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Efficient Stable Genetic Transformation of Pea (Pisum sativum)

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Research Article

Keywords: Pisum sativum, Gene editing, Functional genomics, Genetic engineering

Posted Date: October 29th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-7841977/v1

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Abstract

Pea *(Pisum sativum)* has emerged as a major protein source for meat substitutes due to its high nutritional value, low production costs, and short life cycle. The generation of elite pea cultivars can be achieved via genetic engineering and CRISPR-based gene editing. However, this approach has lagged due to the low efficiency, lack of reproducibility, and cultivar dependency of the reported pea transformation protocols. Due to the challenges in the genetic engineering of pea, we employed a transient expression approach to identify optimal conditions for gene expression with the expectation that these conditions would enhance the efficiency of stable transformation. The highest transient expression was achieved when the *Agrobacterium* suspension was used at 1.6 optical density, combined with a co-cultivation time of one hour. With the optimized conditions and a staggered antibiotic selection protocol, genetic perturbations, including ectopic and antisense expression and CRISPR/Cas9 editing of the flavanone 3-hydroxylase (F3H) gene, were performed in a purple-seeded pea line. We report an efficient, stable transformation protocol for pea with a mean efficiency of 2.9%. Greenhouse-adapted seed-bearing transgenic plants were obtained in eight months. The T2 transgenic lines were verified using PACE-PCR and RT-qPCR analysis, which confirmed the transgenic status of the plant and altered expression of the F3H gene demonstrating successful genetic engineering in *Pisum sativum*.

Key message

An efficient genetic engineering approach in *Pisum sativum* will enable functional genomics studies in a crop that is the second most important source of plant-based protein in human diet.

Introduction

Pea (*Pisum sativum*) is an important cool-season crop grown worldwide for various uses, including food and feed. Due to the high content of lysine and overall high nutritional value, peas have become invaluable for the plant-derived protein market (do Carmo et al., 2016; Peng et al., 2016; Xiong et al., 2018). Additionally, pea protein offers functional biochemical properties required for the development of meat analogs (Pietrysiak et al., 2018). The pea protein market was valued at $2 billion in 2022 and is projected to have a compound annual growth rate (CAGR) of 12.0% from 2023 to 2030 (Grand View Research, 2023). The increase in the consumption of plant-derived protein is expected to contribute to mitigating the global greenhouse gas emissions caused by livestock production, which supplies most of the dietary protein (Stehfest et al., 2009).

Although peas have been used as a model to study genetics since Mendel's time, breeding advancements have lagged. Therefore, the pea industry relies on a limited number of cultivars, used primarily due to their seed quality, consumer familiarity, and acceptance, as well as other important agronomic characteristics such as plant height. With the need to develop elite cultivars in a shorter time frame, genetic engineering represents an expedient strategy for improving pea cultivars. However, the utilization of this approach is limited since the few reported genetic transformation protocols are of low

efficiency and lack reproducibility, and are mostly cultivar dependent (Bean et al., 1997; Grant et al., 1995a; Jordan & Hobbs, 1993a; Nadolska-Orczyk & Orczyk, 2000a; Pniewski & Kapusta, 2005a; Polowick et al., 2000a; Schroeder et al., 1993a).

Efficient genetic transformation protocols can enable CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) gene editing, which has proven to be precise, highly effective, and versatile in numerous crop species (Ghogare et al., 2019; Hu et al., 2016; Liang et al., 2017; Lu & Zhu, 2017; Michno et al., 2015; Svitashev et al., 2016). Improved and non-GMO pea cultivars can be obtained via CRISPR editing, as this approach introduces changes to DNA intrinsic to the target species, and the resulting lines are not considered GMO products (Shew et al., 2018). The high demand for pea protein presents a timely opportunity to utilize pea genetic engineering and genome editing for rapid and efficient improvement, as has occurred for soybeans over the last few decades.

As with most other crops, the primary focus of crop improvement in peas is to overcome specific biotic and abiotic stresses, ensuring profitable production. In this study, the goal was to develop a highly efficient transformation system for which we evaluated several parameters, including *Agrobacterium* strain and optical density, duration of co-cultivation, and antibiotic selection. Since we used a purple-pigmented genotype, we utilized flower pigmentation as a secondary visual marker to track the successful genetic transformation of pea by targeting genetic perturbations in the F3H gene that codes for flavanone 3-hydroxylase. The enzyme catalyzes the conversion of naringenin into dihydroflavanones, which serve as precursors for anthocyanin biosynthesis (Sreevidya et al., 2006).

Materials and Methods

Plant material

The purple-pigmented pea line PI 175226 was used for genetic transformation purposes, selected based on the highest resistance to root rot (Bodah et al., 2016; Porter et al., 2014). Preliminary laboratory results indicated that this genotype exhibits the best *in vitro* regeneration performance among root rot-resistant, purple-pigmented lines (PI 125673, PI 175226, and ‘Melrose’). PI 175226 plants were grown in the greenhouse with an 18/6 hr. day/night photoperiod and 22/18°C Day/night temperatures. The source of explants for tissue culture was immature seeds obtained from 9-12-day-old immature pods. Immature pods at this stage are known as the "eating pea’ stage. At this stage, the seeds had reached their maximum size but had not yet begun to dry. Pods were collected for one to two weeks and maintained at 4°C before their use for initiating the cultures.

For the confirmation of transgenic status, seeds from each putative transgenic line were imbibed in water and grown in a growth room maintained at 25°C under white light. After 10 days of germination, leaf tissue was harvested for downstream analyses, and subsequently, confirmed transgenic plants were transferred to a greenhouse and grown at 25–28°C with 50% humidity until the pods developed.

Transient expression of the uidA gene to standardize the transformation protocol

Surface sterilization

Pea pods were surface sterilized in 70% (v/v) ethanol for 2 minutes under constant agitation, followed by a wash with sterile MilliQ water. Pods were then immersed in a 1% (w/v) sodium hypochlorite with 1ml/L of Tween-20 for 20 minutes at 200 rpm, followed by three washes with sterile MilliQ water.

Extraction of embryonic axes

Immature seeds were isolated from the pods, and the testa was removed. The embryonic axis of the seeds was excised in half, separating the cotyledons. Thereafter, 80–90% of the cotyledon, distal from the embryonic axis, was excised. The root end of the embryo axis was cut off. Explants were pre-conditioned on *RM1* media (Table S1) for eight days.

Agrobacterium-mediated transient expression: Agrobacterium strain GV3850 carrying pCAMBIA1304 binary plant transformation vector was used for co-cultivation with embryo axis explants. After pre-conditioning on *RM1* for eight days, embryo axes were co-cultivated with *Agrobacterium* resuspended in *CM-L* (Table S1). Transformation treatments (*T1-T4*) were determined and modified from previous reports on pea transformation by (Nadolska-Orczyk & Orczyk, 2000b; Schroeder et al., 1993b). Pea explants were co-cultivated with *Agrobacterium* suspension, which also included acetosyringone (50 µM), of OD of 0.001 (*T1*), 0.01 (*T2*) for 48 hrs. on a shaking platform at 120 rpm, 22°C; or an OD of 0.8 (*T3*) or 1.6 (*T4*) for one hr. on a shaking platform at 120rpm, 22°C. All explants were placed on *CM-S* (Table S1) and incubated for 48 hrs. at 22°C. Explants were rinsed with sterile MilliQ water, and subsequently, β-glucuronidase (GUS) staining of the explants was performed as reported by (Jefferson, 1987). This experiment was repeated twice. In addition, the effect of *Agrobacterium* suspension of OD of 1.6 or 2.4 with 200-mm Hg vacuum infiltration for 10- and 20 minutes during co-cultivation was evaluated with *T4* as a positive control (OD:1.6, no vacuum). The effects of the length of pre-conditioning on *RM1* (2, 8, and 15 days) and four different *Agrobacterium* strains (EHA105, AGL-0, GV3101, GV3850) were also assessed. GUS staining of explants was performed after co-cultivation, as described previously.

Construction of Plant Transformation Vectors

All recombinant DNA manipulations were performed in *Escherichia coli* strain TOP10. The DNA plant transformation vectors described in this work were confirmed via restriction digest and subsequent Sanger sequencing. The DNA sequences of all the oligonucleotides used in the study are listed in Table S2.

F3H ectopic and antisense expression plant transformation vectors

For F3H overexpression, full-length F3H mRNA from *Medicago truncatula* (XM_003629275.3) was synthesized (Synbio Technologies, NJ, USA) with an *Nco*I and *Xba*I restriction site at the 5’ and 3’ ends (Supplementary data 1), respectively. The full-length F3H gene was cloned via restriction digestion into

the pAD120 shuttle vector described previously and derived from pAVA121 (Jiwan et al., 2013; von Arnim et al., 1998). The pAD120 vector contains a double CaMV35S promoter, Tobacco Etch Virus translational enhancer, Nopaline synthase (Nos) terminator, and smGFP, which is digested out and replaced with the gene of interest.

For F3H-antisense expression, first, the two F3H isoforms were amplified with oligonucleotides BWBp_1-BWBp_4 (Table S2) and sequenced using DNA from the PI 175226 line by (Williamson-Benavides et al., 2020). Thereafter, a 332 nt region of the F3H gene (Supplementary data 1) was amplified from cDNA using oligonucleotides BWBp_5-BWBp_6 (Table S2) using Q5 High-Fidelity DNA Polymerase (New England Biolabs, MA, USA). The cDNA was synthesized from mRNA derived from PI 175226 adult plants using the SuperScript ® Vilo Kit (ThermoFisher Scientific, MA, USA). The 332 nt section represents exon 2 of the F3H gene (Fig. 1) and is shared between both F3H isoforms identified from PI 175226. For antisense orientation, specific oligonucleotides (Table S2) were used to introduce *Nco*I and *Xba*I restriction enzyme sites at the 3' and 5' ends of the gene, respectively. The 332 nt-F3H fragment representing the antisense orientation was cloned via restriction digest into the pAD120 as described above.

The cassettes containing 35S promoter, enhancer, gene of interest (full-length-F3H or antisense-F3H), and TNos were released and cloned via restriction digestion with *Hind*III into pCAMBIA2300 to produce two new vectors. The resulting plasmids were referred to as pBWB4 (full-length F3H) and pBWB5 (antisense-F3H) (Fig. 1).

CRISPR Cas9 Construct: Three guide RNAs (gRNAs) of 20 bp each were used to target exon 2 of the F3H gene (Fig. 1). The Blue Heron Biotech website (http://www.blueheronbio.com/Services/CRISPR-Cas9.aspx) was used to identify highly active gRNAs. A specific region of 23 nt (AACAAAAGCATGTGTTGATA**TGG**) was designated as the target region of gRNA 1 (AACAAAAGCATGTGTTGATA). This 23 nt sequence is conserved among the two F3H isoforms in PI 175226 (Supplementary data 1). gRNA2 (ACCAAAGAGACTATTCAAGG) and gRNA3 (AGCAAAGAGACTATTCAAGG) target the same locus (ASCAAAGAGACTATTCAAGG**TGG**) for F3H isoform 2 and 1, respectively. The gRNA sequences were aligned via BLAST analysis against previously generated transcriptome data (Williamson-Benavides et al., 2020) to minimize off-target effects. Following the manufacturer's instructions, an *in vitro* cleavage assay was performed to assess the functionality of each gRNA on the PCR product of each F3H isoform (EnGen® sgRNA Synthesis Kit, *Streptococcus pyogenes*; NEB, MA, USA). Each *in vitro* cleavage reaction included 300 ng of Cas9 Nuclease, 525 ng of gRNA (gRNA1, 2, or 3), and 125 ng of PCR product (F3H isoform 1 or 2). *In vitro* cleavage reactions were incubated for four hrs. at 37°C.

Plasmid pRWC42.6 (provided by Dr. Ryan Christian in the Dhingra Lab), with a pUC backbone, was used to clone each gRNA separately. Each gRNA was integrated into the pRWC42.6, using the *Hind*III restriction enzyme to produce three new vectors. gRNAs were multiplexed into the binary vector pKSE401 (Xing et al., 2014) via the BioBrick cloning method (Shetty et al., 2008). pKSE401 carried the

CRISPR/Cas9 machinery and the three gRNAs. The expression of each gRNA was driven by the ATU6 promoter. The resulting vector was referred to as pBWB6 (Fig. 1).

Stable Transformation via Agrobacterium tumefaciens and Plant Regeneration

After pre-conditioning on *RM1*, explants were co-cultivated with A. *tumefaciens* GV3850 carrying pCAMBIA2300, pBWB4, pBWB5, and pBW6 binary plant transformation vectors. Pea explants were immersed in the *CM-L* (Table S1) with an *Agrobacterium* OD of 1.6 for one hr. at 120 rpm, 22°C (*T4*). All explants were placed on *CM-S* (Table S1) medium for 48 hours at 22°C.

After co-cultivation, the explants were washed three times with sterile MilliQ water and subsequently immersed in a washing solution of 400 mg/L Timentin and 400 mg/L Cefotaxime diluted in MilliQ water for one hr. at 120 rpm, 22°C. After removing the washing solution, explants were placed on *RM2* for regeneration and selection (Table S1). Further subcultures were performed from *RM2* to *RM3* and *RM4* for shoot regeneration under an increasing amount of geneticin selection (20–30 mg/L) (Table S1). After *RM4*, explants were moved to *EM* and *RM5* media for shoot elongation (Table S1). Rooting of shoots was performed on *RtM* media. Shoots that did not root on *RtM* media were grafted *in vitro* onto WT seedlings using *s*ilicone grafting clips (0.5 x 1.0 cm).

DNA extraction, PCR confirmation, and Inverse PCR of transgenic lines

Total cellular DNA was isolated using the CTAB method from putative transgenic lines. DNA from lines harboring pCAMBIA 2300, pBWB4, pBWB5, and pBW6 were PCR-screened with oligonucleotide sets BWBp_7–8, BWBp_9–10, BWBp_11–12, BWBp_13–14, and BWBp_15–16, respectively (Table S2).

The number of T-DNA insertions per transgenic line was determined via inverse PCR following a previously published protocol (Kim et al., 2011). The choice of restriction endonuclease for *Ps*-pCAMBIA2300, *Ps*-pBWB4, and *Ps*-pBWB5 lines was *Bgl*II, and for the *Ps*-pBW6 lines, it was *Xba*I. Inverse PCRs and nested PCRs were performed using the DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, MA, USA). Oligonucleotides for inverse PCRs and nested PCRs are listed in Table S2.

Transgene Confirmation in T2 Lines via PACE (PCR Allele Competitive Extension) Genotyping

The transgenic nature of the T2 lines was confirmed using PACE (PCR Allele Competitive Extension). PACE assays were conducted following the manufacturer's instructions. DNA from leaf samples was isolated using the 96-Well SYNERGY™ Plant DNA Extraction Kit according to the manufacturer's instructions (OPS Diagnostics, NJ, USA). Briefly, 50 mg of plant tissue was homogenized using the Plant

Homogenization Buffer provided by the manufacturer and then centrifuged at 2,100 × g for 10 minutes. The supernatant was collected, filtered using a filter plate, and treated with RNase. The lysates were then mixed with isopropanol, passed through binding plates, washed with 70% ethanol, and eluted using Molecular Biology Grade Water.

PCR reactions were performed using a reaction mix that included 3µL PACE Assay Mix, 1µL sterile water, 0.0824 µL primer mix (12 μ M Forward-fam and 30µM Reverse + 12µM Forward-hex and 30µM Reverse) and 2µL DNA template diluted to 10ng/µL for a total volume of 6uL. 1 µL of genomic DNA, 3 µL of nuclease-free water, and 4 µL of PACE Assay Mix, including primers (Table S2). The reactions were conducted in 384-well plates, with each well containing DNA from a single plant. Wild-type plants served as controls to represent untransformed plants. The primer for the *npt*II gene, fused with a FAM binding site, was used to detect the transformed plants, while the primer for the polyubiquitin gene, fused with a HEX binding site, was used as an internal control (Table S2).

Real-time quantitative PCR

RNA was extracted from leaves using the RNeasy Plant DNA Extraction Kit (Qiagen, Mainz, Germany). RNA was obtained from wild-type (WT), as well as PCR-confirmed F3H-ecotopic expression lines (*Ps*-pBWB4), F3H-antisense expression line (*Ps*-pBWB5), and F3H-CRISPR/Cas9 gene-edited lines (*Ps*-pBWB6). After extraction, RNA was subjected to DNase treatment. First-strand cDNA synthesis was performed using RNA samples with the SuperScript ® Vilo kit (ThermoFisher Scientific, MA, USA).

The flavanone-3-hydroxylase (F3H) gene was selected for RT-qPCR analysis. Using homologous regions, a set of primers was designed to amplify the four expected isoforms of the F3H gene (Table S2). The *Pisum sativum* root border cell-specific protein (GenBank accession AF1139187.1) was used as an internal reference control. Primers for RT-qPCR were designed with the Primer3 software (Rozen & Skaletsky, 2000).

The QUBIT 3.0 fluorometer (Invitrogen, CA, USA) was used to quantify the concentration of the cDNA library.. The reaction mix contained 5.5µL of PowerTrack™ SYBR Green Master Mix for qPCR (Thermo Fisher Scientific, USA), 0.55uL Primer (8µM forward + 8µM reverse), 2.575µL water, and 2µL cDNA. Three biological replicates representing three independent transgenic events (n = 3) were used with four technical replicates using the Azure Cielo instrument (Azure Biosystems, Inc., USA). Raw fluorescence data were used as input for crossover threshold (Ct) calculations, and reaction efficiencies were adjusted using Azure Cielo Manager software (Azure Biosystems, Inc., USA). The ΔΔCt method offered by PE Applied Biosystems (Perkin Elmer, Foster City, CA) was used to obtain relative differential expression values after reaction efficiencies were adjusted.

Results

Transient expression of the uidA gene to standardize the transformation protocol

Treatments *T1* to *T4* resulted in transient expression efficiencies that ranged from 0% to 18.8% (Table S3). Treatments *T1* and *T2*, adapted from the method described by (Nadolska-Orczyk and Orczyk, (2000), resulted in the lowest efficiency (0.0–2.0%) (Table S3). After 48 hrs. of co-cultivation in liquid media, the explants were vitrified, which may have negatively impacted the transient expression efficiency. *T3* and *T4* treatments, based on the method described by (Schroeder et al., (1993), resulted in the highest efficiency (3.1–18.8%) (Table S3). The treatment with the highest transient expression efficiency was *T4*, which resulted in a mean efficiency of 13.5% and a range of 8.2–18.8%. The effect of different variables such as vacuum application, *Agrobacterium* O.D. during co-cultivation, length of preconditioning before co-cultivation, and *Agrobacterium* strain on transient expression efficiency is presented in Tables S4 - S6.

Sequencing of two isoforms of the F3H gene

The two predicted F3H isoforms were successfully amplified from PI 175226 genomic DNA and sequenced using the Sanger method (Supplementary data 1). Based on the recently reported transcriptome information, the homology between the two full RNA-seq predicted F3H isoforms was 88.07% (Williamson-Benavides et al., 2020). PCR amplified sequence (415 bp) of the F3H isoforms from PI 175226 showed an 89.59% homology. The PCR-amplified isoform 1 showed 99.52% homology with the RNA-seq predicted isoform 1. The PCR amplified isoform 2 showed 99.29% homology with the RNA-seq predicted isoform 2.

In vitro cleavage assay

The *in vitro* cleavage assay confirmed the effectiveness of each gRNA in cleaving both the isoforms of F3H at the target sites (Fig. 2). As expected, assays with gRNA1 & gRNA3, and gRNA1 & gRNA2, completely cleaved F3H isoform 1 and 2, respectively (Fig. 2).

Confirmation of transgenic events

Transgenic plants were successfully regenerated via direct organogenesis. The regeneration and elongation media described by (Nadolska-Orczyk and Orczyk, (1994) were adapted for this study with certain modifications. The media supported the regeneration (Fig. 3A, B) and elongation (Fig. 3C) of shoots from pea embryonic axes from PI 175226 breeding line. Primary shoots produced on *RM1, CM-S, RM2*, and *RM3* were excised and discarded because it was assumed they arose from the apical meristem. Importantly, hyperhydricity was not observed during the regeneration process.

For the induction of roots, several media combinations were tested, including MS with high concentrations of NAA over BAP (5:1 and 10:1 ratios). These combinations used sucrose or glucose as a carbon source and gelrite or agar as gelling agent. However, the combinations of NAA and BAP resulted

in slow, erratic, and generally unreliable rooting. The RtM media was most efficient in inducing rooting (68.97%) in the regenerated shoots (Fig. 3D) (Table S1).

Confirmation of stable integration of the T-DNA was performed via PCR. The mean transformation efficiency rate for the five plasmids used for transformation was 2.9%, while the range was 2.5–3.6% (Table S7). The DNA isolated from four independent, primary transformants was subjected to inverse PCR. These four independent lines contained a single copy of T-DNA insertion (Fig. 4).

The parameters for antibiotic selection described in this study were determined empirically. During protocol standardization, using kanamycin as a selection agent at high levels of 100–200 mg/L failed to impose a selection pressure, resulting in the continued development of apical meristems from the embryonic axes. PCR analysis revealed that only 12 of 60 regenerants were transgenic, resulting in 20.0% efficiency. Using geneticin as a selection agent yielded 48 positive transgenics out of 68 regenerants, resulting in an efficiency of 70.6%.

T1 transgenic events in the greenhouse demonstrated changes in flower color, indicating perturbation of the F3H gene was achieved (Fig. 5). Flower phenotypes of *Ps-pB*WB4, *Ps-pB*WB5, and *Ps-pB*WB6 transgenic lines are presented in Fig. 5B-D, respectively.

Identification of Transgenic Offspring

The offspring of transgenic lines were assessed using PACE-PCR across different generations. The number of transgenic plants detected in each line is summarized in Supplementary Table 8. A total of 37 positive plants were identified for *Ps*-pCAMBIA2300, 175 for *Ps*-pBWB4, 19 for *Ps*-pBWB5, and 130 for *Ps*-pBWB6.

F3H Gene Expression

To evaluate the expression of the F3H gene, RT-qPCR analysis was performed on WT and *Ps-pB*WB4 (ectopic expression), *Ps-pB*WB5 (antisense expression), and *Ps-pB*WB6 (CRISPR/Cas9 editing) transgenic lines. Gene expression analysis was performed on three independent transgenic lines per construct, with four biological replicates for each line. RT-qPCR results indicated reduced expression of the F3H gene in the antisense lines compared to WT. In the overexpression and knockout lines, two out of three lines showed the expected regulation—upregulation in the overexpression lines and downregulation in the knockout lines. Surprisingly, the control vector line also showed consistent downregulation of the F3H gene (Fig. 6).

Discussion

By standardizing various parameters, including the use of geneticin as a selection agent, an efficient Agrobacterium-mediated transformation system was established in *P. sativum*. A transient expression analysis of GUS activity was used to determine the optimal conditions for achieving high transformation

efficiency. The most optimal conditions that were identified were the use of *Agrobacterium* at a concentration of 1.6 O.D., and an hour of co-cultivation time.

As reported previously (Schaerer and Pilet, 1991), a similar frequency of transformation was obtained with all four strains of *A. tumefaciens*. In this study, EHA105 and GV3850 performed somewhat better. Strain EHA105, which is hypervirulent, was difficult to eliminate using Timentin. In that regard, the GV3850 strain performed the best.

A common challenge with pea transformation is the low recovery of transgenic events. In this study, the mean transformation efficiency was 2.9% with a range of 2.5–3.6% (Table S7), which is comparable to or higher than previously reported in pea. Previous studies have reported transformation efficiencies of 1.1% (Bean et al., 1997), 1.5–2.5% (Schroeder et al., 1993b), 0.6% (Polowick et al., 2000b), 3.6% (Nadolskaean-Orczyk & Orczyk, 2000b), 0-4.1% (Pniewski & Kapusta, 2005b), 1% (Jordan & Hobbs, 1993b), and 0.7-2% (Grant et al., 1995b).

The method described here enabled the recovery of greenhouse-adapted seed-bearing transgenic plants in approximately 35.5 weeks (approximately 8 months) post-co-cultivation. This is a significantly faster method for producing transgenic pea plants compared to previous reports of 15 months (Puonti-Kaerlas et al., 1992a) and 9 months (Polowick et al., 2000b; Schroeder et al., 1993b). This protocol should be evaluated for other pea genotypes to test the reproducibility of the relatively successful transformation efficiencies in a shorter timeframe.

The *npt*II gene codes for the aminoglycoside 3'-phosphotransferase enzyme, which inactivates a range of aminoglycoside antibiotics such as neomycin, kanamycin, paramomycin, ribostamycin, butirosin, and geneticin through phosphorylation. While kanamycin has been used as a selectable marker for some pea varieties (Grant et al., 1995b; Polowick et al., 2000b), few studies have found a high level of tolerance of pea lines to kanamycin (Puonti-Kaerlas et al., 1992b; Schroeder et al., 1993b). In fact, these latter studies have highlighted that kanamycin selection was ineffective for pea transformation. Furthermore, kanamycin selection has been found to generate phenotypically abnormal plants (Bean et al., 1997; Nadolska-Orczyk & Orczyk, 2000b). In this study, preliminary data found kanamycin to be an unsatisfactory method of selection for transgenics because of the large percentage of escapes (80%).

Due to the inefficiency of kanamycin to select for pea transgenics, the suitability of geneticin selection was tested in this study. Geneticin proved to be much more efficient than kanamycin selection. Almost 71% of the putative transgenic events selected on geneticin tested positive for the transgene. The effectiveness of selection on geneticin compared to kanamycin had already been reported for rice and soybean transgenic plants expressing *npt*II gene (Dekeyser et al., 1989; Itaya et al., 2018a; Twyman et al., 2002a). In a recent study, among > 600 events obtained using geneticin selection, no escapes were found (Itaya et al., 2018b). The high efficiency of selection is likely since geneticin is more toxic than kanamycin to plant cells (Twyman et al., 2002b).

Another challenge in generating pea transgenics was the development of roots from regenerated pea shoots. Root production on regenerated pea shoots has been described as challenging (Bean et al., 1997; Böhmer et al., 1995). However, after an empirical screening of media treatments, a 70% root induction rate was achieved by removing all hormones and changing the carbohydrate source from sucrose (30g/L) to glucose (16g/L). The presence of glucose has proven to facilitate the production of roots in difficult to root species (Calamar & De Klerk, 2002; da Rocha Correˆa et al., 2005; Yasodha et al., 2008). Glucose is found to have growth hormone-like activities, interacts positively with auxin signaling, and is one of the signaling molecules for gene expression, cell proliferation, and root inflorescence growth (Cho et al., 2006; Rolland et al., 2006; Yanaglsawa et al., 2003).

Lastly, transgenic flowers represent a range of petal pigmentation compared to the control (Fig. 5). We have identified *Ps-pB*WB6 independent lines with decreased pigmentation content in flowers (Fig. 5D). Interestingly, overexpression lines show an intense silencing of the anthocyanin resulting in totally white flowers (Fig. 5B). The change in anthocyanin content is also verified with the expression of F3H gene using RT-qPCR (Fig. 6). These phenotypic results have to be validated with molecular techniques, as well as with *Fsp* bioassays, to determine if the anthocyanin synthesis pathway is associated with defense resistance against *Fsp*. The transformation protocol presented in this study provides a streamlined approach for the rapid production of transgenic plants from immature seeds. The highly regenerable nature of the chosen target material increases the probability of obtaining transgenic plants, which is further enhanced by the use of an efficient antibiotic for selecting transgenic lines. In this study, CRISPR/Cas9 gene editing was achieved in pea; however, editing efficiency remains to be tested and reported. The presence of integrated T-DNA remains to be tested in future generations to assess its transmissibility and stability. The series of F3H overexpressing and silenced transgenic lines and gene edited lines represents a reverse genetics resource to address the hypothesis that anthocyanin is directly responsible for root rot resistance.

Declarations

- **Competing Interests:** Authors declare no competing financial interests.
- **Author Contributions:** AD and BWB designed the study. AD supervised the study. BWB, AP, JM, and LO performed experiments and generated the data. All authors read and approved of the final manuscript.
- **Funding:** This work was supported by Washington State University Hatch Project # WNP00011, Texas A&M AgriLife Hatch Project #TEX0-9950-0 and startup funds from Texas A&M AgriLife Research and Texas A&M University to AD. BWB acknowledges graduate research assistantship support from Washington State University Graduate School.
- **Acknowledgements:** Not applicable.

References

1. Bean, S. J., Gooding, P. S., Mullincaux, P. M., & Davies, D. R. (1997). A simple system for pea transformation. *Plant Cell Reports*, *16*(8), 513–519.
2. Bodah, E. T., Porter, L. D., Chaves, B., & Dhingra, A. (2016). Evaluation of pea accessions and commercial cultivars for fusarium root rot resistance. *Euphytica*, *208*(1), 63–72.
3. Böhmer, P., Meyer, B., & Jacobsen, H.-J. (1995). Thidiazuron-induced high frequency of shoot induction and plant regeneration in protoplast derived pea callus. *Plant Cell Reports*, *15*(1–2), 26–29.
4. Calamar, A., & De Klerk, G.-J. (2002). Effect of sucrose on adventitious root regeneration in apple. *Plant Cell, Tissue and Organ Culture*, *70*(2), 207–212.
5. Cho, Y.-H., Yoo, S.-D., & Sheen, J. (2006). Regulatory functions of nuclear hexokinase1 complex in glucose signaling. *Cell*, *127*(3), 579–589.
6. da Rocha Correˆa, L., Paim, D. C., Schwambach, J., & Fett-Neto, A. G. (2005). Carbohydrates as regulatory factors on the rooting of Eucalyptus saligna Smith and Eucalyptus globulus Labill. *Plant Growth Regulation*, *45*(1), 63–73.
7. Dekeyser, R., Claes, B., Marichal, M., Van Montagu, M., & Caplan, A. (1989). Evaluation of selectable markers for rice transformation. *Plant Physiology*, *90*(1), 217–223.
8. do Carmo, C. S., Nunes, A. N., Silva, I., Maia, C., Poejo, J., Ferreira-Dias, S., Nogueira, I., Bronze, R., & Duarte, C. M. M. (2016). Formulation of pea protein for increased satiety and improved foaming properties. *RSC Advances*, *6*(8), 6048–6057.
9. Ghogare, R., Williamson-Benavides, B., Ramírez-Torres, F., & Dhingra, A. (2019). CRISPR-associated nucleases: the Dawn of a new age of efficient crop improvement. *Transgenic Research*, 1–35.
10. Grant, J. E., Cooper, P. A., McAra, A. E., & Frew, T. J. (1995). Transformation of peas (Pisum sativum L.) using immature cotyledons. *Plant Cell Reports*, *15*(3–4), 254–258.
11. Hu, X., Wang, C., Fu, Y., Liu, Q., Jiao, X., & Wang, K. (2016). Expanding the range of CRISPR/Cas9 genome editing in rice. *Molecular Plant*, *9*(6), 943–945.
12. Itaya, A., Zheng, S., & Simmonds, D. (2018). Establishment of neomycin phosphotransferase II (nptII) selection for transformation of soybean somatic embryogenic cultures. *In Vitro Cellular & Developmental Biology-Plant*, *54*(2), 184–194.
13. Jefferson, R. A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter*, *5*(4), 387–405.
14. Jiwan, D., Roalson, E. H., Main, D., & Dhingra, A. (2013). Antisense expression of peach mildew resistance locus O (PpMlo1) gene confers cross-species resistance to powdery mildew in Fragaria x ananassa. *Transgenic Research*, *22*(6). https://doi.org/10.1007/s11248-013-9715-6
15. Jordan, M. C., & Hobbs, S. L. A. (1993). Evaluation of a cotyledonary node regeneration system forAgrobacterium-mediated transformation of pea (Pisum sativum L.). *In Vitro Cellular & Developmental Biology-Plant*, *29*(2), 77–82.

16. Kim, S.-R., Jeon, J.-S., & An, G. (2011). Development of an efficient inverse PCR method for isolating gene tags from T-DNA insertional mutants in rice. In *Plant Reverse Genetics* (pp. 139–146). Springer.
17. Liang, Z., Chen, K., Li, T., Zhang, Y., Wang, Y., Zhao, Q., Liu, J., Zhang, H., Liu, C., & Ran, Y. (2017). Efficient DNA-free genome editing of bread wheat using CRISPR/Cas9 ribonucleoprotein complexes. *Nature Communications*, *8*, 14261.
18. Lu, Y., & Zhu, J.-K. (2017). Precise editing of a target base in the rice genome using a modified CRISPR/Cas9 system. *Molecular Plant*, *10*(3), 523–525.
19. Michno, J.-M., Wang, X., Liu, J., Curtin, S. J., Kono, T. J. Y., & Stupar, R. M. (2015). CRISPR/Cas mutagenesis of soybean and Medicago truncatula using a new web-tool and a modified Cas9 enzyme. *GM Crops & Food*, *6*(4), 243–252.
20. Nadolska-Orczyk, A., & Orczyk, W. (1994). New aspects of soybean somatic embryogenesis. *Euphytica*, *80*(1–2), 137–143.
21. Nadolska-Orczyk, A., & Orczyk, W. (2000). Study of the factors influencing Agrobacterium-mediated transformation of pea (Pisum sativum L.). *Molecular Breeding*, *6*(2), 185–194.
22. Peng, W., Kong, X., Chen, Y., Zhang, C., Yang, Y., & Hua, Y. (2016). Effects of heat treatment on the emulsifying properties of pea proteins. *Food Hydrocolloids*, *52*, 301–310.
23. Pietrysiak, E., Smith, D. M., Smith, B. M., & Ganjyal, G. M. (2018). Enhanced functionality of pea-rice protein isolate blends through direct steam injection processing. *Food Chemistry*, *243*, 338–344.
24. Pniewski, T., & Kapusta, J. (2005). Efficiency of transformation of Polish cultivars of pea(Pisum sativum L.) with various regeneration capacity by using hypervirulent Agrobacterium tumefaciens strains. *Journal of Applied Genetics*, *46*(2), 139–147.
25. Polowick, P. L., Quandt, J., & Mahon, J. D. (2000). The ability of pea transformation technology to transfer genes into peas adapted to western Canadian growing conditions. *Plant Science*, *153*(2), 161–170.
26. Porter, L. D., Kraft, J. M., & Grünwald, N. J. (2014). Release of pea germplasm with Fusarium resistance combined with desirable yield and anti-lodging traits. *Journal of Plant Registrations*, *8*(2), 191–194.
27. Puonti-Kaerlas, J., Eriksson, T., & Engström, P. (1992). Inheritance of a bacterial hygromycin phosphotransferase gene in the progeny of primary transgenic pea plants. *Theoretical and Applied Genetics*, *84*(3–4), 443–450.
28. Rolland, F., Baena-Gonzalez, E., & Sheen, J. (2006). Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.*, *57*, 675–709.
29. Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics methods and protocols* (pp. 365–386). Springer.
30. Schaerer, S., & Pilet, P.-E. (1991). Roots, explants and protoplasts from pea transformed with strains of Agrobacterium tumefaciens and rhizogenes. *Plant Science*, *78*(2), 247–258.

31. Schroeder, H. E., Schotz, A. H., Wardley-Richardson, T., Spencer, D., & Higgins, T. J. V. (1993). Transformation and regeneration of two cultivars of pea (Pisum sativum L.). *Plant Physiology*, *101*(3), 751–757.
32. Shetty, R. P., Endy, D., & Knight, T. F. (2008). Engineering BioBrick vectors from BioBrick parts. *Journal of Biological Engineering*, *2*(1), 5.
33. Shew, A. M., Nalley, L. L., Snell, H. A., Nayga Jr, R. M., & Dixon, B. L. (2018). CRISPR versus GMOs: Public acceptance and valuation. *Global Food Security*, *19*, 71–80.
34. Sreevidya, V. S., Srinivasa Rao, C., Sullia, S. B., Ladha, J. K., & Reddy, P. M. (2006). Metabolic engineering of rice with soybean isoflavone synthase for promoting nodulation gene expression in rhizobia. *Journal of Experimental Botany*, *57*(9), 1957–1969.
35. Stehfest, E., Bouwman, L., Van Vuuren, D. P., Den Elzen, M. G. J., Eickhout, B., & Kabat, P. (2009). Climate benefits of changing diet. *Climatic Change*, *95*(1–2), 83–102.
36. Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K., & Cigan, A. M. (2016). Genome editing in maize directed by CRISPR–Cas9 ribonucleoprotein complexes. *Nature Communications*, *7*, 13274.
37. Twyman, R. M., Stöger, E., Kohli, A., Capell, T., & Christou, P. (2002). Selectable and screenable markers for rice transformation. In *Testing for Genetic Manipulation in Plants* (pp. 1–17). Springer.
38. von Arnim, A. G., Deng, X. W., & Stacey, M. G. (1998). Cloning vectors for the expression of green fluorescent protein fusion proteins in transgenic plants. *Gene*, *221*(1), 35–43. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=9852947
39. Williamson-Benavides, B. A., Sharpe, R. M., Nelson, G., Bodah, E. T., Porter, L. D., & Dhingra, A. (2020). Identification of Fusarium solani f. sp. pisi (Fsp) Responsive Genes in Pisum sativum . In *Frontiers in Genetics* (Vol. 11, p. 950).
40. Xing, H. L., Dong, L., Wang, Z. P., Zhang, H. Y., Han, C. Y., Liu, B., Wang, X. C., & Chen, Q. J. (2014). A CRISPR/Cas9 toolkit for multiplex genome editing in plants. *BMC Plant Biology*, *14*(1). https://doi.org/10.1186/S12870-014-0327-Y,
41. Xiong, T., Xiong, W., Ge, M., Xia, J., Li, B., & Chen, Y. (2018). Effect of high intensity ultrasound on structure and foaming properties of pea protein isolate. *Food Research International*, *109*, 260–267.
42. Yanaglsawa, S., Yoo, S. D., & Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signaling in plantas. *Nature*, *425*(6957), 521–525.
43. Yasodha, R., Kamala, S., Kumar, S. P. A., Kumar, P. D., & Kalaiarasi, K. (2008). Effect of glucose on in vitro rooting of mature plants of Bambusa nutans. *Scientia Horticulturae*, *116*(1), 113–116.

Figures

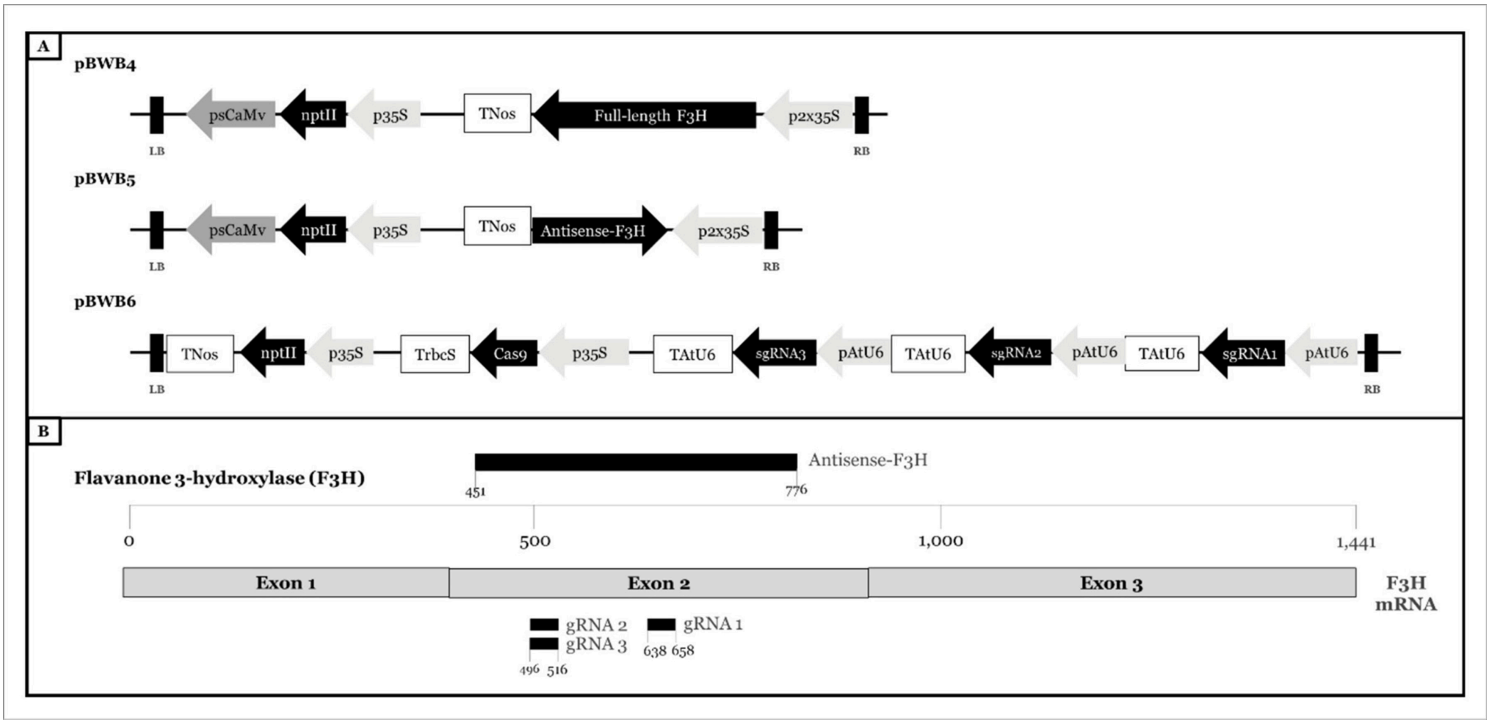


Figure 1

Structure of the T-DNA region in the binary vectors of pBWB4, pBWB5, and pBWB6 (a) and visual representation of the targeted regions in the flavanone 3-hydroxylase (F3H) gene (b).

Cas9: CRISPR associated protein 9; gRNA; guide RNA; LB and RB: left and right border of the T-DNA; *npt*II: neomycin phosphotransferase II; p35S: CaMV35S promoter; pAtU6: AtU6 promoter; psCaMv: cauliflower mosaic virus polyA signal; tAtU6: AtU6 terminator; TNos: nopaline synthase gene terminator; and trbcS: rbcS terminator.

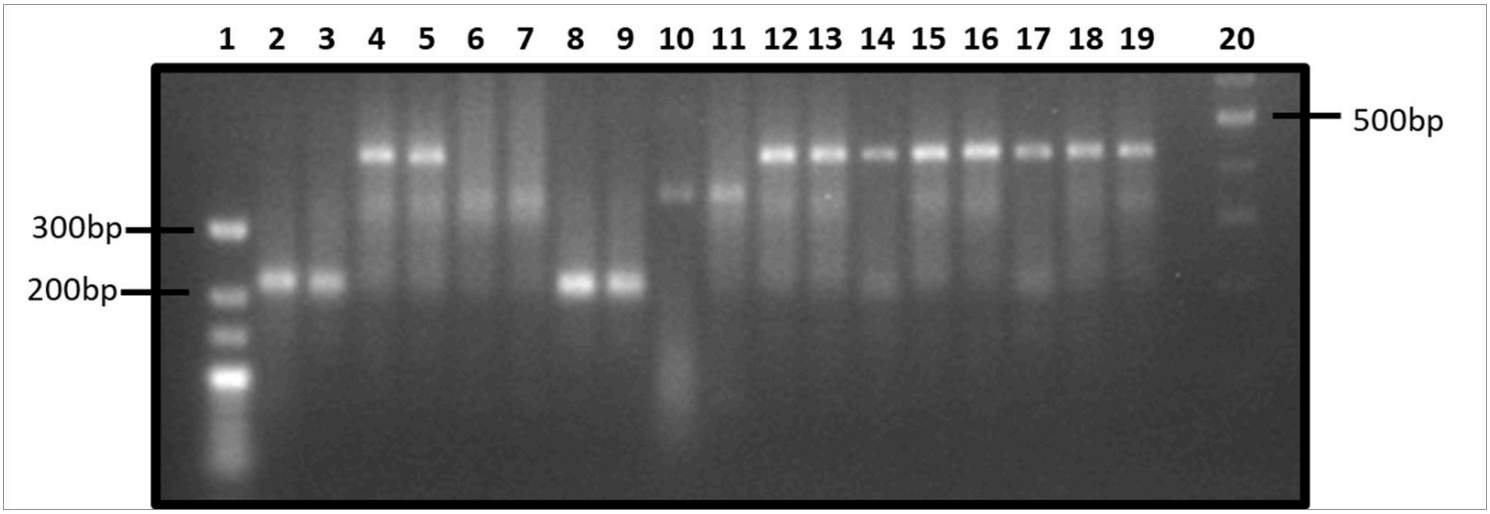


Figure 2

In vitro cleavage assay to assess the functionality of gRNAs on PCR amplified F3H isoform 1 (lanes 2-7) and isoform 2 (lanes 8-13). Assays with gRNA1 are presented in lanes 2, 3, 8, and 9. Assays with gRNA2

are presented in lanes 4, 5, 10, and 11. Assays with gRNA3 are presented in lanes 6, 7, 12, and 13. Control samples for F3H isoform 1 (lanes 14-16) and isoform 2 (lanes 17-19) were electrophoresed in absence of the Cas9 nuclease. Lanes 14 and 17 correspond to gRNA1; lanes 15 and 18 correspond to gRNA2; and lanes 16 and 19 correspond to gRNA3. Lane 1: Invitrogen Ultra Low Range DNA Ladder. Lane 20: exACTGene 1kb DNA Ladder.

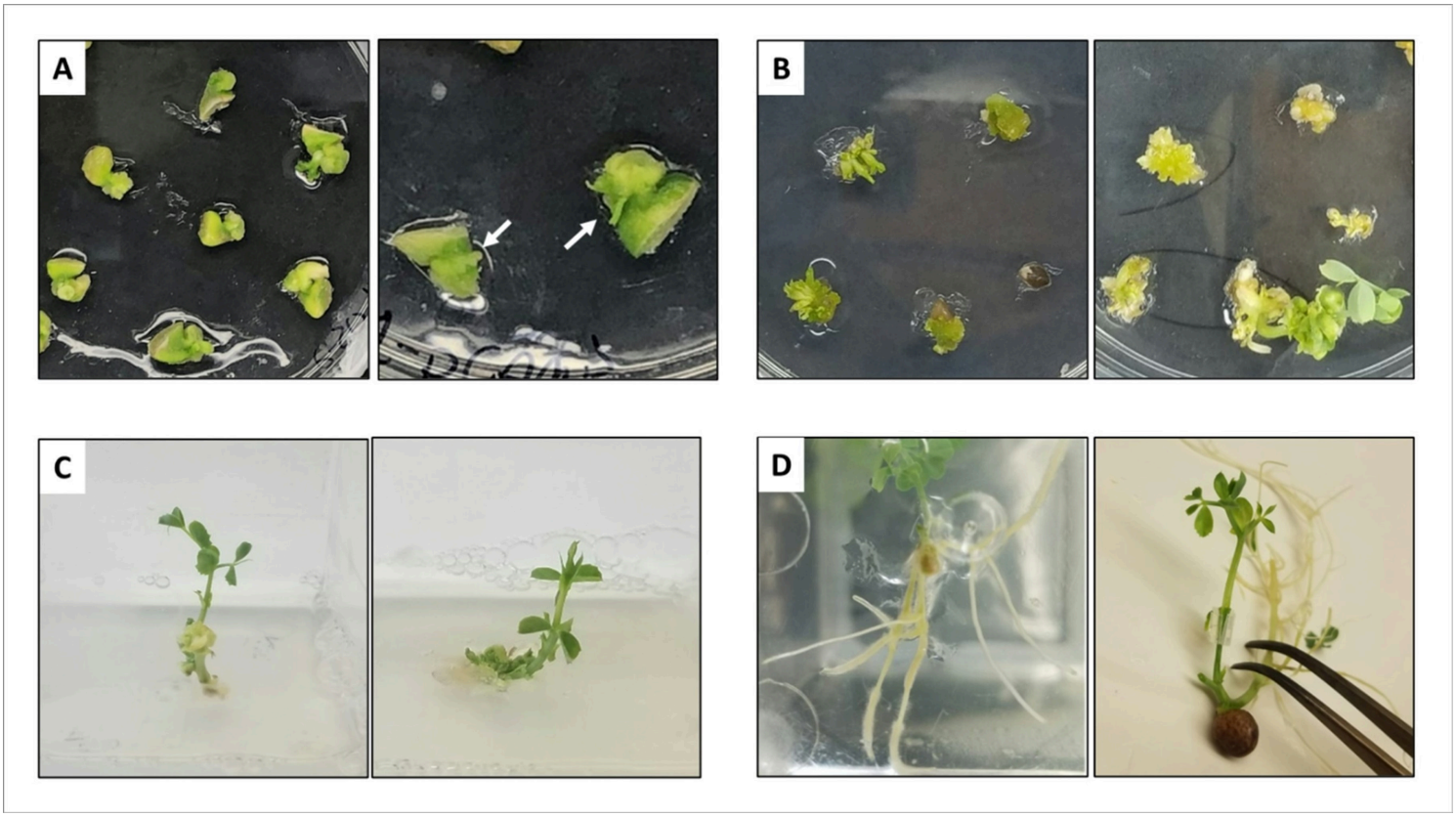


Figure 3

Regeneration (a), elongation (b), and rooting (c and d) of pea transgenic lines. White arrows in image A point at shoots regenerating from embryo axes. Image d (right panel) shows a transgenic line grafted onto a wild type seedling.

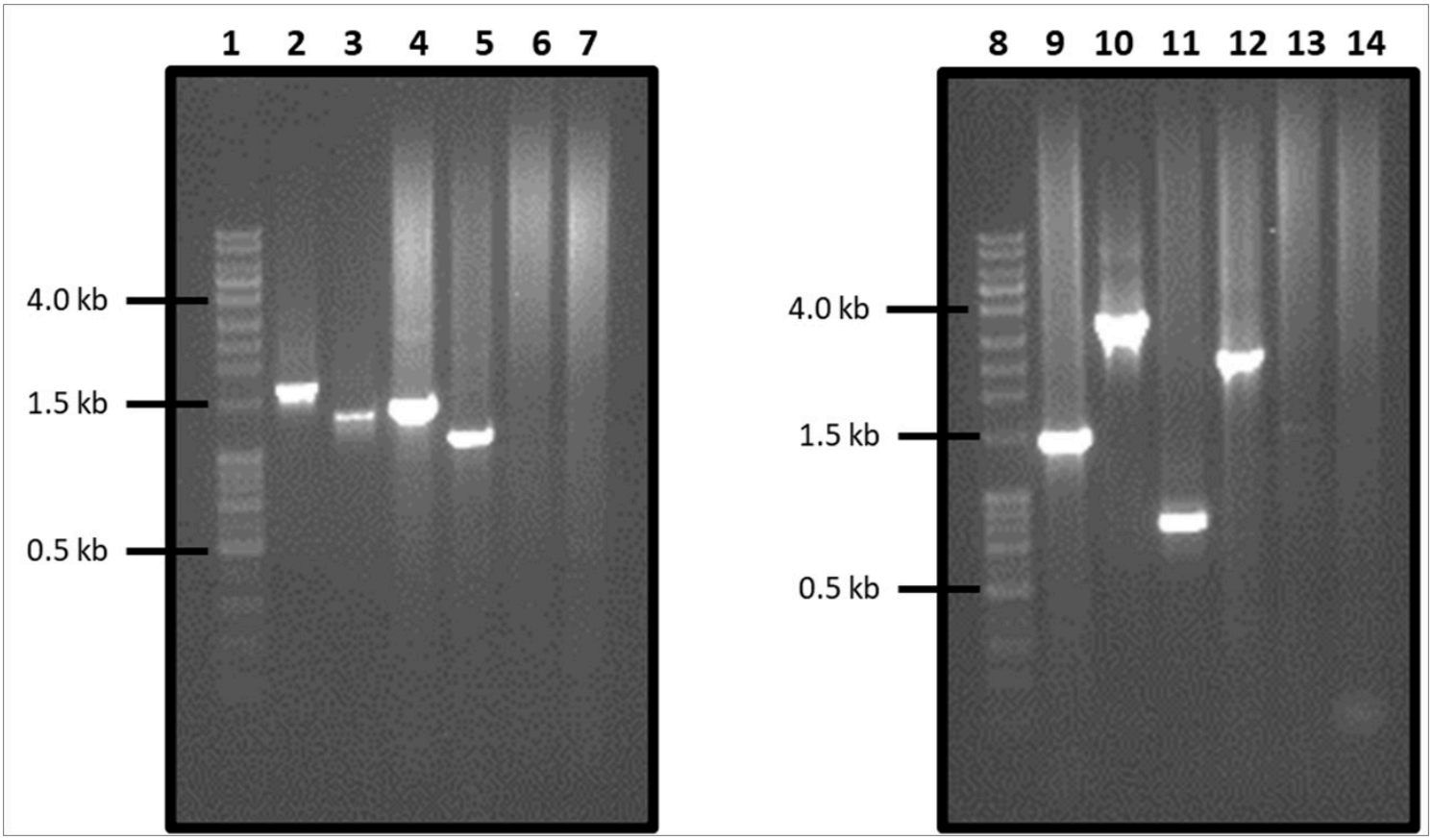


Figure 4

Inverse PCR assay to determine the number of T-DNA copies in transgenic lines. Represented samples are transgenic lines 59.1.0 (pBWB4) (lanes 2-3), 70.1.0 (pBWB4) (lanes 4-5), 63.1.0 (pBWB6) (lanes 9-10), and 66.1.0 (pBWB6) (lanes 11-12), and wild type (lanes 6, 7, 12, 13). Nested PCR for the left borders is represented by lanes 2, 4, 6, 9, 11, and 13. Nested PCR for the right border is represented by lanes 3, 5, 6, 10, 12, and 14. Oligonucleotides for nested PCRs are listed in Table S.1. Lanes 1 and 8: exACTGene 1kb DNA Ladder.



Figure 5

Flowers of wild type (a) and transgenic lines (b-c) transformed with pBWB4 – ectopic expression (b), pBWB5 – antisense expression (c), and pBWB6 – CRISPR/Cas9 edited line (d). Note the variation of anthocyanin accumulation in the wing part of the flower in the transgenic lines (b-d) compared to the WT flower (a).

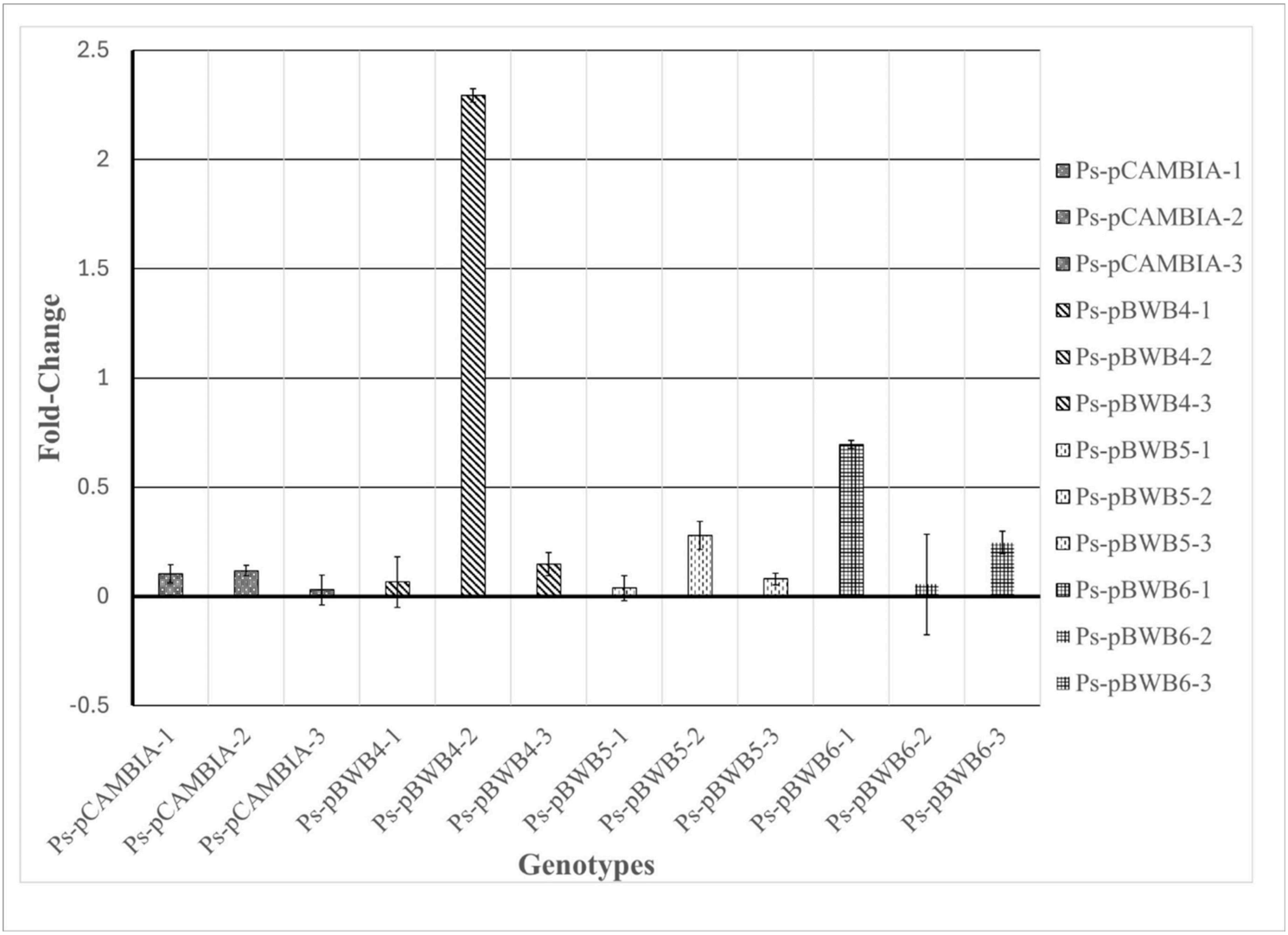


Figure 6

RT-qPCR analysis of F3H gene expression in the leaves of pea transgenic lines: Gene expression values were normalized to WT (Y axis = 1), and fold change expression of F3H in transgenic lines was plotted. Three biological replicates with four technical replicates for each event were used for the analysis. Error bars represent standard error. *Ps*-pCAMBIA2300 1-3 lines represent the empty vector, *Ps*-pBWW4 1-3 lines represent ectopic expression of the F3H gene, *Ps*-pBWB5 1-3 lines represent antisense expression of the F3H gene, and Ps-pBWB6 1-3 lines represent the CRISPR/Cas9 gene-edited individuals.

Supplementary Files

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