RapidOut DNA removal kit (Thermo Scientific™, Waltham, Massachusetts, USA) to avoid any presence of genomic DNAs and DNase in the samples. Electrophoresis was used to validate the integrity of RNA as described [**24**]. RNA concentrations were recorded with a Nanodrop spectrophotometer (ThermoScientific™). Primers for housekeeping genes were designed through Primer3 software. RT-qPCR was performed with the CFX96 Touch Deep Well Real-Time PCR Detection System (Biorad, Hercules, California, USA), using SuperScript™ III Platinum™ SYBR™ Green One-Step qRT-PCR Kit (Invitrogen™, Waltham, Massachusetts, USA) with total volume of 20 µl containing 0.4 µl SuperScript® III RT/Platinum® *Taq* Mix (includes RNaseOUT™), 10 µl 2X SYBR® Green Reaction Mix, 0.4 µl of each primer (10 µM), 8 µl total RNA (10ng), and 0.8 µl nuclease-free water. Reaction conditions were 50℃ for 3 minutes, followed by 40 cycles of 95℃ for 15 seconds and 60℃ for 30 seconds, with a final cycle of 40℃ for 1 minutes. Five biological and three technical replicates were used for the housekeeping gene, *actin* and the GUS gene (160bp) (**Table 5**). Since all three housekeeping genes were expressed in the two pepper species, we arbitrarily only selected *actin* for the housekeeping gene and used GUS gene to demonstrate the normalized gene expression level for two different genotypes of transformed peppers under qPCR.

Statistical analysis

Differences between the transformed and non-transformed groups were analyzed using Student's *t*-test with IBM SPSS Statistics version 20. *P*-values of less than 0.05 were considered statistically significant.

Results

Detection of EGFP and GUS genes in transformed pepper leaves by PCR.

The leaves of two chili peppers, A108 and Hab51p2-1, were infiltrated with two different types of *Agrobacterium* strains harboring CGEL-21 vectors encoding the EGFP and GUS genes. Genomic DNA was isolated from the transformed leaves of peppers after 5 days of infiltration. The EGFP and GUS genes were amplified using gene-specific primers (**Table 5**) (**Fig. 7 & Fig. 8**). The 160 bp and 861 bp fragments of the GUS and EGFP genes were amplified by PCR, respectively (**Fig 7. & Fig. 8**).

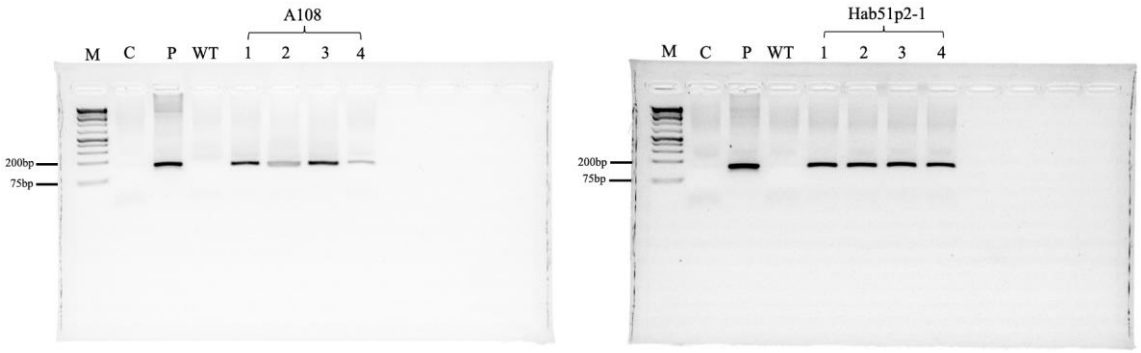

Figure 7. The detection of the GUS gene in transformed pepper leaves

Genomic DNA was collected from transformed leaves after 5 days of agroinfiltration. PCR was performed using GUS-specific primers. The PCR product size was 160 bp. M= 1kb plus DNA ladder (ThermoFisher Scientific™), C = Negative control, P= Plasmid DNA. WT = non-transformed.

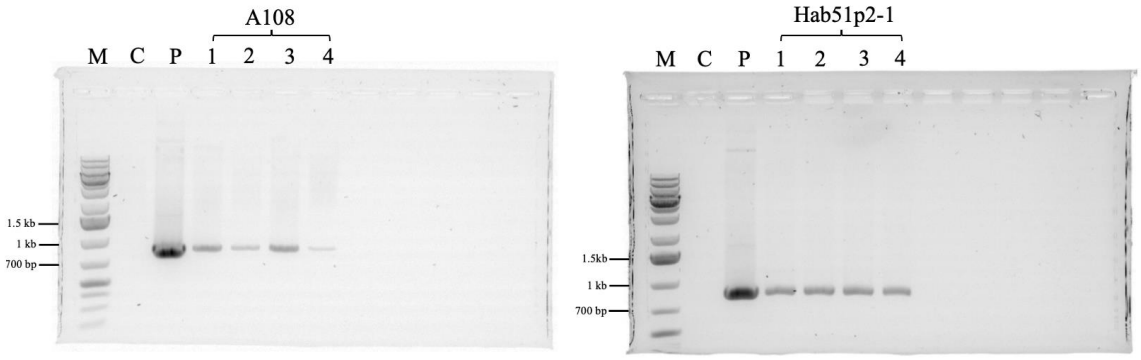

Figure 8. The detection of the EGFP gene in transformed pepper leaves

Genomic DNA was collected from transformed leaves after 5 days of agroinfiltration. PCR was performed using EGFP-specific primers. The PCR product size was 861 bp. M= 1kb plus DNA ladder (ThermoFisher Scientific™), C = Negative control, P= Plasmid DNA.

Morphology and detection of EGFP and GUS protein expression in the transformed pepper leaves.

The leaves of two chili peppers, A108 and Hab51p2-1, were infiltrated with *Agrobacterium* strain AGL-1 or GV3101 harboring the CGEL-21 vector, which encodes the EGFP and GUS genes. Expression of both genes were measured after 5 days of infiltration using a microscope under bright filter with an exposure time of 500ms and with GFP/GFPA filters with 1s of exposure time.

The transformed leaves of two peppers showed necrosis, while no necrosis was observed in non-transformed leaves of the two different peppers (**Fig. 9**)

The expression of EGFP was detected under the fluorescence microscope using GFP and GFPA filters with an exposure time of 1s. Yellow fluorescence was expressed in transformed A108 which was infiltrated with the AGL-1 *Agrobacterium* strain containing the CGEL-21 vector encoding the EGFP and GUS genes (**Fig. 10**.). GV3101 *Agrobacterium* strain containing the CGEL-21 vector encoding the EGFP and GUS genes also showed similar expression in A108 pepper leaves (**Fig.11**). Leaves of Hab51p2-1 pepper which were transformed with AGL-1 *Agrobacterium* strain containing the CGEL-21 vector encoding the EGFP and GUS genes showed fluorescence (**Fig. 12**). Leaves of Hab51p2-1 pepper which were transformed with *Agrobacterium* strain GV3101 CGEL-21 vector encoding EGFP and GUS genes showed similar expression (**Fig. 13**). However, no fluorescence was detected in non-transformed pepper leaves (**Fig. 10, 11, 12 & 13**).

GUS protein expression was recorded under bright filter of the microscope with an exposure time of 500ms after the GUS assay. Blue color was detected in the

transformed leaves of A108, and Hab51p2-1 pepper with either AGL-1 or GV3101 *Agrobacterium* strain harboring CGEL-21 vector encoding the EGFP and GUS genes, while no blue color was detected in non-transformed leaves of the two peppers (**Fig. 14**).

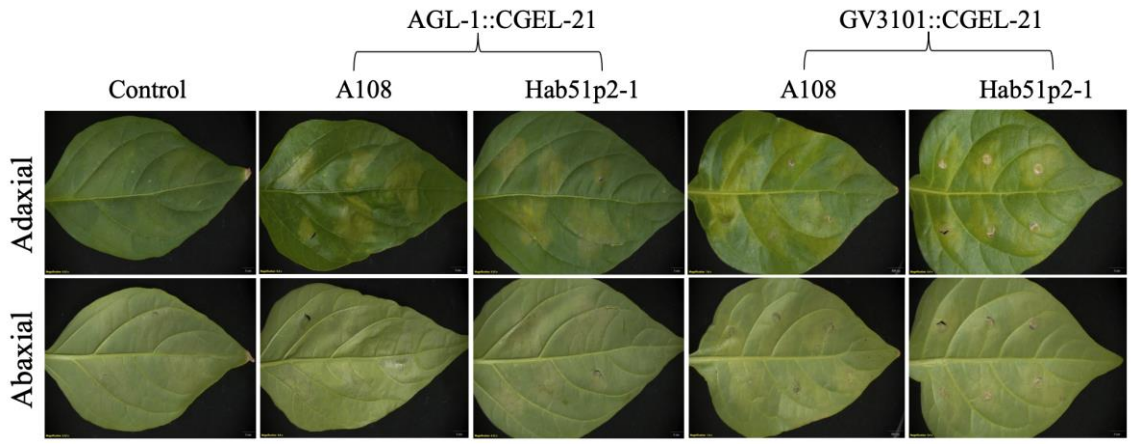

Figure 9. Morphology of transformed pepper leaves.

A. tumefaciens AGL-1 containing CGEL-21 vector encoding EGFP and GUS or *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS was infiltrated into A108 and Hab51p2-1 pepper leaves. Morphology of leaves were taken after 5 days of agroinfiltration.

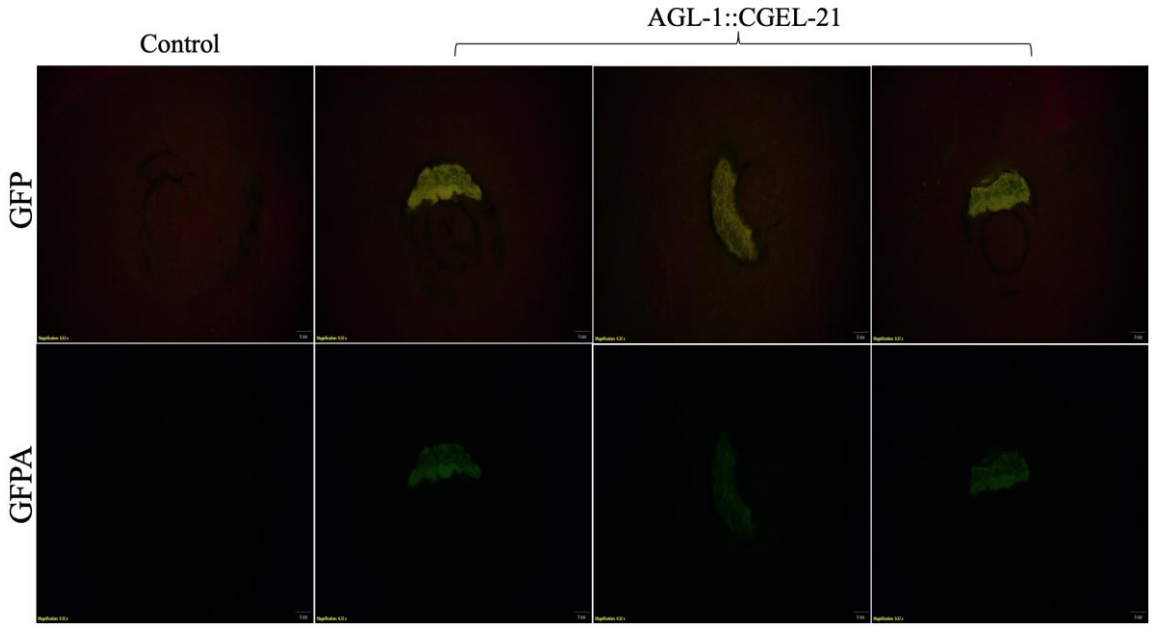

Figure 10. EGFP expression in transformed A108 pepper leaves.

A. tumefaciens AGL-1 containing CGEL-21 vector encoding EGPF and GUS was infiltrated into A108 pepper leaves. The EGFP gene was expressed after 5 days of agroinfiltration.

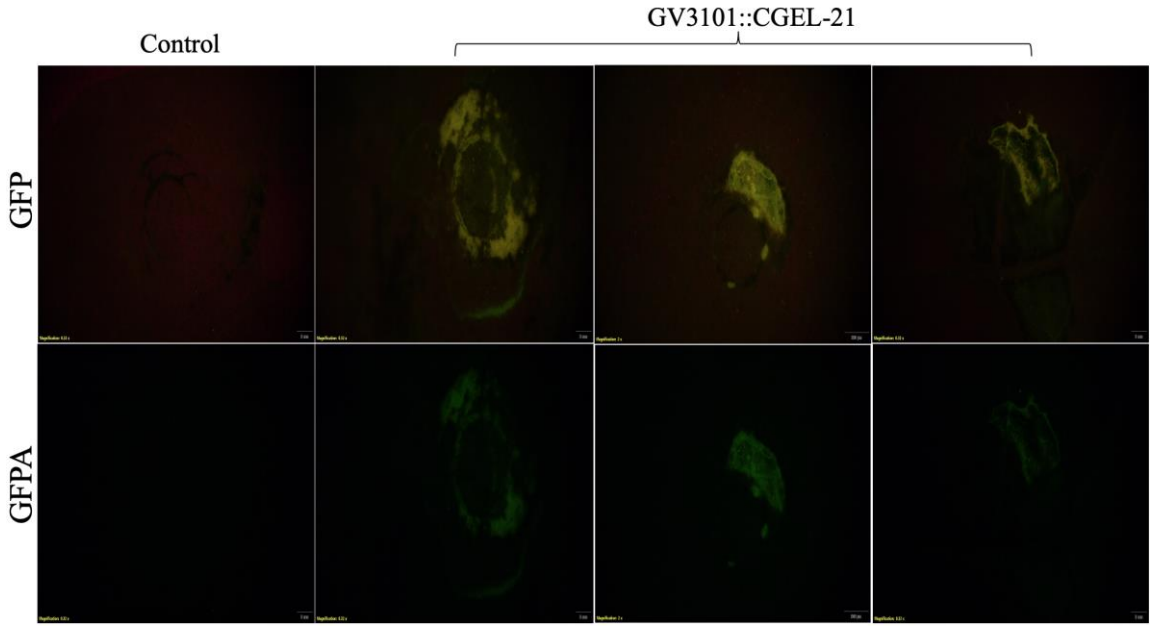

Figure 11. EGFP expression in transformed A108 pepper leaves.

A. tumefaciens GV3101 containing CGEL-21 vector encoding EGFP and GUS was infiltrated into A108 pepper leaves. The EGFP gene was expressed after 5 days of agroinfiltration.

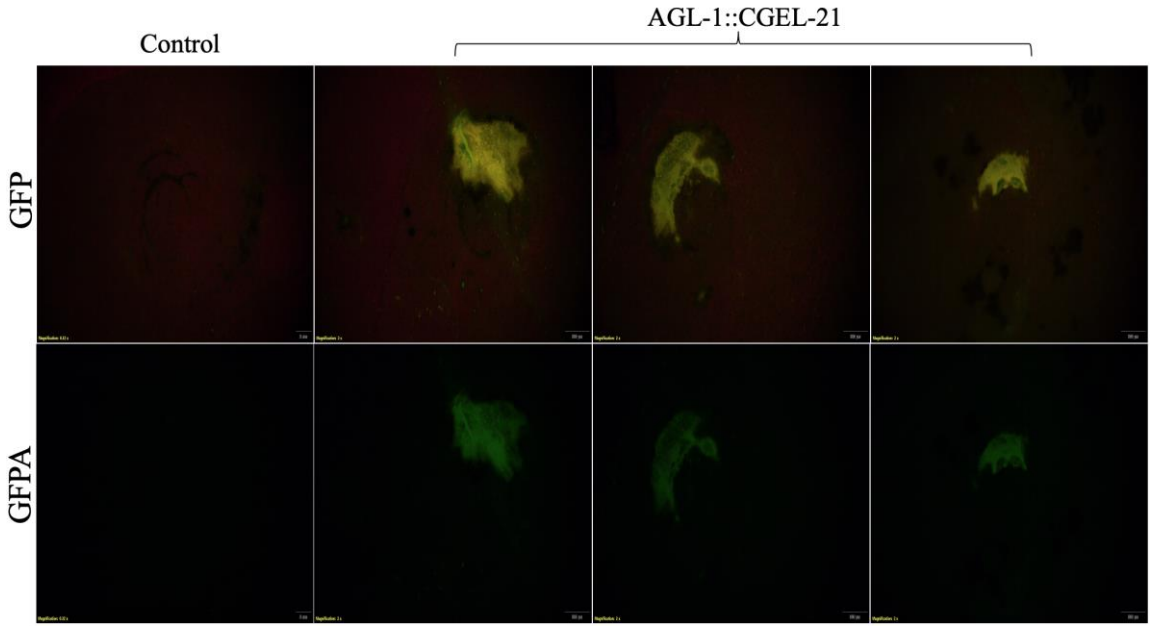

Figure 12. EGFP expression in transformed Hab51p2-1 pepper leaves.

A. tumefaciens AGL-1 containing CGEL-21 vector encoding EGFP and GUS was infiltrated into Hab51p2-1 pepper leaves. The EGFP gene was expressed after 5 days of agroinfiltration.

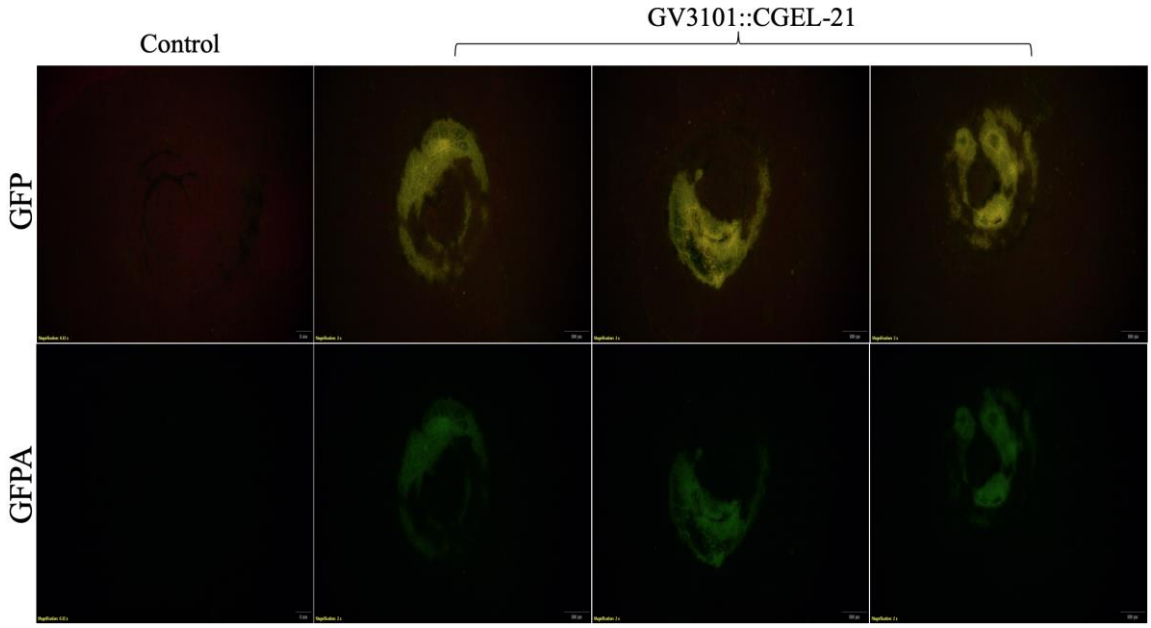

Figure 13. EGFP expression in transformed Hab51p2-1 pepper leaves.

A. tumefaciens GV3101 containing CGEL-21vector encoding EGFP and GUS was infiltrated into Hab51p2-1 pepper leaves. The EGFP gene was expressed after 5 days of agroinfiltration.

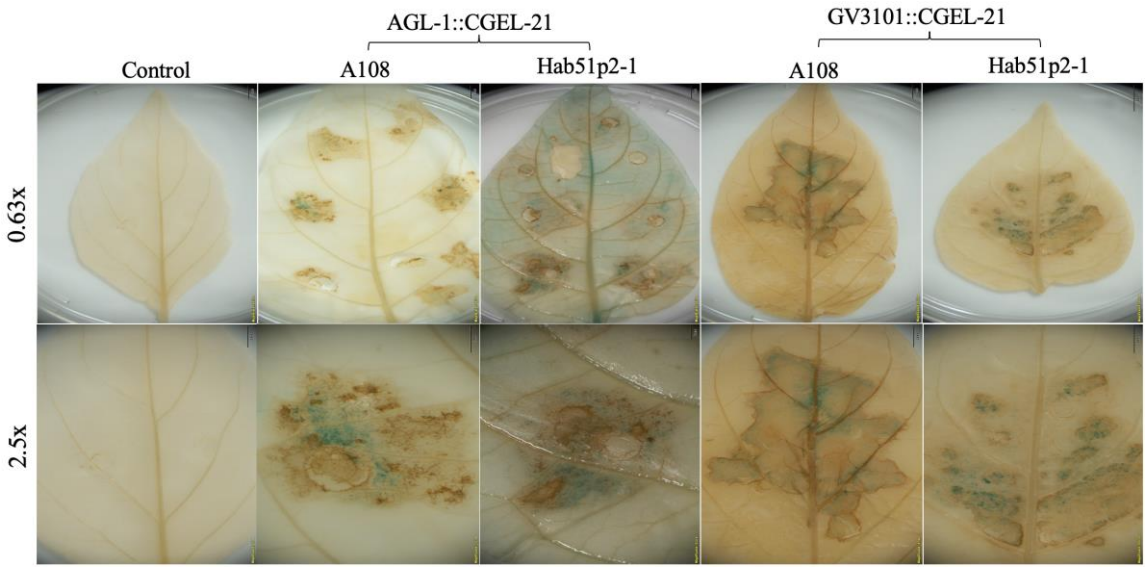

Figure 14. GUS expression in transformed pepper leaves.

A. tumefaciens AGL-1 containing CGEL-21 vector encoding EGFP and GUS or *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS was infiltrated into A108 and Hab51p2-1 pepper leaves. GUS expression in leaves was taken using a Bright filter of microscope after 5 days of agroinfiltration.

Quantification of transformed peppers by Real-time qPCR.

We confirmed the designed primers for three housekeeping genes (Actin, β-tubulin, EF1α) using genomic DNAs of two wild-type pepper species by PCR before performing quantitative real-time PCR. PCR reactions using specific primers for Actin, β-tubulin, and EF1α (**Table 5**) amplified fragments with the expected size (200 bp) (**Fig. 15**).

The expression levels of the GUS gene in the pepper leaves were quantified with qPCR using the actin housekeeping gene for normalization. The mean values of Cq among 72 samples are shown in **Table 6**. Means of GUS Cq values for A108 pepper ranged from 15.98 to 18.29, while those for actin Cq values were 24.74 to 25.26. Means of GUS Cq values for Hab51p2-1 pepper ranged from 19.29 to 21.14 , while those for actin Cq values were 24.19 to 25.36 (**Table 6**). GUS gene expression level for A108 pepper which were transformed with AGL-1 and GV3101 strain was 2.03359±0.54354 and 0.79618±0.28541 respectively. (**Fig. 16**). However, GUS gene expression level in transformed Hab51p2-1 pepper was relatively lower than transformed A108 peppers showing 0.10078±0.02815 and 0.03894±0.00681 that were transformed with AGL-1 and GV3101 strain respectively.(**Fig. 16**).

Table 6. Cq values of GUS and actin genes detected by quantitative real-time PCR among 72 samples

Sample	Gene	Cq Value (mean)
A108-AGL	Actin	25.21
	GUS	15.98
A108-GV	Actin	24.74
	GUS	16.85
A108-WT	Actin	25.26
	GUS	18.29
Hab51p2-1-AGL	Actin	24.19
	GUS	19.29
Hab51p2-1-GV	Actin	24.67
	GUS	21.14
Hab51p2-1-WT	Actin	25.36
	GUS	20.39

A108-AGL, A108 pepper transformed with *A. tumefaciens* AGL-1 containing CGEL-21 vector encoding EGFP and GUS; A108-GV, A108 pepper transformed with *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS; A108-WT, non-transformed A108 pepper; Hab51p2-1-AGL, Hab51p2-1 pepper transformed with *A. tumefaciens* AGL-1 containing CGEL-21 vector encoding EGFP and GUS; Hab51p2-1 -GV, Hab51p2-1 pepper transformed with *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS; Hab51p2-1 -WT, non-transformed Hab51p2-1 pepper.

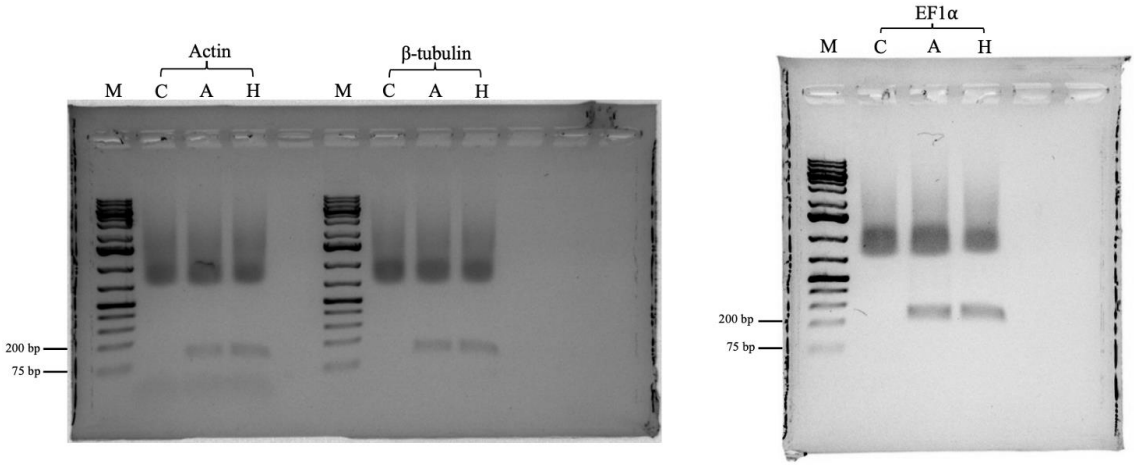

Figure 15. The detection of housekeeping genes in wild-type pepper leaves.

Genomic DNA was collected from wild-type A108 and Hab51p2-1 pepper leaves, and primers for three specific housekeeping genes including Actin, β-tubulin, and EF1α were used for gene detection. The PCR product sizes were 100~200bp. M= 1 kb plus DNA ladder (ThermoFisher Scientific™), C = Negative control, A= A108, H= Hab51p2-1.

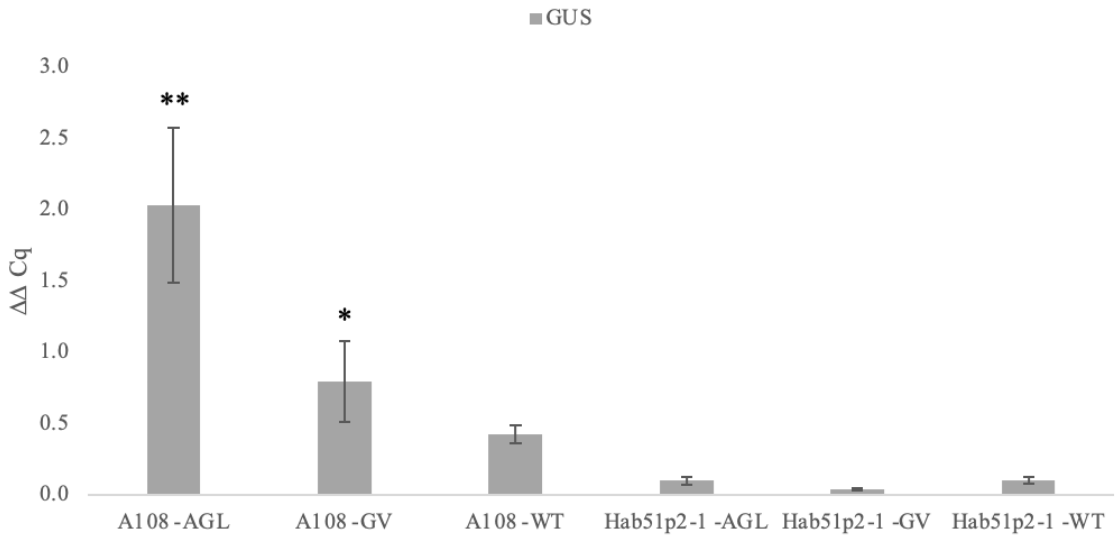

Figure 16. Detection of the GUS gene in pepper leaves.

A. tumefaciens AGL-1 containing CGEL-21 vector encoding EGFP and GUS or *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS were infiltrated into A108 and Hab51p2-1 pepper leaves. GUS expression in leaves was quantified using RNA by quantitative real-time PCR. Expression level fold change was normalized to actin expression. A108-AGL, A108 pepper transformed with *A. tumefaciens* AGL-1 containing CGEL-21 vector encoding EGFP and GUS; A108-GV, A108 pepper transformed with *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS; A108-WT, non-transformed A108 pepper; Hab51p2-1-AGL, Hab51p2-1 pepper transformed with *A. tumefaciens* AGL-1 containing CGEL-21 vector encoding EGFP and GUS; Hab51p2-1 -GV, Hab51p2-1 pepper transformed with *A. tumefaciens* GV3101 containing CGEL-21 vector encoding EGFP and GUS; Hab51p2-1 -WT, non-transformed Hab51p2-1 pepper. ** $P<0.01$; * $P<0.05$

Discussion

Pepper is consumed around the World. Many efforts have been made to transform peppers to insert useful genes. Transformation of peppers is known to be recalcitrant. We tried to develop a transient expression system in two peppers, A108 and Hab51p2-1, using *Agrobacterium tumefaciens* AGL-1 and GV3101 transformed with the CGEL-21 vector harboring the EGFP and GUS genes by agroinfiltration to establish a transient pepper transformation system using the infiltrated pepper leaves.

PCR detection using GUS and EGFP-specific primers showed that the expected nucleotide sizes, 160 bp and 861p of GUS and EGFP genes were amplified, respectively in two peppers, A108 and Hab51p2-1. In addition, a microscope observation showed that EGFP and GUS were expressed in the infiltrated leaves in the two peppers.

Our results showed that expression of the GUS gene was confirmed in A108 pepper genotype which was transformed with *Agrobacterium tumefaciens* AGL-1 and GV3101 strains, which contain the CGEL-21 vector harboring the EGFP and GUS genes, by fluorescence microscopy, PCR, GUS assay and quantitative real-time PCR.

We used the agroinfiltration method to express the GUS and EGFP genes in peppers. In *Nicotiana benthamiana*, agroinfiltration was used for the transient expression of the GUS gene [**12**]. This study used the benchmark pEAQ-HT deconstructed virus vector system and the GUS reporter gene to enhance *Agrobacterium*-mediated transformation and to improve protein production capacities. *Agrobacterium tumefaciens*-mediated transient genetic transformation of Habanero pepper leaf explants was also

reported [**2**]. This study used two empty binary vectors (pCAMBIA2301 and pCAMex) and cDNAs of *C. chinense* encoding the pathogenesis-related protein 10 and esterase.

When we quantified GUS gene expression in the transformed leaves by real-time qPCR, we could detect GUS gene expression in A108 pepper, but not in Hab51p2-1 pepper. The reason that expression of the GUS gene was not expressed well in Hab51p2-1 pepper may be related to the genetic traits of Hab51p2-1 pepper. It seems that GUS expression in the infiltrated leaves of Hab51p2-1 pepper was false- positive. More studies may be needed to address the reason that RNA was not detected in Hab51p2-1 pepper by real-time qPCR. In addition, we need to quantify EGFP gene expression in the transformed leaves of peppers by real time qPCR, and to detect the protein expression of GUS and EGFP in the transformed peppers by Western blot or enzyme-linked immunosorbent assay (ELISA).

Our agroinfiltration system in pepper can be used for studying gene function [**29**], protein production [**27**], host–pathogen interaction [**23**], protein–protein interaction [**7**] and in protein localization [**3**].

Conclusions

In this study, we confirmed the transgene expression of EGFP and GUS genes in pepper leaves of A108 (*Capsicum annuum* x *C. baccatum*) and Hab51p2-1(*Capsicum chinense*), which were infiltrated with two different *Agrobacterium* strains that were transformed with EGFP and GUS gene by fluorescence microscopy, PCR, histochemical

GUS assay, and quantitative real-time PCR. We hope that this transformation system can be applied to the functional study of genes of interest in peppers.

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