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A robust *in planta Agrobacterium*-mediated transformation in red chili (*Capsicum annuum* L.)

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ABSTRACT Plant improvement through *in vitro* culture and genetic engineering is a significant aspect of breeding programs aimed at producing disease-resistant cultivars of disease-prone red chili (*Capsicum annuum* L.). However, the *Capsicum* genus is recalcitrant to genetic transformation and **in vitro** regeneration. Moreover, developing a universal transformation protocol is difficult due to its highly genotype-dependent nature. Therefore, this study aimed to develop an *Agrobacterium*-mediated *in planta* transformation method applicable to various red chili cultivars. Two open-pollinated varieties, Tanjung 2 and Ciko, were subjected to transformation. The young seedlings were immersed in transformation medium containing *Agrobacterium tumefaciens* strain GV3101 harboring the binary vector pCAMBIA1301, which carries the β-glucuronidase (GUS) gene. GUS histochemical analysis revealed that all the primary transformants of Tanjung 2 and Ciko were identified as chimeric. The average staining in the body of the seedlings was 88.63 + 26.33% in Tanjung 2, and 90.65 + 16.77% in the Ciko variety. More than 50% of the seedlings continued to express GUS in their shoot areas 10 days after *Agrobacterium* infection, indicating the possibility of transgene inheritance in the following generation. The *in planta* transformation approach is notably genotype independent, making it a promising standard transformation protocol for different red chili varieties.

KEYWORDS Ciko; GUS staining; *In planta* transformation; Red chili; Tanjung 2

1. Introduction

Red chili (*Capsicum annuum*, family Solanaceae) is an economically important crop worldwide, including in Indonesia. Due to its pungent taste, color, and aroma, red chili has been widely used in culinary applications and is therefore cultivated extensively, with a high annual production rate. However, chili peppers are susceptible to various pathogens, including bacteria, fungi, and viruses.

For disease-prone chili peppers, plant improvement through *in vitro* culture and genetic engineering is an important aspect of the breeding program aimed at producing disease-resistant chili cultivars (Bagga et al. 2019). This is essential to ensure sustainable cultivation and food security. However, this approach remains challenging for red chili. Unlike other members of the Solanaceae family, the genus *Capsicum* is recalcitrant to genetic transformation (Kothari et al. 2010; Kumar et al. 2012; Mate et al. 2021). The success rate of the most widely used approach, Agrobacterium-mediated transformation, results in generally low efficiency rates. For instance, Pusa Jwala has an efficacy level of 5-12.2% (Kumar et al. 2012), and California Wonder shows a rate of 1.3-2.9% (Verma et al. 2013).

Recalcitrance to genetic transformation is mainly due to the inability of explants cultured in vitro to regenerate functional plants, through somatic embryogenesis or de novo organogenesis (Heidari-Zefreh et al. 2019; Xu et al. 2022). Although a few successful cases of *in vitro* chili regeneration have been reported (Kumar et al. 2012; Maligeppagol et al. 2016; Bagga et al. 2019; Heidari-Zefreh et al. 2019), significant barriers to high-efficiency regeneration of chili peppers still exist. Previous studies have identified various problems associated with the difficulty of carrying out *in vitro* regeneration in chili peppers, such as low morphogenetic potential (Kothari et al. 2010). Chili pepper explants often form rosette shoots or ill-defined shoot structures, which are resistant to the elongation process (Kothari et al. 2010; Verma et al. 2013).

The existence of high genotypic dependence is another major factor inhibiting organogenesis in genus *Capsicum*. The ability to regenerate in chili pepper varies considerably, even between cultivars and genotypes (Kumar et al. 2009, 2012; Heidari-Zefreh et al. 2019). There is also variation in the effect of phytohormones (Kothari et al. 2010).

Optimization of the transformation and regeneration protocols for different cultivars is always necessary to take advantage of transgenic methods (Kumar et al. 2012; Verma et al. 2013). Thus, the *Capsicum* genus remains difficult to work with for genetic engineering. Although *in vitro* regeneration has been successful in some red chili cultivars (Kumar et al. 2012), tissue culture is laborious and often leads somaclonal variations in the regenerated plants (Saifi et al. 2020).

In planta transformation refers to the direct transformation into the plant cells without involving *in vitro* culture and plant regeneration (Saifi et al. 2020; Pandey et al. 2016). It is an easy, simple, efficient, and cost-effective transformation method for recalcitrant plants, such as *Capsicum* (Kumar et al. 2012; Arthikala et al. 2014), horse gram (Amal et al. 2020), peanut (Karthik et al. 2018), switchgrass (Xu et al. 2022), and indica rice (Basavaraju et al. 2020; Saifi et al. 2023). *Agrobacterium* with the required transgene is allowed to infect plant meristematic tissues directly, thus eliminating some stages of tissue culture (Niazian et al. 2017; Saifi et al. 2020). Although *Agrobacterium*-mediated stable transformation has been reported in other plants (Marwani et al. 2013; Pandey et al. 2016; Karthik et al. 2018; Basavaraju et al. 2020; Saifi et al. 2023), the reports on successful *Agrobacterium*-mediated *in planta* transformations to generate stable transformants in red chili are still scarce (Hamdani et al. 2021). In addition, very few *in planta* transformation methods have been tested for chili peppers. In the study by Kumar et al. (2009), piercing on shoot apical meristem with needle, followed by incubation in *Agrobacterium* EHA105 culture resulted in 11.4% - 17.8% of the T0 bell pepper being chimeric, and 29.7% - 35.0% were identified in the T1 generation as stable transformants (Kumar et al. 2012). In Arthikala et al. (2014), the method resulted in 26.4% and 24.2% transgenic bell peppers in T0 and T1 generations, respectively. Although this method improved the efficiency of *Capsicum* transformation, it remains time-consuming and highly labor-intensive for a large-scale transformation. Additionally, the correct amount of bacteria for the transformation is difficult to measure (Mate et al. 2021).

Improving the transformation method for red chili is crucial to ensure sustainable red chili production. Since *in planta* transformation is genotypically independent, this approach has high potential for recalcitrant red chili (Hamdani et al. 2021). Therefore, the present study was conducted to develop a simplified and efficient method for *Agrobacterium*-mediated *in planta* transformations of red chili pepper varieties.

2. Materials and Methods

2.1. Plant material

The seeds of red chili pepper were obtained from Balai Pengujian Standar Instrumen (BPSI) Tanaman Sayuran, Lembang, West Java. Red chili pepper seeds of open-pollinated varieties viz., Tanjung 2 and Ciko were used in this study. Prior to germination, the seeds underwent surface sterilization using 70% (v/v) ethanol for 1 min, followed by 30% sodium hypochlorite/bleach solution (Bayclin®; SC Johnson & Son, Inc.) for 10 min. The seeds were then thoroughly rinsed 3 to 4 times with sterile aquadest.

2.2. Agrobacterium culture

Agrobacterium tumefaciens strain GV3101, harboring the plasmid pCambia 1303 containing the GUS reporter gene, was used in the transformation. The *Agrobacterium* strain was cultured in Luria-Bertani (LB) medium supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin. The bacterial culture was incubated for 24–48 h at 28 °C with agitation (150 rpm) on a shaker, or until achieving an optical density (OD_{600}) of 1. Next, 15 mL of bacterial culture was centrifuged, and the pellet was resuspended in 50 mL of transformation medium (1/2× MS medium, 2% sucrose, 100 µM acetosyringone, pH 5.6–5.8).

2.3. Transformation of leaf explants and the integration of GUS gene

In the preliminary stage, optimization of the conditions for *Agrobacterium*-mediated transformation in red chili leaves (Nugroho 2022) involved the variation in OD_{600} of *A. tumefaciens* culture (OD_{600} = 0.25, 0.5, 1, and 1.5), the concentration of acetosyringone (0, 50, 100, and 200 µM), and the cocultivation time (24, 48, 72, and 96 h). The highest transformation efficiency of red chili leaves was obtained under conditions of OD_{600} = 1, 100 µM acetosyringone, and 72 h co-cultivation.

We carried out the transformation using leaf explants of the Tanjung 2 variety based on the optimized condition in the preliminary study, to further confirm the reproducibility of this method. The Tanjung 2 seeds were germinated for seven days in germination medium (1× MS, 3% sucrose, 0.8% agar, pH 5.6–5.8) under dark conditions at 25 °C for a week, followed by 16-hour photoperiods for 3–4 weeks. Five 30-day-old leaves from different plants were used for transformation. The leaves were incised slightly and soaked in transformation medium for 60 min at 28 °C. The infected leaves were then transferred into co-cultivation media (1× MS medium, 3% sucrose, 0.8% agar, 100 µM acetosyringone, pH 5.6) and incubated at 28 °C for 72 h in the dark. Following co-cultivation, the leaves were subjected to GUS histochemical staining to observe GUS expression. The presence of GUS gene in the leaf explants was detected by PCR, using CaMV 35S promoter forward primer (5′-ATAGAGGACCTAACAGAACTCGC-3′) and GUS reverse primer (5′-GGCTTTCTTGTAACGCGC-3′). To amplify the 668 bp GUS gene fragment in the putative transformants, PCR was begun by a hot start at 95 °C for 5 min, followed by 35 cycles of 95 °C/30 s, 60 °C/30 s, and 72 °C/40 s with a 72 °C/5 min final extension. The DNA extracted from plants transformed with empty *A. tumefaciens* was used as a negative control. The pCAMBIA1301

vector was a positive control, and the reaction mix was without DNA as a water-blank. The products were run on a 1% agarose gel.

2.4. *In planta transformation and recovery of transformant seedlings*

The seeds for *in planta* transformation were germinated in a petri dish with germination medium (1× MS, 3% sucrose, 0.8% agar, pH 5.6–5.8) under dark conditions at 25 °C. Each plate contained 20–22 seeds, with a total of six plates per variety. Within 7–10 days healthy seedlings with radicles and cotyledons were each collected from the germination media.

A total of 80 and 101 seedlings of Tanjung 2 dan Ciko, respectively, were collected for transformation. The seedlings were immersed in the transformation medium containing *Agrobacterium* and incubated in a shaker (50 rpm) at 28 °C for 30 min (29 Tanjung 2 and 50 Ciko seedlings) and 60 min (51 Tanjung 2 and 61 Ciko seedlings). Next, the infected seedlings were transferred into cocultivation medium (1× MS medium, 3% sucrose, 0.8% agar, 100 µM acetosyringone, pH 5.6–5.8) and incubated at 28 °C for 72 h in the dark.

After 72 h cocultivation, half of the seedlings were directly stained by GUS histochemical to detect the transient expression of GUS gene. The remaining seedlings were allowed to grow for at least a week to determine the percentage of seedling recovery after transformations, and to detect stable GUS expression. The seedlings used for observing seedlings recovery were rinsed 3 to 4 times with sterile water containing 500 ppm cefotaxime. Subsequently, the seedlings were transferred into MS medium (1× MS medium, 3% sucrose, 0.8% agar, 250 ppm cefotaxime, pH 5.6) to grow at 28 °C under a 16 h photoperiod for approximately 7 d. The survived seedlings were then subjected to GUS staining to detect stable GUS expression.

Phenotypic GUS expression was determined by GUS histochemical staining. The seedlings were immersed for approximately 24 h in a staining solution containing 50 mM each of NaH_2PO_4 and Na_2HPO_4 (pH 7.0), 1 µM X-Gluc (5-bromo-4-chloro-3-indoly-b-D-glucuronide, Themofischer), and 0.1 mM each of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ at RT, under dark conditions. The tissues were later immersed in 95% ethanol for 24–48 h to clear chlorophyll.

Following the phenotypic GUS expression, the transformation frequency was calculated as follows: The mean frequency (%) of GUS expression = The number of seedlings producing blue spots/The total number of seedlings × 100

The blue-colored area in seedlings was measured using ImageJ Version 1.54k (National Institutes of Health). The area of GUS expression in seedlings was calculated as follows: The mean area (%) of GUS expression = The area of seedlings producing blue spots/the total area of seedlings × 100

3. Results and Discussion

3.1. *Phenotypic GUS expression in leaf explants and the integration of GUS gene*

In the preliminary study, the efficiency of the transformation method was evaluated by looking at the frequency of infection with *Agrobacterium* and the intensity of GUS expression. In this study, the efficiency of transformation was evaluated based on the leaf area producing blue spots and the presence of GUS gene fragments in the genome. The esults showed that the leaves transformed with *A. tumefaciens* harboring pCAMBIA 1303 under conditions of OD_{600} = 1, 100 µM acetosyringone and 72 h cocultivation (Figure 1a) produced homogenous GUS staining. The PCR of GUS gene fragment also showed the integration of the GUS gene into the genome in all samples (Figure 1b). Therefore, the *Agrobacterium*-mediated transformation in Tanjung 2 leaf explants were used as the initial standardization experiments for in planta transformation in seedlings.

3.2. *In planta transformation and recovery of transformant seedlings*

In planta transformation was carried out on all planted seeds grown within 7–10 days, regardless of their growth stage. The results showed that the optimized condition for

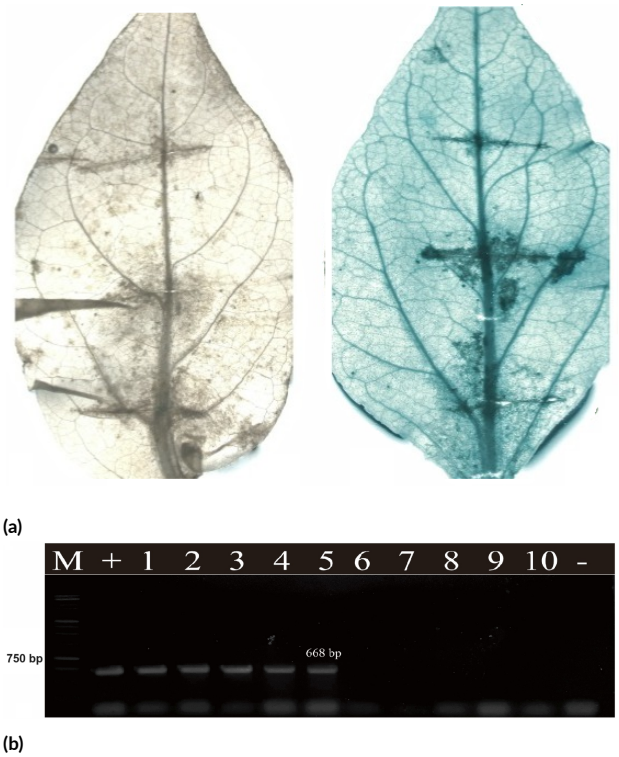

FIGURE 1 GUS histochemical analysis. a. Leaf of Tanjung 2 transformed with empty *Agrobacterium tumefaciens* (left) and *A. tumefaciens* containing pCAMBIA 1303 (right), b. PCR using GUS gene primers: M = marker, + = pCAMBIA1301 vector , 1–5 = PCR positive Tanjung 2 transformed with *A. tumefaciens* containing pCAMBIA 1303, 6–10 = Tanjung 2 negative control, – = water-blank (reaction mix with out DNA).

leaf transformation was successfully employed for carrying out *Agrobacterium*-mediated *in planta* transformation in the young seedlings of Tanjung 2 and Ciko. All tested seedlings produced blue spots (Table 1). The largest mean area of transient GUS expression (3 days post-infection) was observed in Ciko treated with 60 min immersion in transformation medium (90.65%). Meanwhile, the mean area of GUS expression in Tanjung 2 and Ciko seedlings treated with 30 min immersion was similar. However, different trend found in potentially stable GUS expression (10 days post-infection). The largest mean area of GUS expression was observed in Tanjung 2 treated with 60 min immersion (92.89%), while other treatment groups showed that the average stained area was decreased. The average percentage of infected seedlings that recovered and continued to grow for each cultivar was relatively high, except in Ciko treated with 30 min immersion (Table 1). Generally, the survival rates of transformants were lower in the Ciko variety compared to Tanjung 2. The infection time of 60 min did not considerably reduce the survival of infected seedlings in both cultivars. In addition, those seedlings only showed slight cases of browning for both treatments.

The extent of transformation into some tissues of these seedlings was ascertained based on GUS histochemical assay. Transient GUS expression (3 days post-infection) in the seedlings of the primary transformants was observed in hypocotyl, cotyledon and root (Figure 2 and 3). Moreover, the survived seedlings showed positive GUS expression when the seedling was subjected to GUS staining approximately 10 days post-infection (Figure 2 and 3). Thus indicates the integration and expression of the transgene in both red chili varieties. Based on the color intensity observed after 30 and 60 min of incubation with *Agrobacterium*, GUS expression was not significantly different. Even though it has a lower color intensity, but more than half of seedling express GUS in shoot area. The highest number of seedlings that express GUS in the shoot was found in Tanjung 2 treated with 60 min immersion, at 88.89%.

3.3. Discussion

Previous work on the transformation of red chili pepper used hypocotyls and cotyledons as explants, where recalcitrance has been the major problem for efficient genetic transformation and *in vitro* regeneration (Kothari et al. 2010). Pioneering efforts on the *Agrobacterium*-mediated transformation method, followed by *in vitro* regeneration of explants, resulted in somewhat limited results with substantial variability in different red chili pepper cultivars (Kumar et al. 2012; Verma et al. 2013).

Several approaches have been studied to break recalcitrancy, such as de novo meristem induction using various developmental regulators (Drs) and *in planta* transformation methods. Overexpression DRs, such as WUSCHEL (WUS), ISOPENTENYL TRANSFERASE (IPT), and BABYBOOM (BBM) to promote plant regeneration of host cells has been explored (Hamdani et al. 2021). However, the use of DRs still involves tissue culture. Alternatively, *in planta* transformation is more appealing to be developed as a routine protocol for genetic transformation in many varieties of red chili, since the method is tissue culture-free, easy, less-laborious, and cost-effective.

Key parameters for reproducible and efficient genetic transformation include the active log phase of bacterial growth (OD value), acetosyringone concentration, and co-cultivation time. The OD_{600} value depicts the active log phase of bacterial growth and is commonly used to ensure that cells are harvested with an appropriate density of live cells for efficient transformation (Kumar et al. 2012). Acetosyringone is a phenolic chemical that acts as a powerful *vir* gene inducer, enhancing the cell's transformation capacity. Appropriate concentration of acetosyringone improves transformation efficiency (Karthik et al. 2018). The period of co-cultivation had an effect on transformation efficiency as well. A suitable time for co-cultivation

TABLE 1 Number and percent of GUS positives in primary transformants and recovered transformants after infection.

Variety	**Tanjung 2**	**Ciko**	**Tanjung 2**	**Ciko**
Treatment	**30 min immersion in the transformation medium**		**60 min immersion in the transformation medium**	
Total number of transformant	29	50	51	61
The mean frequency of transient GUS expression (3 dpi)	100%	100%	100%	100%
Average percentage of blue areas on seedlings	88.63 + 26.33	88.64 + 20.33	78.01 + 26.75	90.65 + 16.77
Percent of seedlings survived after infection	86.67%	57.69%	100%	83.87%
The mean frequency of survived seedlings showing GUS expression (10 dpi)	100%	100%	100%	100%
Average percentage of blue areas on seedlings	78.82 + 27.20	84.43 + 26.35	92.89 + 10.08	74.31 + 20.42
Percent of survived seedlings showing GUS expression in shoots	57.14%	46.67%	88.89%	65%

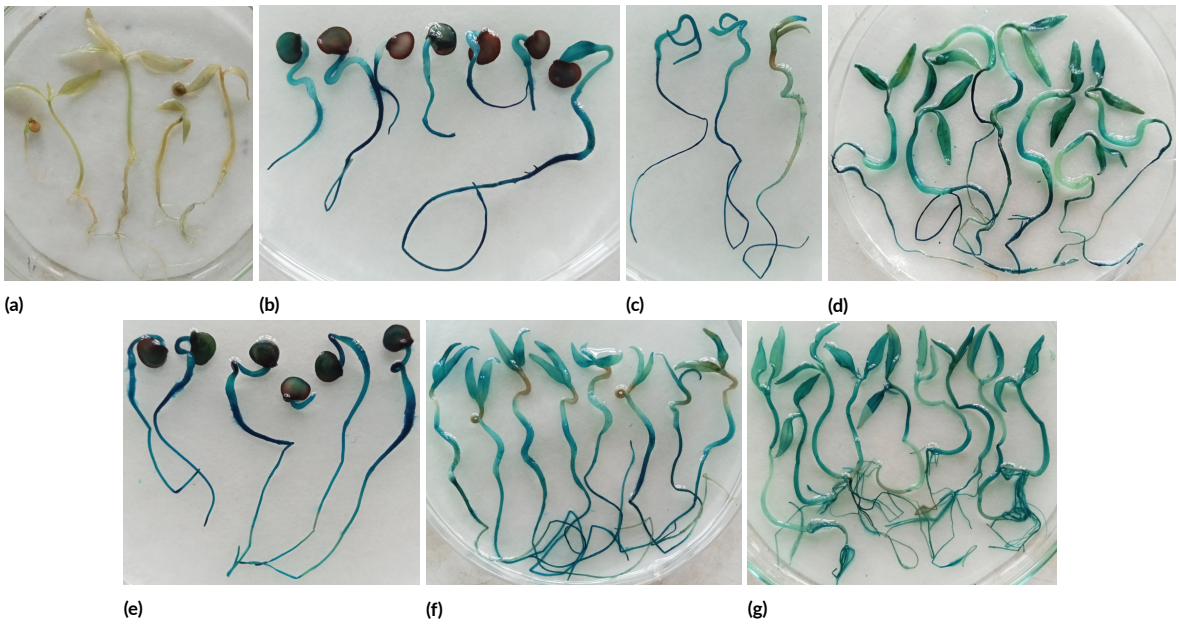

FIGURE 2 GUS histochemical staining of the primary transformants in Tanjung 2 seedlings. a. Wild-type, b-c. Transient GUS expression in 3 days post-infection seedlings (30 min incubation), d. Stable GUS expression in 10 days post-infection seedlings (30 min incubation), e-f. Transient GUS expression in 3 days post-infection seedlings (60 min incubation), g. Stable GUS expression in approximately 10 days post-infection seedlings (60 min incubation).

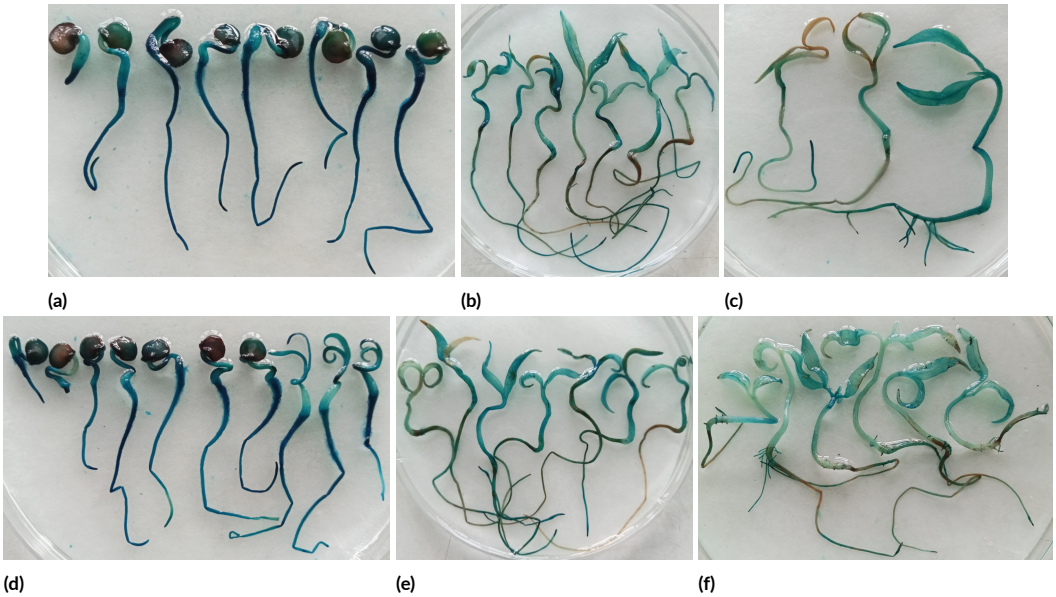

FIGURE 3 GUS histochemical staining of the primary transformants in Ciko seedlings. a-b. Transient GUS expression in 3 days post-infection seedlings (30 min incubation), c. Stable GUS expression in 9 days post-infection seedlings (30 min incubation), d-e. Transient GUS expression 3 days post-infection seedlings (60 min incubation), f. Stable GUS expression in approximately 10 days post-infection seedlings (60 min incubation).

allows the explants to have a high transformation frequency with maximum survivability of explants (Karthik et al. 2018; Amal et al. 2020).

The optimal condition of OD_{600}, acetosyringone concentration and co-cultivation time for *Agrobacterium*-mediated transformation to the leaf explants were then applied to the red chili seedlings. In this study, GUS histochemical analysis of the primary transformants was used as the first confirmation of the amenability of different red chili cultivars to the *in planta* transformation methods. Interestingly, despite the common practice of wounding the plant material to increase transformation frequency (Kumar et al. 2012; Arthikala et al. 2014; Mate et al. 2021), the results showed that successful infection of seedlings by *Agrobacterium* was achieved even without mechanical injury.

The use of young seedlings for *Agrobacterium*-mediated transient expression assays has been successfully

done in *Arabidopsis* and several crops, such as tomato and rice (Li et al. 2009; Wu et al. 2014), demonstrating efficient transformation approach and stable gene expression. In *Arabidopsis*, 100% of analyzed seedlings were successfully transformed, with homogenous GUS staining in cotyledon, and 4-fold higher GUS activity in transformants than in non-transformant seedlings (Wu et al. 2014).

In this study, GUS histochemical analysis revealed positive results in all Tanjung 2 and Ciko seedlings. Most of the seedlings exhibited strong and homogenous GUS staining. The transformation frequency is also significantly higher compared to the initial standardization experiments in bell pepper Arka Gaurav and Arka Mohini, which accounted for 70% of the seedlings that were positive for GUS histochemical analysis (Kumar et al. 2009). Moreover, no tissue injuries were needed, which greatly simplifies and amplifies the power of the transformation method.

The transformation efficiency in Ciko was generally higher in Ciko than in Tanjung 2. However, the standard efficiency of *in planta* transformation cannot be set for any crop or experiment, since many factors influence its transformability using this protocol. For primary transformants, it depends on the number of chimeras that arise from the total number of T0 plants. The number of chimeras depends on the number and type of cells that integrate the transgene (Kumar et al. 2009). Also the survivability of seedlings after transformation was lower in Ciko than that in Tanjung 2. It shows that Tanjung 2 and Ciko showed different degrees of susceptibility to *A. tumefaciens* strain GV1301.

Another noticeable aspect is the ability of seedlings to express potentially stable GUS expression, especially in the shoot area. It indicates that this method can also produce a high frequency of stable transformation in both varieties. The inheritance of the transgenes into the T1 generation to produce stable transformants requires that the cells targeted for transformation be likely to develop into germ cells (Kumar et al. 2009). The simplified method developed in this study did not specifically target the meristematic cells, so the transgene inheritance was probably limited. However, with the presence of more than 50% seedlings expressed GUS in the shoot area 10 days after *Agrobacterium* infection, the probability of successful transgene delivery into the shoot apical meristem is relatively high in red chili cultivars. The inheritance of transgenes through the development of plant reproductive organs and seeds from the transformed apical meristematic cells should be further confirmed in the T1 and T2 generations.

A high frequency of transient transformation will also be highly potential to be further developed into a screening system for CRISPR/Cas9-mediated gene editing in red chili. The effectiveness of CRISPR-based gene editing tools is a crucial precondition for successful precision gene editing. However, recalcitrance to transformation is an obstacle for gene editing in several plants (Nivya and Shah 2023). One of the approaches used to determine the efficient guide RNAs for CRISPR/Cas9 is protoplast technology (Lin et al. 2018; Kim et al. 2020), including in Capsicum. Although Kim et al. (2020) reported that protoplast-based CRISPR guide-RNA screening is a robust method for assessing the effectiveness of designed CRISPR tools in *Capsicum*, the protoplast technology is costly and technically delicate.

Plant transformation is very species- and genotype-dependent and is frequently one of the main obstacles in implementing genetic engineering for crop trait enhancement (Altpeter et al. 2016; Nagle et al. 2018; Maren et al. 2022). The expression of GUS was not significantly different in both cultivars. It shows that this *in planta* transformation method is genotype-independent and thus can be extended to different cultivars. Therefore, the *in planta* transformation method used in this study would be beneficial to improving red chili peppers using transgenic technology, especially in indigenous varieties.

4. Conclusions

Due to the resistant and genotypically-dependent nature of red chili peppers, there was no current approach able to attain high transformation efficiency for transient gene expression with minimal modification. However, this study successfully established a simplified and effective *Agrobacterium*-mediated *in planta* transformation method for young seedlings of red chili pepper varieties Tanjung 2 and Ciko. The technique also highlighted the differences in transformability and survivability among different red chili varieties, with Ciko showing higher efficiency but lower seedling survivability compared to Tanjung 2. The method also showed promising results for stable transformation, though further study is needed to determine transgene inheritance in the next generation. Notably, *in planta* transformation approach exhibited genotypic independence, making it a potential standardized transformation protocol for different red chili pepper varieties.

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Authors' contributions

ADH, SS, RRE designed the study. ADH, SN carried out the laboratory work. ADH, SN, SS, RRE, AF analyzed the data. ADH, SN, SS, RRE wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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