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Research Article

Development of a direct transformation method by GFP screening and *in vitro* whole plant regeneration of *Capsicum frutescens* L.

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ABSTRACT

Background: *Capsicum* is a genus of an important spice crop that belongs to the chili lineage. However, many *Capsicum* species (family Solanaceae) are known to be recalcitrant to genetic transformation and *in vitro* regeneration, thus hampering the effort in using *Capsicum* species for detailed biological investigation. In this study, we have developed an optimized protocol for the direct transformation of *Capsicum frutescens* L. cv. Hot Lava *using* a biolistic particle delivery system. In addition, a procedure for *in vitro* whole plant regeneration from the hypocotyl explants of *C. frutescens* was established.

Results: In this study on the biolistic system, explant target distance, bombardment helium (He) pressure, and the size of the microcarrier were the key parameters to be investigated. The optimized parameters based on the screening of GFP expression were determined to have a target distance of 6 cm, helium pressure of 1350 psi, and gold particle (microcarrier) size of 1.6 μm. The greatest number of shoots was obtained from hypocotyls as explants using Murashige and Skoog medium supplemented with 5.0-mg/L 6-benzylaminopurine and 0.1-mg/L 1-naphthaleneacetic acid. On an average, five shoots per explant were formed, and of them, one shoot managed to form the root and developed into a whole plant.

Conclusions: We obtained an optimized protocol for the biolistic transformation of chili and *in vitro* regeneration of chili plantlets. The establishment of the protocols will provide a platform for molecular breeding and biological studies of chili plants.

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1. Introduction

Capsicum constitutes the genus of an important group of spices — the chili peppers. Much active research has been carried out on *Capsicum* to explore its growth and developmental physiology in different environments, as well as its potential for genetic improvement, metabolite utilization, and preharvest and postharvest enhancements [1,2,3,4,5,6]. Genetic engineering techniques and plant tissue culture are useful tools that can advance improvements in *Capsicum*. In many recombinant DNA techniques that are commonly used, an effective plant transformation procedure is required alongside a protocol for plant regeneration [7]. However, the development of such strategies for *Capsicum* is progressing more slowly than those for other Solanaceae members owing to the recalcitrant nature and high genotypic dependence of *Capsicum* [2,8]. Although chili is an economically important crop, the regeneration of *in vitro* cultures of *Capsicum* has not been fully established. Some studies have reported success in the regeneration of several pepper species from the shoot tip, shoot bud, node, leaf, stem, root, hypocotyl, cotyledon, and zygotic embryo [9,10,11,12,13,14,15,16]. Despite these studies, many of the studies indicated the existence of limitations that are yet to be overcome regarding the tissue culture of *Capsicum*.

The genetic engineering of *Capsicum* is hampered by its low morphogenetic capability [8]. Thus far, most of the transformation studies on *Capsicum* centered on *Agrobacterium*-mediated gene transfer [5,17,18,19,20], whereas reports on the transformation of *Capsicum* by particle bombardment remain lacking, more so for *Capsicum frutescens*. Gilardi et al. [21] described the introduction of pepper mild mottle virus coat protein into *Capsicum chinense* by biolistics. More recently, the same group reported the introduction of tobamovirus coat protein into *C. frutescens* L. cv. Tabasco, also by biolistics [22]. In another study, a biolistic hand gun was used to

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deliver the beta-glucuronidase (GUS) reporter gene into *Capsicum annuum* L. [23].

In the present study, three main parameters, namely, microcarrier size, explant target distance, and bombardment He pressure, were optimized for the direct transformation of *C. frutescens* L. cv. Hot Lava by particle bombardment, with green fluorescent protein (GFP) as the reporter protein. Despite several reported drawbacks such as susceptibility to photobleaching and sensitivity to pH changes [24,25,26], GFP is advantageous because it does not rely on any exogenous cofactor to fluoresce and is useful as a nondestructive reporter assay for *in vivo* visualization [27]. Apart from the optimization of the biolistic parameters, an alternative procedure for the regeneration of whole plant from hypocotyls is also reported.

2. Materials and methods

2.1. Plant materials and growth media

Seeds of *C. frutescens* L. cv. Hot Lava were obtained from Green World Genetics Pte. Ltd. (Kepong, Malaysia). Surface sterilized seeds were germinated on standard Murashige and Skoog (MS) solid agar containing 30-g/L sucrose and 3.5-g/L Phytagel™ (Sigma-Aldrich, St. Louis, USA). The same MS agar was used for the optimization of biolistic parameters and *in vitro* regeneration studies.

2.2. Incubation conditions of plant cultures

All the plant cultures in the studies were maintained in 16-h light/8-h dark photoperiod with 40 μmol/m^2/s (or 2960 lx) light intensity from cool white fluorescent lamps. The temperature and humidity of the incubation room were kept at approximately 24°C and 42%, respectively.

2.3. Optimization of biolistic parameters

2.3.1. Plasmid preparation

A plasmid construct, pcDNA6.2::35S-GFP, which harbored the gene for GFP driven by the cauliflower mosaic virus (CaMV) 35S promoter, was used. The backbone of the plasmid contained a blasticidin-S resistance (*bsr*) gene, which served as a positive selectable marker. The plasmid was propagated using OneShot® ccdB Survival™ 2 $T1^R$ *Escherichia coli* (Invitrogen, Carlsbad, USA) by heat shock transformation. *E. coli* colonies carrying the plasmid were cultured in Luria-Bertani (LB) broth containing 100-mg/L ampicillin. Finally, the plasmid was extracted using GeneAll® Hybrid-Q Plasmid Rapidprep kit (GeneAll Biotechnology, Songpa-gu, South Korea) according to the manufacturer's instructions.

2.3.2. Explant preparation

One day before the bombardment procedure, hypocotyls from 14-day-old *C. frutescens* seedlings were excised to the length of 5 mm and were laid on standard MS solid agar in Petri plates. Fifteen explants were arranged at the center of each Petri plate. Each experiment for every set of the studied biolistic parameters was conducted three times, with 45 hypocotyl explants per parameter treatment.

2.3.3. Microcarrier preparation

For each of the gold particle sizes (0.6, 1.0, and 1.6 μm), gold suspension in 50% (v/v) glycerol (3 mg of gold particles per six bombardments) was mixed with pcDNA 6.2::35S-GFP plasmid (5 μg of plasmid per six bombardments) as well as calcium chloride ($CaCl_2$) and spermidine under constant vortex to mix well. The gold-DNA suspension was then pelleted, and the supernatant was removed. The gold-DNA pellet was washed once with 70% (v/v) ethanol, followed by another washing with 100% ethanol before finally resuspending in 100% ethanol.

2.3.4. Microprojectile bombardment

For each of the gold particle sizes tested, *i.e.*, 0.6, 1.0, and 1.6 μm, the He pressure was set at 900, 1100, and 1350 psi, respectively. For each of the gold particle sizes and each of the He pressures used, the target distance (distance between the stopping screen and explants) was set at 3, 6, and 9 cm. The macrocarrier travel distance was constant at 8 mm, whereas the rupture disk–macrocarrier gap distance was fixed at 0.64 cm. The vacuum pressure in the bombardment chamber was fixed at 94.82 kPa. Delivery of plasmid-coated microcarriers was performed using the Bio-Rad PDS-1000/He particle delivery system (Hercules, USA).

2.3.5. Selection and regeneration of plant transformants

Bombarded explants were incubated in the dark for 48 h before being transferred to standard MS agar medium containing 0.25-mg/L blasticidin-S, 2.0-mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.5-mg/L kinetin for antibiotic selection and regeneration. The explants were incubated for one month and subsequently were transferred to the same, fresh medium for another month of selection and regeneration.

2.3.6. Screening of plant transformants

Bombarded explants were observed for GFP expression using a Nikon SMZ1500 stereomicroscope under blue light illumination. Observations were made every day up to five days postbombardment, and then for every five days for up to 60 d. Microscopic images were captured, and the intensity of GFP fluorescence was measured using NIS Elements D v3.22.00 (Build 710) software. Mean green fluorescence intensity, which was used as the measurement of GFP fluorescence, was determined quantitatively from the signal of the green channel (excluding red and blue channels) from the microscopic images (Fig. 1). Mean green fluorescence intensity was calculated by the software as the sum of the green fluorescence intensities across a given area.

2.3.7. Extraction of plant genomic DNA

After two months postbombardment, genomic DNA of the selected callus was extracted. For each sample, approximately 200 mg of callus tissues was ground in a microcentrifuge tube with a Kimble pestle until the tissues became mushy. An extraction buffer solution containing 200-mM Tris-Cl (pH 7.5), 250-mM sodium chloride (NaCl), 25-mM ethylenediaminetetraacetic acid (EDTA), and 0.5% (w/v) sodium dodecyl sulfate (SDS) was added, and the sample was homogenized. Saturated phenol was added to the homogenized sample and mixed. Then, this sample was subjected to centrifugation at the maximum speed for 10 min at 4°C; the aqueous phase was collected, and a solution of chloroform/isoamyl alcohol (24:1, v/v) was added to it. After a repeat centrifugation process, isopropanol was added to the aqueous phase and the DNA was allowed to precipitate at room temperature for 30 min. The DNA was then pelleted, isopropanol was removed, and the pellet was washed with 70% v/v ethanol. Following centrifugation and removal of ethanol, the DNA pellet was dried in a vacuum desiccator for 2 min and then dissolved in autoclaved water.

2.3.8. Confirmation by polymerase chain reaction (PCR)

The detection of the *gfp* gene in the extracted genomic DNA was done by PCR using the 5'-ATGGTGAGCAAGGGCGAGGAG-3′ forward primer and 5'-TTACTTGTACAGCTCGTCCAT-3′ reverse primer. Each of the 20-μl PCR mix consisted of 1× PCR buffer, 3.0-mM magnesium chloride ($MgCl_2$), 0.3-mM deoxynucleotides (dNTPs), 0.3-μM forward primer, 0.3-μM reverse primer, 1 unit of recombinant *Taq* polymerase (Invitrogen, Carlsbad, USA), and 50 ng of template DNA. The PCR

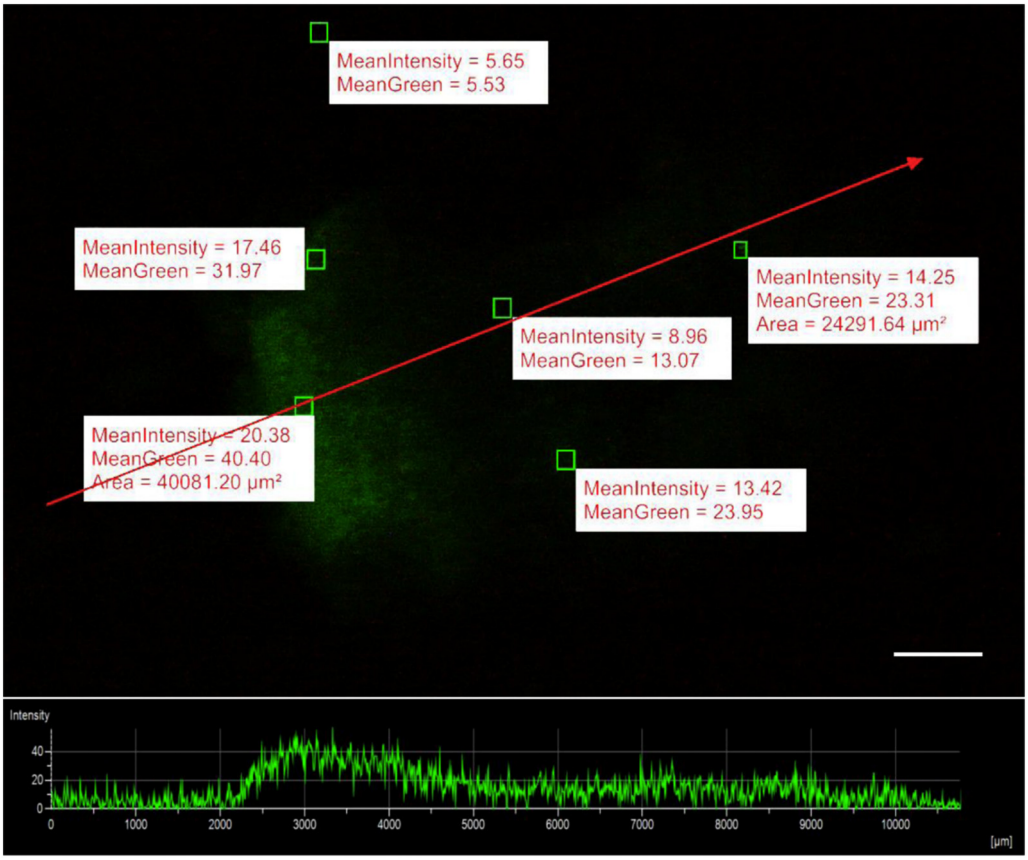

Fig. 1. Microscopic diagram showing the measurement of GFP intensity across an explant. The graph below the diagram shows the intensity plot of green signals across the distance as indicated by the red arrow in the diagram. Mean intensity indicates the sum of intensities, combining red, green, and blue channels, across a given area. Mean green intensity indicates the sum of intensities from the green channel only across a given area. Scale bar represents 1 mm.

thermal cycling conditions were as follows: 1 cycle of initial denaturation at 95°C for 3 min; 30 cycles of denaturation (95°C, 30 s), annealing (53°C, 30 s), and elongation (72°C, 45 s); and 1 cycle of final elongation at 72°C for 5 min. Gel electrophoresis of the PCR products was performed in 1% (w/v) agarose gel (First Base, Malaysia) with 1× Tris-acetate-EDTA (TAE) buffer and SYBR Safe DNA stain (Invitrogen) at 90 V for 45 min.

2.4. In vitro whole plant regeneration

2.4.1. Explant preparation on regeneration media

Hypocotyls and cotyledons from 14-day-old seedlings were excised to the length of 5 mm. They were cultured on standard MS agar supplemented with (a) 5.0-mg/L 6-benzylaminopurine (BAP) and 0.1-mg/L 1-naphthaleneacetic acid (NAA), (b) 10.0-mg/L BAP and 0.1-mg/L NAA, (c) 3.0-mg/L BAP and 1.0-mg/L indole-3-acetic acid (IAA), (d) 10.0-mg/L BAP and 1.0-mg/L IAA, (e) 5.0-mg/L BAP, and (f) 10.0-mg/L BAP. For each regeneration treatment, the experiment was conducted in triplicate, with each replicate consisting of 20 explants.

2.4.2. Subculture, acclimatization, and transplantation

Explants that developed callus and primordium-like structures followed by shoots and roots were subcultured onto their respective fresh regeneration media. Newly proliferated whole plants were allowed to grow to the full height of a culture jar (culture jar dimension: 62-mm outer diameter, OD, × 127-mm height). Each plant was then acclimatized in its culture jar with a punctured cover for one week. After that, the culture jar was moved to a normal room (approximately 27°C temperature, 54% humidity, and 10-h light/14-h dark photoperiod with unknown light intensity) and was held for one week. Finally, the plant was removed from the culture medium and transplanted to a pot of peat compost for further growth and development.

2.5. Numerical and statistical analyses

All numerical data and statistical analyses were performed with GenStat 17th Edition (v17.1.0.14713). Unless otherwise stated, the data presented are represented as mean ± standard deviation (SD) of the mean. The logistic regression of binomial proportions based on generalized linear models was performed for results of the optimization of biolistic parameters. Analysis of variance (ANOVA) with Tukey's *post hoc* test of least significant difference (LSD), where multiple comparisons were applicable, was performed for the results of *in vitro* whole plant regeneration. The level of significance or *P*-value of the test of hypotheses was set at 0.05.

3. Results and discussion

3.1. Optimization of biolistic parameters

The combination of 1.6-µm size of gold particles, 6-cm target distance, and 1350-psi He pressure gave the highest number of transformants and the highest GFP intensity (Table S1). These parameters provided up to 18% of transformation efficiency, which was calculated as the percentage of effective number of transformed calli (DNA integration confirmed by PCR) divided by the total number of bombarded explants. The transformation efficiency across the different combinations of parameters was generally low. This could be due to the nature of transgene integration in biolistic transformation, which is complex and could be affected by random occurrences such as DNA breakage and premature ligation of DNA before integration

Table 1
Accumulated analysis of deviance of the parameters microcarrier size, target distance, and He pressure by logistic regression within groups and between groups. Mean values with the same superscript letters are not significantly different at $P = 0.05$ within their respective groups, where F probability <0.001.

Comparison of parameters	Mean number of transformants	Accumulated analysis of deviance		
		Degree of freedom	Mean deviance	F probability
Within groups				
Microcarrier size (μm)		2	17.466	<0.001
0.6	0.185[b]			
1.0	0.556[a,b]			
1.6	1.519[a]			
Target distance (cm)		2	27.636	<0.001
3	0.00[b]			
6	1.444[a]			
9	0.815[a,b]			
He pressure (psi)		2	31.029	<0.001
900	0.000[b]			
1100	0.667[a,b]			
1350	1.593[a]			
Between groups				
Microcarrier size and target distance	0.753	4	0.174	0.816
Microcarrier size and He pressure	0.753	4	0.087	0.941
Target distance and He pressure	0.753	4	0.259	0.681
Microcarrier size, target distance, and He pressure	0.753	8	0.236	0.830

[28]. Analysis within each of the respective groups (microcarrier size, target distance, and He pressure) showed significant interactions (F probability <0.05) (Table 1). The microcarrier with a size of 1.6 μm performed better than the microcarriers with a size of 0.6 and 1.0 μm; the 6-cm target distance was significantly more effective than the 3- and 9-cm target distances; and the 1350-psi He pressure was significantly more efficient than the 900- and 1100-psi He pressures. According to our study results, a subsequent combination of these outcomes showed that 1.6-μm gold particle size, 6-cm target distance, and 1350-psi He pressure were the most suitable parameters for the biolistic transformation of the hypocotyl explants of *C. frutescens* L. It is to be noted that the biolistic transformation method is relatively easy to operate, as it is a direct method and does not require long time to prepare for bombardment. However, the downstream regeneration and analysis is still taking a relatively long duration, similar to the duration in many other plants.

The highest GFP intensity values showing the highest achievable GFP expression by a given set of biolistic parameters are presented in Table S1. In standard fluorescence microscopy, noise from autofluorescence can cause errors in the measurement of GFP intensity when the GFP intensity-to-noise ratio is less than 1.5, which is considered low [29,30]. Our reported data of the highest GFP intensities had signal-to-noise ratios of more than 1.5, a threshold below which an error is said to occur [31]. This shows that GFP signals detected in this study were significantly higher than those detected from the autofluorescence or background. A transient expression of GFP was observed to start from 15 d postbombardment using the best reported parameters, *i.e.*, 1.6-μm gold particle size, 6-cm target distance, and 1350-psi He pressure. GFP intensity increased slowly across the days, and the highest level of intensity was seen at 50 d postbombardment, after which the GFP intensity declined sharply (Fig. 2). This could be due to the slow proliferation of transformed tissues, as the presence of a selective antibiotic in the regeneration medium caused stress to the explants. Although slow, the increase in GFP intensity up to 50 d suggests that the rate of GFP production had increased with the proliferation of transformed tissues. From the time-point observations, it was hypothesized that the onset of transgene expression to a detectable level (as in the case of GFP) had started 15 d postbombardment. Assuming a similar expression pattern for the blasticidin-S resistance selectable marker, it was thought that the bombarded explants should be transferred to the selective media 15 d postbombardment to minimize selection against actually transformed explants that had yet to express the resistance gene to a level sufficient to confer resistance on the explants.

The detection of the *gfp* gene of 720 bp by PCR two months postbombardment confirmed the presence of the gene in the genome of transformants (Fig. 3). Although the gene was present, the drastic decline in GFP fluorescence intensity after 50 d postbombardment suggests that the protein might have ceased expression or the protein was still expressed, but it had lost its fluorescence. The GFP reporter gene used in the plasmid construct was a modified *gfp* gene that encodes a protein in which serine was replaced with threonine by chromophore mutation at position 65 of the amino acid sequence to give a fluorescence signal 100-fold higher than that of the original jellyfish GFP [32]. Transcription of this reporter gene was driven by a reliable, constitutive CaMV 35S promoter to give constant expression of GFP in cells where it was expressed. Nevertheless, there could have

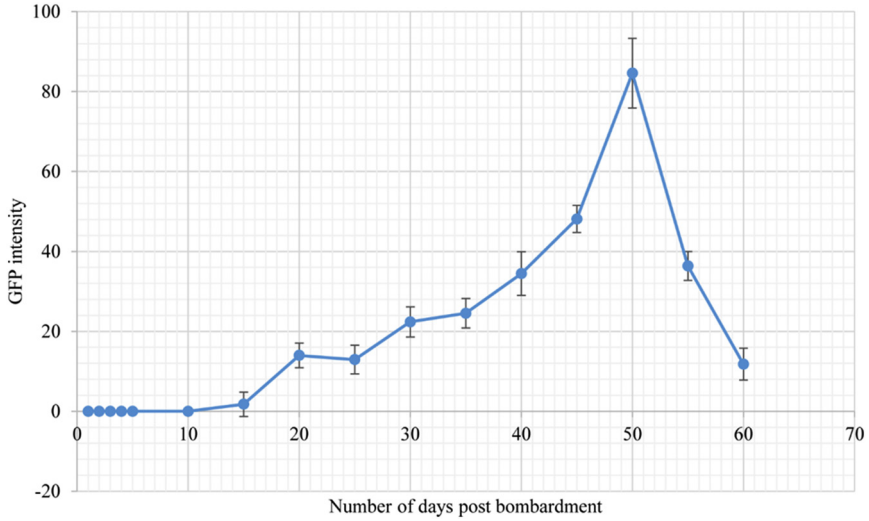

Fig. 2. Plot of the highest GFP intensity averaged from three experiments across 60 d after bombardment. Vertical error bars represent standard deviation of the mean values. The highest peak was observed at 50 d after bombardment.

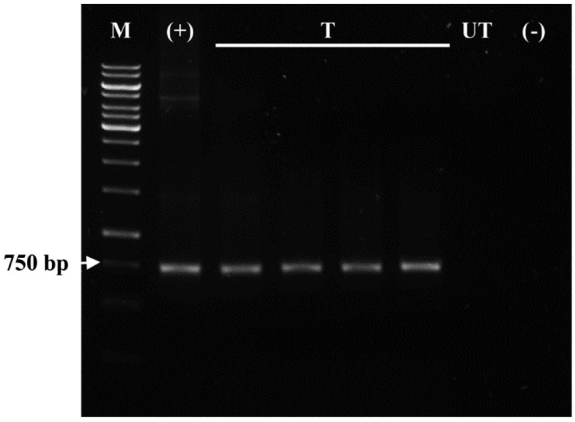

Fig. 3. PCR results of the gfp gene (720 bp) in the genomic DNA of the transformed calli (T). M – 1 kb DNA ladder; (+) PCR positive control using the pcDNA 6.2::35S-GFP plasmid as the DNA template; UT – PCR using the genomic DNA of untransformed calli as the DNA template; (−) PCR negative control without any DNA template.

been loss of expression with time because of systemic gene silencing by the host's defense mechanism. Transcribed messenger RNA (mRNA) of GFP was possibly removed with the production of small interfering RNA (siRNA) to target the mRNA of GFP for degradation [33,34]. A small amount of siRNA could also trigger spontaneous local silencing of GFP where there was no systemic silencing [35]. Additionally, the chromophore of GFP could have lost its fluorescence following constant light irradiation, a phenomenon known as photobleaching that is seemingly irreversible [24]. Light irradiation of GFP generates endogenous singlet oxygen ($^1O_2$), which induces damage to the GFP chromophore [25]. However, the rate of photobleaching of GFP could be lower than that of other fluorescent proteins because the key GFP chromophore is located in the core of a β-barrel structure, thus somewhat protecting it from reaction with $^1O_2$ [36,37]. In addition to irradiation that causes photobleaching, the spectral properties of GFP *in vitro* are also sensitive to pH. For example, the fluorescence of wild-type GFP is stable at pH 6–10, but a decrease below pH 6 causes a reduction in the fluorescence [26]. Therefore, any changes in the ionic strength of the tissue culture that lead to pH change could result in the loss of fluorescence. In the case that the GFP did not cease expression, it was possible that the rate of GFP production could not overcome the rate of fluorescence decline because of photobleaching or pH change after 50 d.

It was observed that the GFP intensity was not consistent throughout a transformed explant (Fig. 4), thus indicating that GFP was randomly distributed and was not expressed at the same level across the explant. This could be due to the random uptake of the exogenous plasmid into the cells, as well as random integration of the GFP DNA into the genome of the host cells. Integration of target DNA into the genome as a nuclear transformation takes place by random recombination [38,39]. In addition to depending on the nature of the DNA, gene expression also depends on the position of the target DNA in the genome where it is integrated. Integration of the gene into a transcriptionally active region may result in high gene expression, whereas positioning the gene in a nonactive region may cause reduced or no gene expression [40]. Moreover, the number of copies of the GFP gene that was integrated was uncertain, although

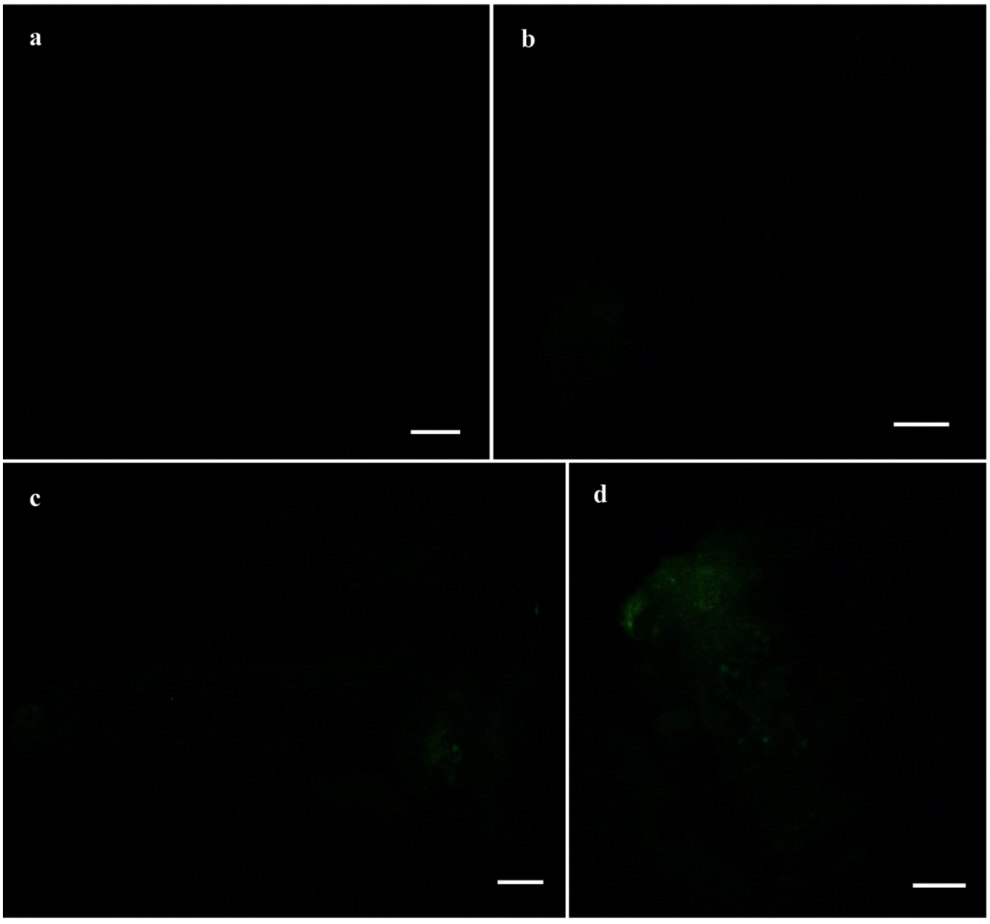

Fig. 4. Regenerated tissues of calli expressing GFP at different intensities after bombardment. Callus not expressing GFP is shown in a (negative control), and calli expressing GFP at varying intensities are shown in b (lower) and c (higher). The distribution of GFP spots was not consistent throughout the explants.

Table 2
Comparison of the number of cotyledon and hypocotyl explants forming shoot and root between the different combinations of BAP and NAA or IAA in the regeneration media. Mean values (±standard deviation) with the same superscript letters are not significantly different at $P = 0.05$ within their respective columns.

BAP concentration (mg/L)	NAA or IAA concentration (mg/L)	Cotyledon explant		Hypocotyl explant	
		Number forming shoot	Number forming root	Number forming shoot	Number forming root
5.0	NAA, 0.1	1.33 ± 1.53^{a}	0.33 ± 0.58^{a}	4.67 ± 0.58^{a}	1.33 ± 0.58^{a}
10.0	NAA, 0.1	2.67 ± 0.58^{a}	0.00^{a}	3.33 ± 1.53^{ab}	0.00^{b}
3.0	IAA, 1.0	1.67 ± 1.52^{a}	0.00^{a}	1.33 ± 1.15^{b}	0.00^{b}
10.0	IAA, 1.0	1.00 ± 0.00^{a}	0.00^{a}	3.67 ± 1.53^{ab}	0.00^{b}
5.0	–	2.00 ± 1.00^{a}	0.00^{a}	3.33 ± 0.58^{ab}	0.00^{b}
10.0	–	2.00 ± 1.73^{a}	0.00^{a}	4.00 ± 1.00^{ab}	0.00^{b}

transformants from the biolistic method usually take in multiple copies of the transgene into their nuclear genome [39]. Different copy number could result in difference in the expression level. With regard to this, a high copy number does not always correspond to a high expression level. The occurrence of multiple copies of a foreign gene could trigger posttranscriptional gene silencing in the host cell, which in turn could lead to the loss of gene expression [41,42,43].

3.2. In vitro whole plant regeneration

A procedure for whole plant regeneration by tissue culture has been shown to be successful, although with low frequency. The results from a number of hormone combinations leading to whole plant regeneration are reported in this section (Table 2).

Comparison of data between the regeneration treatments regarding the number of cotyledon explants forming shoots and roots was performed statistically by ANOVA with Tukey's multiple comparisons. The number of cotyledon explants forming shoots did not differ significantly across all the treatments tested. A similar result was observed on the number of cotyledon explants forming roots. On the other hand, the number of hypocotyls forming shoots with 5.0-mg/L BAP and 0.1-mg/L NAA was generally higher than the numbers in the other treatments, although the differences were insignificant except when compared with 3.0-mg/L BAP and 1.0-mg/L IAA and with control treatment without any hormone. For the formation of root from hypocotyl explants, the most promising treatment was 5.0-mg/L BAP and 0.1-mg/L IAA, whereas the rest of the treatments did not result in root formation at all. On average, little root formation was observed among the cotyledon explants. Therefore, it is important to note that explants that produced a shoot did not necessarily produce a root and *vice versa*. The former occurrence was more prominent in this study. In addition, the occurrence of such characteristic development was random and inconsistent based on our observation.

Each of the explants that regenerated into both shoot and root produced only one main shoot at one end of the explant and one root at the other end instead of multiple adventitious shoots and roots (Fig. 5). This observation shows that the regenerated parts exhibited apical dominance. Such characteristic ensures that the resources are focused toward the main axis during plant growth [44,45]. Auxin is known to be involved in shoot apical dominance, as it represses the local biosynthesis of cytokinin, which stimulates the extension of axillary buds [46]. On the other hand, although auxin is known to promote root development, cytokinin promotes root apical dominance [47]. The explants underwent dedifferentiation and differentiation processes to produce callus and then primordium-like structures at

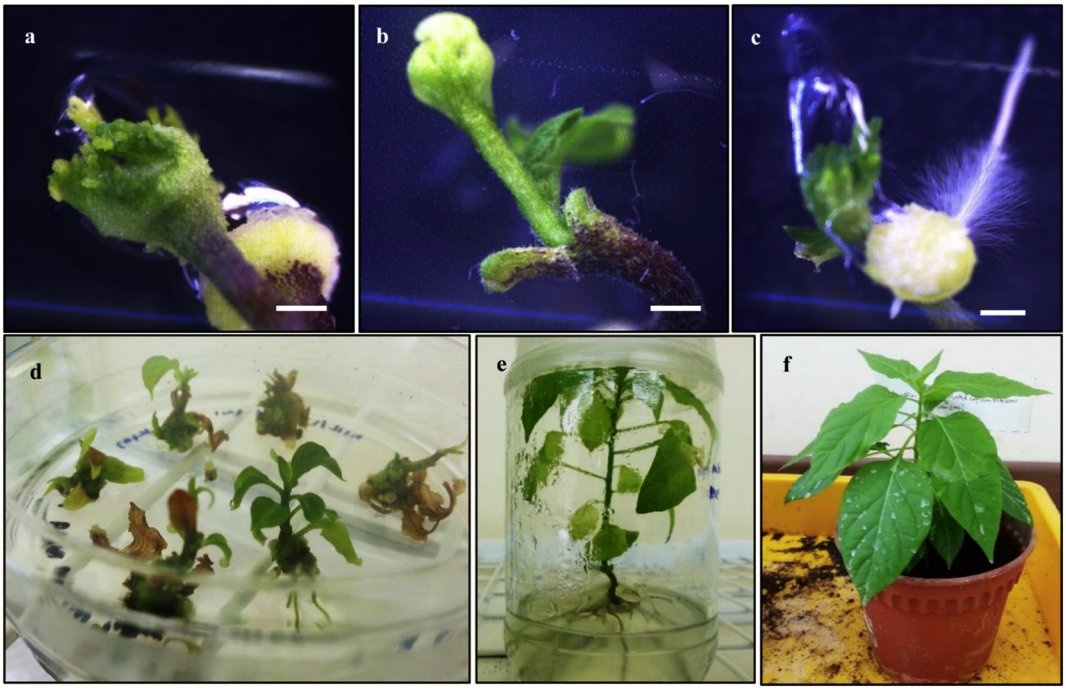

Fig. 5. Hypocotyl explants on standard MS agar with 5.0 mg/L BAP and 0.1 mg/L NAA. (a, microscopic) Both ends of the explant formed callus and then primordium-like structures after 10 d; (b, microscopic) shoot apex at one end of the explant after 20 d; (c, microscopic) root at another end of the explant after 20 d; (d) newly developed whole plant after 40 d, subcultured; (e) grown whole plant after 2 months, subcultured into a tall culture jar; and (f) after 3 months, transplanted into the soil. Scale bar represents 1 mm.

both ends before developing into shoot and root, respectively. These observations are the result of the initial attainment of competence, followed by determination to form either a shoot or root, and finally outgrowth to a shoot or root [48]. Furthermore, the development of a shoot at one end and a root at the other end shows that apical–basal polarity developed during the regeneration process (Fig. 5). The formation of callus could have been caused by disruption of the flow of polar signals, for example, auxin, from young tissues in the shoot to the root. Callus formation in relation to polarity is a complex process that depends on not only the stability of gene expression but also the cell type and orientation, cell competence, vascular differentiation, and cellular responses linked to the synthesis and transport of such signals [49,50].

The formation of the callus and primordium-like structures was observed within 9 d after culturing (Fig. 5a), followed by the development of the main shoot and the first leaves, and a root within 20 d (Fig. 5b, c). After two months of culture, the whole plant that had developed (Fig. 5d, e) was ready for acclimatization. Acclimatization was successfully achieved after the exposure of the plant to the tissue culture room environment (outside of the culture jar) for one week, followed by another week of exposure to normal room conditions at ~27°C and 54% humidity before transplanting to soil (Fig. 5f). Although a number of whole plants that were acclimatized to soil had been regenerated through this procedure, the success rate was low, with an average regeneration frequency of only 6.65%.

Overall, based on GFP expression, we suggested that the optimized parameters for particle bombardment of chili were 6-cm target distance, 1350-psi He pressure, and 1.6-μm gold particle (microcarrier) size. In addition, an *in vitro* whole plant regeneration procedure for hypocotyl explants of *C. frutescens* is suggested.

Conflict of interest

The authors declared that they have no conflict of interest.

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Supplementary data

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