



Establishment of regeneration, transformation, and genome editing procedures for a seed-propagated carnation (*Dianthus caryophyllus* L.) variety

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Abstract

Carnations (*Dianthus caryophyllus* L.) are amongst the three most commercially valuable cut flowers worldwide. However, traditional breeding methods are often time-consuming and labor-intensive. Although genome editing is used as an alternative method for creating new varieties, the high heterozygosity of carnations inhibits the ability to maintain varietal characteristics in null segregants except for target-derived traits. The use of homozygous lines is a possible solution. Therefore, this study aimed to establish regeneration, transformation, and genome editing methods using seed-carnation varieties. The effects of four auxins (indole-3-butyric acid, IBA; a-naphthaleneacetic acid, NAA; 2,4-dichlorophenoxyacetic acid, 2,4-D; and 3-indoleacetic acid, IAA) and five cytokinins (6-benzyladenine, BA; thidiazuron, TDZ; kinetin, KT; zeatin, ZT; and N⁶-2-isopentenyl adenine, 2IP) on callus and shoot induction were evaluated. The combination of 0.05 mg/l 2,4-D and 4 mg/l TDZ had the highest shoot formation rate at 28%. In addition, shoot hyperhydricity was reduced by increasing the size of culture vessels. Sucrose, agar, and AgNO₃ concentrations, as well as pH, were optimized to facilitate regeneration. Hygromycin at 12.5 mg/l was subsequently used as the selection agent after *Agrobacterium*-mediated transformation. Finally, the phytoene desaturase gene was knocked out using the CRISPR/Cas9 system. The obtained albino shoot had a one-base deletion or two-base insertion in the genome sequence. To our knowledge, this is the first study to establish a system for genome editing of callus-derived shoots from a homozygous seed-propagated carnation, which may contribute to the rapid breeding of the new varieties.

Key message

This is the first report to establish regeneration, transformation, and genome editing methods using homozygous seed-carnation cultivars to maintain varietal characteristics in null segregants.

Keywords CRISPR/Cas9 · Stable transformation · Homozygote · Hyperhydricity · Callus induction · Shoot induction

Abbreviations

IBA	Indole-3-butyric acid
NAA	a-naphthaleneacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	3-indoleacetic acid, IAA
BA	6-benzyladenine
TDZ	Thidiazuron
KT	Kinetin
ZT	Zeatin
2IP	N ⁶ -2-isopentenyl adenine

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Introduction

Carnation (*Dianthus caryophyllus*), belongs to the *Caryophyllaceae* family, originates from Mediterranean, and is currently cultivated worldwide (Anonis 1985; Reddy 2016). It is among the top three commercially valuable cut flowers. Carnations have been bred for improved color and shape, sensitivity of floral differentiation to day length and low temperatures, as well as resistance to pathogens. Crossbreeding, natural variation, and artificially induced variation, such as radiation and chemical substances, are predominantly used to obtain new varieties (Henderson and Salt 2017). To date, crossbreeding has been the main approach used to create new carnation varieties to meet commercial demands. However, this methodology requires a great amount of labor and is time-consuming. Recently, genome editing has appeared as a novel technology to overcome these problems, making possible the release of new plant genotypes with accurate trait modifications in less time. Furthermore, CRISPR/Cas9 genome editing can prevent transgenesis, which may help to avoid public and administrative concerns about cultivation and commercialization of genetically modified crops. This approach has been applied on more than 10 ornamental flowers and is reported in three review papers (Ramirez-Torres et al. 2021; Mekapogu et al. 2023; Liu et al. 2024). Regarding the application of gene editing techniques to carnation, two reports have been published until date. One described the use of protoplasts but shoots were not obtained (Adedeji et al. 2024). In the other report, authors applied an electroporation based approach to successfully produce mutations in the anthocyanidin synthase gene, but chimerism of the regenerated plants remained a challenge (Mori et al. 2024).

Carnations are typically vegetatively propagated through cutting because most varieties are heterozygotes (Yagi 2015). When heterozygotes are propagated by seed, traits segregate in the next generation and various characteristics cannot be maintained. This is a disadvantage in the process of obtaining null segregants without foreign genes after genome editing. To prevent this, genome editing is performed by transiently expressing foreign genes without introducing them into the genome (Hamada et al. 2017; Adedeji et al. 2024) or by using homozygous lines for seed propagation. In genome editing using homozygotes, foreign genes can be removed by selfing to obtain null segregants with the original traits maintained. Since this material has not yet been tested in carnations, this study developed a genome editing system using seed-propagated variety.

To applied genome editing techniques, it is essential to previously develop tissue culture procedures such as adventitious shoot regeneration and transformation (Bekalu et al. 2023). Callus-mediated (indirect) regeneration is advisable since direct shoot regeneration often produces chimeras,

which is a disadvantage in transformation (Zhang et al. 2010; Jha et al. 2011; Sharma et al. 2011). In carnation, some studies have focused on direct regeneration (Frey and Janick 1991; Fisher et al. 1993; Watad et al. 1996), and some in callus-mediated regeneration (Thakur et al. 2002; Thakur & Kanwar 2018; Jorapur et al. 2018). However, homozygous seed-propagated carnation genotypes have not been used as experimental materials. Another major challenge in carnation in vitro culture is hyperhydricity. Hyperhydric carnation shoots become yellow, swollen, glassy, and curved after being transferred to soil; thus, they do not survive (Yadav et al. 2003). Although callus-mediated regeneration has been extensively studied, homozygous carnations have not been used as experimental materials (Thakur et al. 2002; Thakur & Kanwar 2018; Jorapur et al. 2018).

In the current study, we targeted *phytoene desaturase* gene (*PDS*) for genome editing. *PDS* catalyzes diapophytoene to produce ζ -carotene in the carotenoid synthesis pathway. Plants often exhibit albino and dwarfing phenotypes when *PDS* is suppressed. This gene is widely used to evaluate CRISPR/Cas9 efficiency since *PDS*-silenced plants can be easily detected (Pan et al. 2016; Hooghvorst et al. 2019; Wilson et al. 2019). Therefore, we targeted the *PDS* gene for genome editing in carnations. In this study, we established for the first time the regeneration, transformation, and genome editing of callus-derived shoots from seed-propagated carnations that can consistently acquire null segregants. Our finding is expected to contribute to the rapid breeding of new carnation varieties in the future.

Materials and methods

Plant materials

Seeds of 'Chabaud Giant' were purchased from Fukukaen Nursery & Bulb Co., Ltd. (Aichi, Japan) and stored in a refrigerator at 4 °C until use (Fig S1). This variety blooms with double, fringed, and medium-sized flowers, making it suitable for early harvesting during outdoor cultivation.

In vitro seed germination

Seeds were first surface-sterilized with 70% ethanol for 5 min, followed by 2% sodium hypochlorite with one drop of 10% Tween 20 for 15 min. They were then rinsed thrice with sterilized distilled water. Twenty milliliters of solid medium containing Murashige and Skoog (MS) (1962) basal salts, 30 g/l sucrose, and 8 g/l agar (FUJIFILM Wako Pure Chemical Corporation, Japan) were prepared and adjusted to pH 5.8 ± 0.1 before sterilization with an autoclave at 121 °C for 20 min. Sterilized seeds were sown on

the medium in $\Phi 90 \times 19$ mm Petri dishes. Seeds were cultured in a growth chamber at 26 °C under a 16 h photoperiod provided by cool-white fluorescent lamps at a light intensity of approximately 100 $\mu\text{mol/s/m}^2$.

Determination of the phytohormones involved in callus induction

Seeds sown in the medium germinated in about 3 days. Subsequently, the top four true leaves were taken from each seedling and cut into 5 mm square segments after 18 days of seedling culture. The segments were cultured in the same medium used for germination but with phytohormones. The experiment was divided into two parts. In experiment 1, combinations of various hormones (IBA, NAA, 2,4-D, IAA, BA, TDZ, KT, zeatin, and 2iP) were used at 0.5 and 1.0 mg/l as callus induction medium (CIM). Each treatment combination was tested with 10 explants in triplicate. The growth conditions were the same as those used for germination. After 1 month, the obtained calli were moved to a shoot induction medium (SIM) supplemented with 0.1 mg/l NAA and 1.0 mg/l BA as described by Nontaswatsri et al. (2004). After 0.5 months, shoot induction rates were recorded, distinguishing the CIM origin of the callus. 2,4-D as auxin and TDZ as cytokinin, which had high final shoot induction rates, were selected as the hormones for CIM, and the detailed concentrations were reexamined as Experiment 2. The combinations of 2,4-D and TDZ were examined at concentrations of 0.01, 0.05, 0.1, 1.0, 2.0, 4.0, and 8.0 mg/l. The medium pH was adjusted to 5.8 ± 0.1 before sterilization at 121 °C for 20 min. The culture environment was the same as that described in the subsection above. The segments were cultured for 1.5 months. In addition, each treatment combination was tested with 10 explants in triplicate. Because this hormone combination yielded callus followed by shoot, no independent SIM trials were conducted in Experiment 2.

Effect of other factors in the induction of healthy calli and shoots

Additional factors such as sucrose (20, 30, 40, and 50 g/l), agar (4, 8, 12, and 16 g/l), AgNO_3 (0.5, 1.0, and 2.0 mg/l), and pH (5.0, 5.4, 5.8, and 6.2), were studied. The different treatments were compared with standard conditions based on pH 5.8, as well as 8 g/l agar, 30 g/l sucrose, 0.05 mg/l 2,4-D, and 4 mg/l TDZ. Culture conditions were similar to those described for seed germination. Each treatment combination was tested with 10 explants in triplicate.

Rooting experiments

Newly induced shoots in 1.5 months after inoculation were excised from explants or calli onto rooting medium at pH 5.8 supplemented with MS basal salts, 30 g/l sucrose, and 4 g/l gellan gum, which facilitates observation of the roots better than agar. The medium included combinations of the following phytohormones: 0.01 mg/l KT, 5 mg/l GA_3 ; 0.01 mg/l NAA, 0.01 mg/l IBA. To test the vessels, 50, 200, and 450 ml culture bottles were used, and the medium was filled to 40% of the vessel volume. Three shoots were placed per bottle in triplicate. The culture environment was the same as that described in the former section “in vitro seed germination”. Rooting data was recorded after 1 month.

Effect of antibiotics on shoot regeneration

The optimal antibiotic concentration for selection of transformed tissues after *Agrobacterium* infection was determined. Kanamycin at 25, 50, 75, or 100 mg/l and hygromycin at 5.0, 7.5, 10.0, 12.5, or 15 mg/l were tested with a combination of 0.5 mg/l 2,4-D and 4.0 mg/l TDZ. All the other components in the medium were the same as those used for seeds germination. Leaves of ‘Chabaud Giant’ were used as explants for culture under the same conditions as those described in the former section “in vitro seed germination”. Each treatment combination was tested with 10 explants in triplicate. The formation of callus and shoot were observed after 1 month.

Cloning of *DcPDS*

Genomic DNA of ‘Chabaud Giant’ was extracted from leaves using the Nucleon PhytoPure Plant DNA Extract Kit (Cytiva, UK). Specific primers were designed based on the gene fragment in the carnation ‘Francesco’ database (Yagi et al. 2014). *PDS* genes were found in the Carnation DB and named *DcPDS1*, *DcPDS2*, and *DcPDS3*. Because the *DcPDS2* and *DcPDS3* sequences were identical, we later named them *DcPDS2/3*. We designed primers for the middle and back regions of *DcPDS1* and for the front and back regions of *DcPDS2/3* (Table S1). The PCR reaction system was as follows: 10× PCR Buffer containing 5 μl KOD Plus Neo, 5 μl dNTPs, 3 μl MgSO_4 , 1 μl upstream primer, 1 μl downstream primer, 1 μl KOD plus Neo, 200 ng template DNA, and ddH₂O to complete the final volume. The reaction conditions were as follows: 94 °C for 2 min; 98 °C for 10 s, (Tm) °C for 30 s, and 68 °C for 30 s/kb, 45 cycles; and then hold at 25 °C. The PCR products were detected through 1.2% agarose gel electrophoresis. PCR product purification was performed using the GEL/PCR Purification Mini Kit (FAVORGEN) according to the manufacturer’s

instructions. Next, purified PCR products were cloned with the pTA2 vector, transformed into *Escherichia coli* DH5 α , and identified via PCR. The positive clone was then selected for sequencing.

Construction of the CRISPR/Cas9 vector

Comparison of the PDS amino acid sequence of the carnation with that of other plants revealed the first conserved domains in the second and fourth exons of *DcPDS1* and *DcPDS2/3*, respectively (Fig S2). To knock out all three genes, 20 nt gRNAs were designed adjacent to the PAM sequence (Fig S3). Primers containing the *AarI* restriction site were annealed and ligated into pKIR1.1 (Tsutsui and Higashiyama 2017). pKIR1.1 was a gift from Tetsuya Higashiyama (Addgene plasmid # 85758; <http://n2t.net/addgene:85758>; RRID: Addgene_85758). A vector was constructed and verified through standard cloning and sequencing, respectively.

Transformation by *Agrobacterium* infection

Agrobacterium LBA4404 was used for the transformation experiments. *Agrobacterium* harboring the vector construction for CRISPR/Cas9 was cultured in LB liquid medium supplemented with 100 mg/l rifampicin and 100 mg/l spectinomycin at 28 °C for 2 d. A large-scale culture was performed until OD₆₀₀ $\geq$ 0.6. The centrifuged pellet was then adjusted to OD₆₀₀ = 0.6–0.7 using fresh LB liquid medium supplemented with 0.1 μ M acetosyringone and mercaptoethanol. The leaf segments were soaked in *Agrobacterium* suspension for 7 min, dried with filter paper, and placed onto MS solid medium supplemented with 0.05 mg/l 2,4-D and 4.0 mg/l TDZ. All the other components in the medium, and culture conditions were the same as those used for seeds germination. They were then co-cultivated for 3 d and moved to the selection medium containing antibiotics; 12.5 mg/l hygromycin and 50 mg/l meropenem. Each treatment combination was tested with 10 explants in 80 replicates. The medium was changed every 2 weeks. Regenerated albino shoots were selected as candidate genome-edited plants for further analysis.

Confirmation of the edited genome sequence

To determine the genome editing efficiency of *DcPDSs*, the target sequence was amplified from gDNA using PCR and cloned into the standard sequencing vector (pSKII⁺) using an In-Fusion HD Cloning Kit (Takara Bio). The positive clones were selected for Sanger sequencing. The nucleotide sequences were determined using the dideoxy chain-termination method on 3130, 3130xl Genetic Analyzer (Applied

Biosystems), SeqStudio, or SeqStudio Flex 8 (Thermo Fisher Scientific) automated DNA sequencing system using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit 3.1 (Applied Biosystems Division, Perkin-Elmer, Foster City, CA, USA). Editing of genome sequence was confirmed using the CLC Main Workbench Version 20 sequence analysis programs (QIAGEN Aarhus A/S, Aarhus, Denmark).

Results

Callus and shoot induction with different hormone combinations

In the first experiment, four types of auxins were used in combination with five cytokinins to select the appropriate phytohormone combination for the induction of calli and shoots from explants (Table 1; Fig S4). KT and IBA did not induce callus formation at most concentrations. Similarly, combinations of IAA with BA, KT, or 2IP did not induce callus hardly at all. The other 16 combinations induced calli efficiently, with induction rates ranging from 50.0 to 100%. However, many calli were either too small or hyperhydric (Fig S5A; S5B). Some of them induced roots during one month of culture in CIM (Fig S4), but none of them induced shoots. Therefore, the callus was transplanted to the previously reported SIM supplemented with 0.1 mg/l NAA and 1.0 mg/ BA (Nontaswatsri et al. 2004). However, it was difficult to obtain shoots from calli cultured in this SIM. In 0.5 month after transplanting, shoots only grew from the two calli cultured in a medium supplemented with 0.5 mg/l 2,4-D and 1.0 mg/l TDZ (Fig S5C).

Callus induction with 2,4-D and TDZ

In the experiment 1 in the previous chapter, shoots were induced only on media containing 2,4-D and TDZ. Therefore, as experiment 2, combinations of these phytohormones were tested at various concentrations to improve shoot formation efficiency. Callus formation was observed at all concentrations but decreased at 2,4-D concentrations > 1.0 mg/l (Fig. 1A). At a 2,4-D concentration > 2.0 mg/l, most calli were small, pale yellow, and became hyperhydric without shoot formation (Fig. 1A). The best conditions for the induction of healthy green calli with shoots were 0.05 mg/l 2,4-D and 4.0 mg/l TDZ (Fig. 1B, Table S2).

Effect of other factors in callus and shoot induction

To improve the shoot induction medium from the previous conditions (30 g/l sucrose, 8 g/l agar, pH = 5.8, absence

Table 1 Callus induction rates (%) from carnation leaf explants under different phytohormone combinations

		Auxin (1.0 mg/l)				Mean
		IBA	NAA	2,4-D	IAA	
Cytokinin (0.5 mg/l)	BA	72.2 ± 20.0a	72.2 ± 20.0a	83.3 ± 9.6a	0.0 ± 0.0c	56.9 ± 11.9mn
	TDZ	72.2 ± 14.8a	77.8 ± 22.2a	72.2 ± 5.6a	83.3 ± 9.6a	76.4 ± 6.3mn
	KT	0.0 ± 0.0c	50.0 ± 19.2abc	88.9 ± 11.1a	0.0 ± 0.0c	34.7 ± 12.2n
	ZT	66.7 ± 9.6ab	100.0 ± 0.0a	88.9 ± 11.1a	77.8 ± 11.1a	83.3 ± 5.4 m
	2IP	94.4 ± 5.5a	77.8 ± 11.1a	100.0 ± 0.0a	5.6 ± 5.6bc	69.4 ± 11.8mn
Mean		61.1 ± 9.7xy	75.6 ± 7.6x	86.7 ± 4.0x	33.3 ± 10.7y	
		Auxin (0.5 mg/l)				Mean
		IBA	NAA	2,4-D	IAA	
Cytokinin (0.5 mg/l)	BA	88.9 ± 5.6a	72.2 ± 20.0ab	100.0 ± 0.0a	0.0 ± 0.0d	65.2 ± 12.6mn
	TDZ	94.4 ± 5.6a	94.4 ± 5.6a	88.9 ± 5.6a	88.9 ± 5.6a	91.7 ± 2.5 m
	KT	5.6 ± 5.6d	61.1 ± 14.7abc	100.0 ± 0.0a	11.1 ± 5.6d	44.4 ± 12.2n
	ZT	88.9 ± 5.6a	94.4 ± 5.6a	94.4 ± 5.6a	27.8 ± 5.6 cd	76.4 ± 8.9mn
	2IP	94.4 ± 5.6a	77.8 ± 14.7a	94.4 ± 5.6a	33.3 ± 9.6bcd	75.0 ± 8.6mn
Mean		74.4 ± 9.5x	80.0 ± 6.1x	95.6 ± 2.0x	32.2 ± 8.5y	
		Auxin (0.5 mg/l)				Mean
		IBA	NAA	2,4-D	IAA	
Cytokinin (1.0 mg/l)	BA	55.6 ± 5.6ab	88.9 ± 9.6a	88.9 ± 11.1a	0.0 ± 0.0c	58.3 ± 11.3 nm
	TDZ	94.4 ± 11.1a	100.0 ± 0.0a	83.3 ± 9.6a	77.8 ± 5.6a	88.9 ± 3.8 m
	KT	11.1 ± 11.1bc	72.2 ± 14.7a	83.3 ± 9.6a	0.0 ± 0.0c	41.7 ± 11.9n
	ZT	94.4 ± 5.6a	83.3 ± 9.2a	83.3 ± 9.6a	66.7 ± 1.7a	81.9 ± 5.6 m
	2IP	94.4 ± 5.6a	94.4 ± 5.6a	88.9 ± 5.6a	5.6 ± 5.6c	70.8 ± 11.6mn
Mean		70.0 ± 9.2x	87.8 ± 4.1x	85.6 ± 3.6x	30.0 ± 9.8y	

Data are expressed as the mean rates (%) of callus induction rates ± SE ($n = 6$, $r = 3$) after 1 month. Data followed by different letters are significantly different ($P < 0.05$), as determined using Tukey–Kramer's honestly significant difference (HSD) test

of AgNO_3), various concentrations of sucrose, agar and AgNO_3 , and pH values were investigated. The shoot induction rates of the base conditions were compared in the 2,4-D and TDZ concentration tests and in the tests of the other components. Callus and shoot induction rates did not significantly differ when cultured at different concentrations of sucrose or agar and under varying pH values, however, hyperhydricity was variable (Table 2). In the sucrose treatment, rate of hyperhydric calli was significantly higher at a concentration of 20 g/l. Based on the photos, 50 g/l sucrose resulted in yellowish, brown, and fragile calli (Fig S6A). Agar tended to increase hyperhydricity at lower concentrations, with 4 g/l being significantly lower than with 12 and 16 g/l (Table 2). Calli were dehydrated and their surface appeared white at > 12 g/l agar (Fig S6B). Hyperhydricity tended to increase with advancing acidity and alkalinity, such as 5.0 and 6.2, and was significantly lower at 5.4 and 5.8 than at 5.0. At pH 5.0, there were many light green calli (Fig S6C). These results suggest that the base conditions of 30 g/l sucrose, 8 g/l agar, pH = 5.8 are adequate to reduce hyperhydricity. As the concentration of AgNO_3 increased, the callus induction rate decreased (Table 2). In the AgNO_3 -supplemented medium, callus hyperhydricity was suppressed, but shoots were not induced (Table 2; Fig.S6D).

Therefore, it was not necessary to include AgNO_3 in the medium at the concentrations tested.

Root induction by different hormones and vessels

Different hormones and vessels were evaluated to increase the rooting rate and reduce hyperhydricity. In 200 ml vessels with GA_3 fixed at 5 mg/l, different combinations of three phytohormones (NAA, KT, and IBA) at various concentrations did not significantly affect in rooting rate from shoots and shoot hyperhydricity rate (Table 3). However, comparison of different volume vessels with fixing 5.0 mg/l GA_3 and 0.01 mg/l IBA revealed that rates of rooted shoot was the lowest significantly in 50 ml bottles, less than half of the 200 ml and 450 ml. In addition, the rate of hyperhydric plantlets decreased with an increase in vessel volume. The rate of hyperhydric plantlets was 22% and 90% in 450 and 50 ml bottles, respectively (Table 3). Based on these results, phytohormones were not critical, and larger vessel volumes, such as 450 ml, were more effective in maintaining healthy shoots and promoting rooting. Some rooted regenerants were successfully acclimated and potted in soil (Fig. 2). Through the above experiments, the following conditions were determined to obtain regenerants from seed-propagated carnations. For both callus and shoot induction,

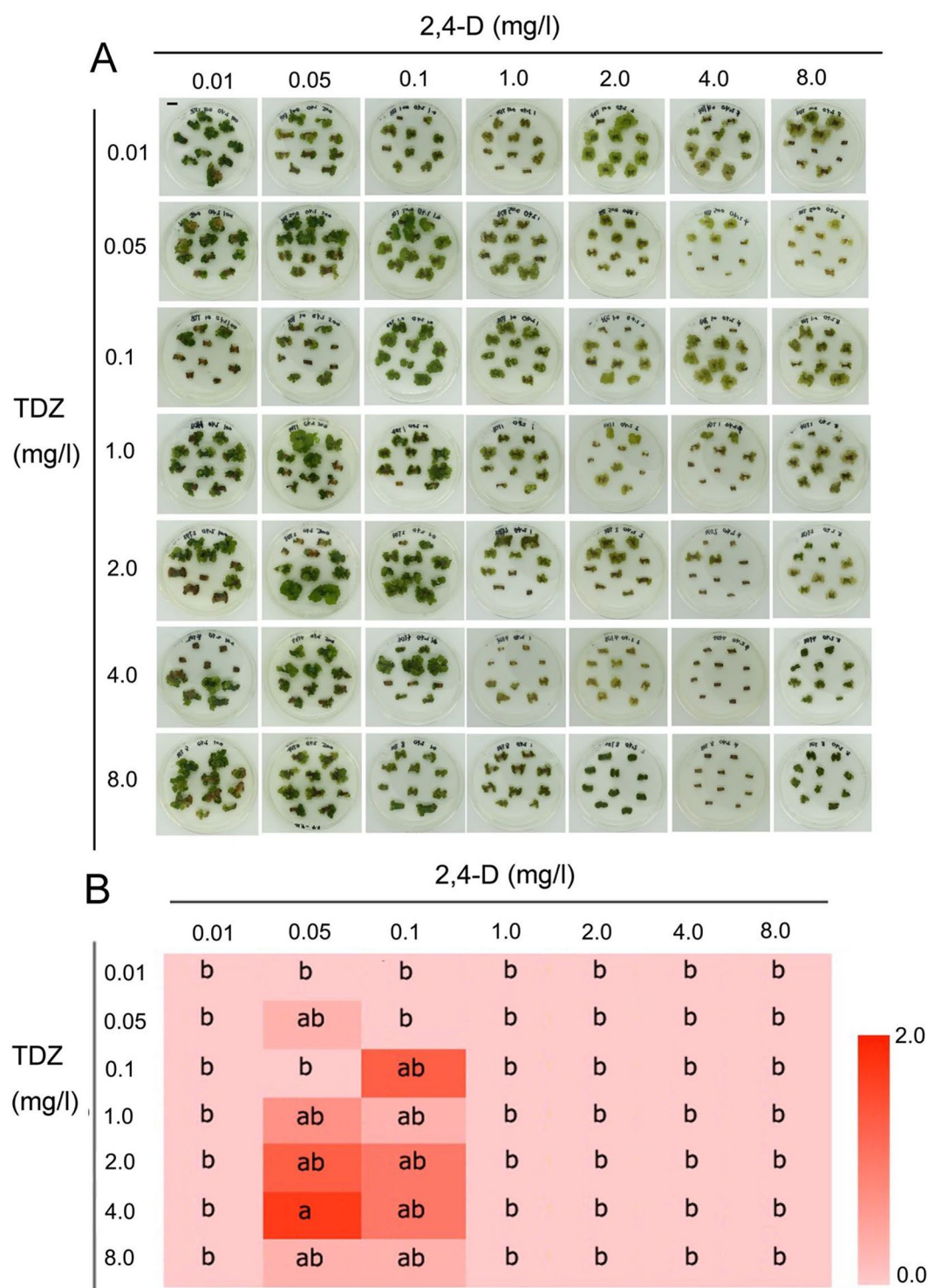


Fig. 1 Effects of different concentration combinations of 2,4-D and TDZ on callus/shoot formation from carnation leaf segments. Photographs of calli/shoots in medium supplemented with 2,4-D and TDZ at different concentrations (A). Significant differences in the number of

explants inducing shoots at various concentrations of 2,4-D and TDZ (B). Ten leaf segments were incubated as explants in each Petri dish ($n = 10$, $r = 3$). Photographs were taken 1 month after inoculation

Table 2 Callus / shoot induction rates under different medium compositions other than phytohormones

Treatment	Callus induction rates (%)	Shoot induction rates (%)	Rates of hyperhydric calli (%)
Sucrose (g/l)	20	86.7 ± 13.3 a	0.0 ± 0.0 a
	30	96.7 ± 3.3 a	7.7 ± 3.3 a
	40	96.7 ± 3.3 a	3.3 ± 3.3 a
	50	96.7 ± 3.3 a	6.7 ± 6.7 a
Agar (g/l)	4	90.0 ± 5.8 a	10.0 ± 6.8 a
	8	96.7 ± 3.3 a	7.7 ± 3.3 a
	12	96.7 ± 3.3 a	0.0 ± 0.0 a
	16	93.3 ± 3.3 a	7.7 ± 3.3 a
pH	5.0	90.0 ± 10.0 a	0.0 ± 0.0 a
	5.4	73.3 ± 17.6 a	0.0 ± 0.0 a
	5.8	93.3 ± 7.7 a	3.3 ± 3.3 a
	6.2	83.3 ± 12.0 a	0.0 ± 0.0 a
AgNO ₃ (mg/l)	0.5	93.3 ± 6.7 a	0.0 ± 0.0 a
	1.0	76.7 ± 12.0 ab	0.0 ± 0.0 a
	2.0	40.0 ± 11.6 b	0.0 ± 0.0 a

Data are expressed as the mean rates (%) of callus / shoot induction ± SE ($n=10$, $r=3$) after 1.5 months. Data followed by different letters are significantly different ($P<0.05$), as determined using Tukey–Kramer's HSD test

leaf segments are placed on MS medium containing 30 g/l sucrose and 8 g/l agar at pH 5.8 as the basal medium, supplemented with 0.05 mg/l 2,4-D and 4.0 mg/l TDZ in petri dishes. For rooting, shoots are grown on the basal medium containing the appropriate phytohormones (e.g., 5.0 mg/l GA₃ and 0.01 mg/l IBA) in 450 ml culture bottles.

Genome editing using *Agrobacterium*-mediated transformation

As we applied *Agrobacterium*-mediated transformation to the regeneration system described in the previous section, we examined the antibiotic conditions for selecting transformants. Calli were induced with <50 mg/l kanamycin or 10.0 mg/l hygromycin after 1 month of leaf segment culture (Fig. S7A; S7B). Accordingly, 75 mg/l kanamycin and 12.5 mg/l hygromycin were used for post-infection



Fig. 2 Appearance of regenerated carnation plantlets. Regenerants were obtained by inducing calli and shoots from leaf segments and rooting using established protocols. Healthy shoots (A) but not hyperhydric shoots (B) were acclimatized and transplanted into pots (C)

screening. The antibiotic kanamycin was initially used to select transformed shoots; however, many of the obtained shoots were only non-transformants in white color. Therefore, hygromycin was selected based on the results of a previous study (Nontaswatsri et al. 2004).

When designing gRNA for the target *DcPDS* gene, it is important to avoid the mutation sequence based on varietal differences between the ‘Chabaud Giant’ material variety and the ‘Francesco’ variety used for reference genome sequences. In this study, we use PCR cloning to obtain two genome sequences for the *DcPDS* of ‘Chabaud Giant’. The

Table 3 Rates of carnation shoots forming roots under different culture conditions

Phytohormone (mg/l)				Vessel volume (ml)	Rates of rooted shoots (%)	Rates of hyperhydric shoots (%)
GA ₃	NAA	KT	IBA			
5.0	0.01	0.01		200	77.8 ± 11.1a	55.6 ± 11.1ab
5.0	0.5	0.5		200	66.7 ± 0.0ab	66.7 ± 19.3ab
5.0			0.5	200	66.7 ± 0.0ab	77.8 ± 11.1ab
5.0			0.01	50	33.3 ± 0.0b	89.9 ± 11.1a
5.0			0.01	200	77.8 ± 11.1a	55.6 ± 11.1ab
5.0			0.01	450	77.8 ± 11.1a	22.2 ± 11.1b
Mean					66.7 ± 4.7	59.2 ± 6.4

Data are expressed as the mean rates of carnation shoots forming roots ± SE ($n=3$, $r=3$) after 1 month. Data followed by different letters are significantly different ($P<0.05$), as determined using Tukey–Kramer's HSD test

resulting DNA sequences encoded both partial *DcPDS1* and full-length *DcPDS2* or *DcPDS3* (hereafter referred to as *DcPDS2/3*). The lengths of the cloned *DcPDS1* and *DcPDS2/3* were 2471 bp and 4337 bp, respectively (Fig. S8). The conserved regions of *DcPDS1* and *DcPDS2/3* were designed for gRNA (Fig. S2). Albino shoots were produced from the explants after infection with *Agrobacterium* containing the pKI1.1R vector (Fig. 3), although the shoot production rate was only 2.5%. Sequence analyses of the albino shoots PCR amplicons revealed two different indels; the deletion of one base (A) or the insertion of two bases (AT) before the PAM site. Therefore, both indels provoked a shift in the reading frame and a non-functional protein (Fig. 4). The probability of genome-edited shoots per regenerated shoots was 6.7%. Since the sequencing was limited to albino shoots, it is possible that the normal shoots with non-silenced *PDS* gene by in-frame editing were missed. If that possibility is included, the true efficiency may be somewhat higher.

Discussion

Tissue culture is an important step in the transgene, necessitating the establishment of an induction protocol of callus and shoots (De Filippis 2014). Thus, combinations of four auxins and five cytokinins were evaluated to establish an in vitro regeneration protocol for homozygous carnations

‘Chabaud Giant’ in this study. Calli were induced by most hormone combinations. However, particularly low levels of callus induction were observed when the auxin IAA was used (Table 1). IAA is a naturally produced plant hormone that is rapidly degraded by many tissues and often cannot support the growth of cultured plant tissues (Hangarter et al. 1980). In addition, the light provided by cool-white, fluorescent bulbs can promote the degradation of IAA. Therefore, IBA, NAA, and 2,4-D are more commonly used than IAA in plant tissue culture (Nissen and Sutter 1990; Stasinopoulos and Hangarter 1990). Although the growth rate of calli was high under many hormone combinations, shoots were only obtained from calli grown in a medium containing 2,4-D and TDZ. Furthermore, many calli were especially small and considerably hyperhydric, which may be the main reason shoots were not obtained. Although 2,4-D is effective in callus induction (Maheshwari et al. 2011; Motte et al. 2011), it is not typically effective in shoot induction (Hangarter et al. 1980). However, 2,4-D induced shoot regeneration in the present study, possibly because of the specificity among species or varieties. In shoot induction from carnation leaves, NAA is the most commonly used auxin (Frey and Janick 1991; Nakano et al. 1994; Abu-Qaoud, 2013), with some use of IBA and IAA (Nontaswatsri et al. 2002; Kanwar and Kumar, 2009). In carnations, TDZ has been successfully used as a cytokinin for shoot induction from leaves. With different varieties from ‘Chabaud Giant’, 0.1–4.0 mg/l of TDZ marked high shoot induction rates ranging

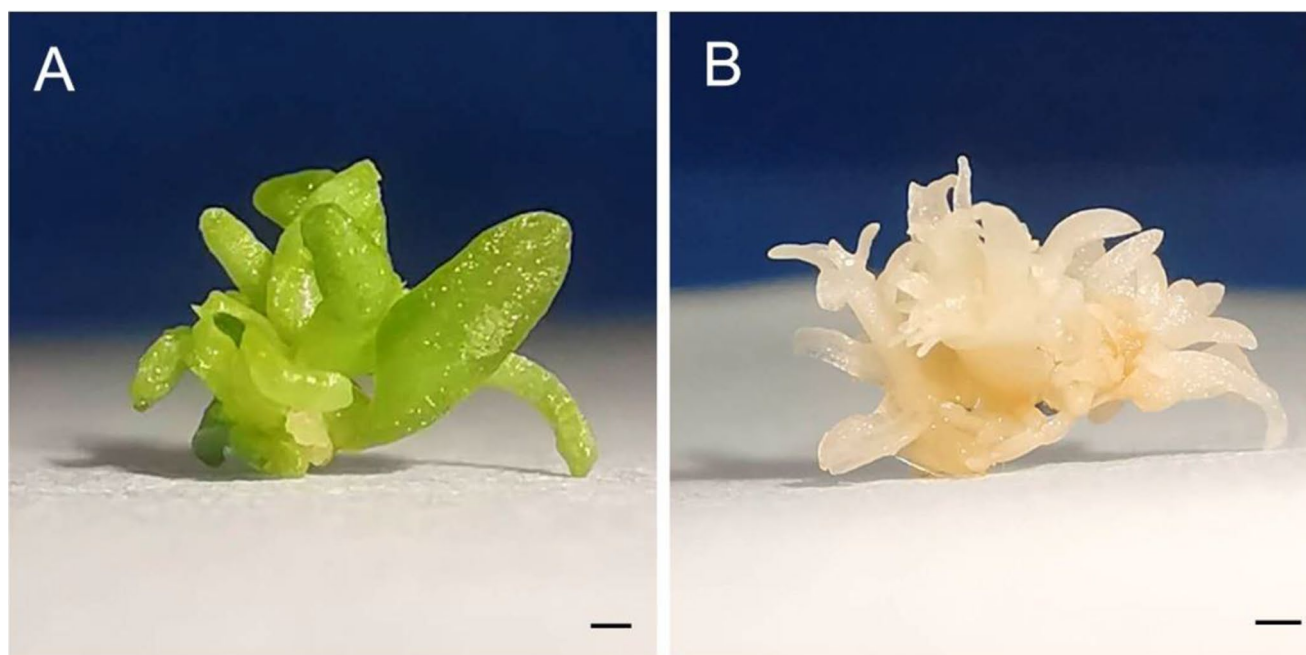


Fig. 3 Appearance of plantlets or calli of carnation after genome editing. Regenerants were obtained from *Agrobacterium*-infected leaf fragments using established protocols. The carnation phytoene desaturase gene (*DcPDS*) was targeted. Photograph show regenerated

plantlets without genome editing (A). Plantlets regenerated following successful genome editing using the pKI1.1R vector containing the hygromycin resistance gene (B)

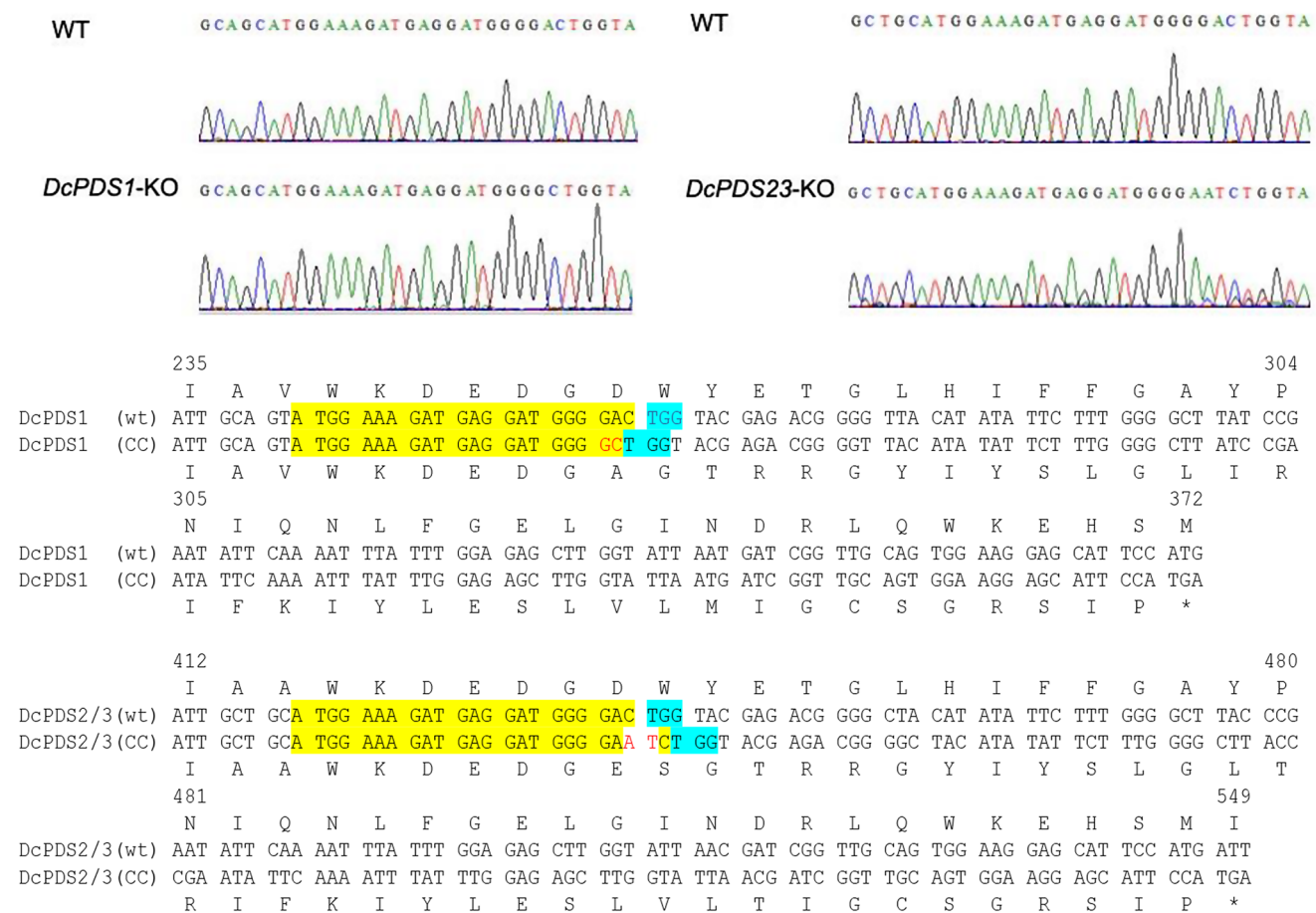


Fig. 4 Altered nucleotide sequence of *DcPDS* by CRISPR/Cas9-derived genome editing. Electropherograms were obtained by sequencing PCR-cloned genome fragments for the wild-type (WT) and two genome-edited strains (*DcPDS1-KO* and *DcPDS23-KO*). Indels other

than multiples of three cause frameshifts and the appearance of an early termination codon or different amino acid. The sequences for gRNA and PAM are highlighted in yellow and light blue, respectively

from 53 to 93% (Frey and Janick 1991; Nontaswatsri et al. 2002; Abu-Qaoud, 2013). The concentrations used in this study were also in the range, suggesting that carnations are generally responsive to TDZ.

Different concentrations of sucrose and agar, as well as different pH values, were tested to obtain high-quality calli, reduce hyperhydricity, and improve shoot induction efficiency. Although the results showed no significant differences in shoot induction efficiency, they provided a basis for selecting a more suitable medium. Shoot regeneration occurred when the concentrations of supplemented sucrose were more than 30 g/l (Table 2). Calli showed obvious hyperhydricity in the medium supplemented with 20 g/l sucrose, and browning was observed when the sucrose concentration was >40 g/l. This result is similar to that observed in previous research showing that increasing concentrations of sucrose can reduce hyperhydria, and that high sucrose concentrations can promote callus browning (Khosroushahi et al. 2011; Liu et al. 2017; Liang et al. 2019). Therefore, a sucrose concentration of 30 g/l was the most appropriate in

the present study. Agar above 12/g drastically reduced the hyperhydricity rate (Table 2). However, the surface of the callus turned white and appeared to be low in water content, inhibiting growth (Fig. S4). Previous studies have reported that high concentrations of agar were less likely to induce hyperhydricity, but were detrimental to shoot regeneration and inhibited growth, which supported our results (Arnold and Eriksson 1984; Kadota et al. 2001; Suthar et al. 2011).

The calli appeared more hyperhydric at pH 5.0 (Table 2; Fig. S6C). pH can affect the hardness of the agar medium: a lower pH softens the medium, whereas a higher pH hardens it (Huang et al. 1995). Although shoots only grew at pH 5.8 in other media, there was no significant effect on the induction rate or appearance of calli and shoots. Hyperhydricity was thought to be due to a reduction in the amount of cellulose and lignin required for cell wall lignification caused by ethylene (Keverse et al. 1984). Silver ions can bind to ethylene receptors and prevent the transduction pathway (Gao et al. 2017). Therefore, AgNO₃ has been used as an anti-hyperhydric agent in several plant species (Mayor et

al. 2003; Vinoth and Ravindhran 2015). This study actually showed that AgNO_3 -supplemented medium suppressed callus hyperhydricity, but did not induce shoots unfortunately (Table 2; Fig. S6D). The carnation ‘Chabaud Giant’ used in this study may be too sensitive to AgNO_3 , as even 0.5 mg/l AgNO_3 was toxic too shoot differentiation.

Various hormones and vessels were evaluated to determine the optimal rooting conditions. There was no significant difference in rooting in the media containing different phytohormones (Table 3). A few examples of rooting from carnation shoots have been reported to be effective with 1.0 mg/l IBA (Lu et al. 2021; Zheng et al. 2020) and 1.0 mg/l NAA (Karami and Kordestani 2007; Ali et al. 2008; Zheng et al. 2020). Although we did not test the effects of these hormones alone in this experiment, it is likely that a number of different auxins were effective to promote rooting in carnations. The use of a large size vessels showed the positive effect on root growth and reduced hyperhydricity (Table 3). It has been reported that nutrient supply increases and air ventilation was improved, all of which were conducive to root growth and reduced hyperhydricity (Lai et al. 2005; Lynch et al. 2012). Some studies have shown that both ethylene and CO_2 are accumulated in a closed vessel (reviewed by Polivanova and Bedarev 2022). In addition, the expression of 1-aminocyclopropane-1-carboxylic acid oxidase gene (*ACO*) involving ethylene synthesis, has been shown to be significantly increased in hyperhydric plants. In a well-ventilated environment, the accumulation of ethylene and CO_2 can be reduced to inhibit the hyperhydricity.

Shoot induction efficiency was reduced following *Agrobacterium* infection, possibly because of altered levels of auxin and other hormones (Bettini et al. 2010; Mashiguchi et al. 2019). The selection of knockout shoots for the *PDS* gene by the antibiotic kanamycin was not successful. This could be because it was difficult to recognize the difference in white color between the drug effect of kanamycin and the loss-of-function effect of *PDS*. Practically many white-colored regenerants were obtained as escapes on the medium containing 75 mg/l kanamycin (Fig. S7C). This indicates that carnations may be somewhat resistant to kanamycin and shoots on the selection medium grow to a certain stage. The calli on the medium containing kanamycin were transparent yellow (Fig. S7D), whereas those with hygromycin were healthy (green) (Fig. S7E). The results show that even in the presence of antibiotic concentrations that weaken the explant, false-positive callus differentiation occurs with kanamycin, whereas it is suppressed with hygromycin. Albinism in non-transformed plantlets due to kanamycin has been also reported in many plant species, such as *Brassica juncea* (Gao et al. 2020), *Vigna mungo* (Saini et al. 2003), Indian cowpea (Chaudhury et al. 2007)

and sugarcane (Rastogi et al. 2018). Therefore, kanamycin was unfavorable for the screening of *PDS* knockouts in carnations.

Two studies on genome editing in carnations have recently been issued. One is a transient system using the method of direct delivery of preassembled CRISPR/Cas9 ribonucleoproteins (RNPs) using protoplasts (Adedeji et al. 2024). Their method is superior in two aspects: elimination of chimerism and foreign gene-free, but organogenesis from protoplasts is difficult and shoots were not obtained. Another study used a method of introducing RNPs into stem sections by electroporation (Mori et al. 2024). With this method, also foreign DNA-free, they obtained regenerated shoots, but the problem of chimerism remained. We successfully developed a genome-editing system with a seed propagation-type carnation in which *PDS* was knocked out. Genome editing of seed-propagated varieties established in this study will accelerate carnation breeding as it will overcome the problems of chimerism, persistence of foreign genes and trait segregation, which are problems in vegetatively propagated varieties.

Conclusions

In this study, a system for shoot regeneration from leaf explants of the homozygous carnation ‘Chabaud Giant’ was established. Of all the treatments tested, the best results of regeneration rate were reached 27.8% in MS medium supplemented with 0.05 mg/l 2,4-D, 4.0 mg/l TDZ, 30 g/l sucrose and 8 g/l agar and adjusted to pH 5.8 after 1.5 month. Moreover, the use of a large size vessels with 450 ml volume was the best condition for root growth and reducing hyperhydricity. Additionally, an *Agrobacterium*-mediated transformation procedure was developed to obtain transformed plants using hygromycin as the selection antibiotic. Gene editing (CRISPR/Cas9) combined with the transformation/regeneration system was investigated using *PDS* as the target gene. Sequencing analyses of the edited albino shoot revealed two different indels: one base deletion or two bases insertion before the PAM sequence. These results demonstrate that the carnation genome editing system was established for the first time with a seed-propagation variety, which will provide convenience for the future study of carnation gene function and the efficient breeding of new varieties.

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Author contributions ZJL performed tissue culture, *Agrobacterium*-mediated transformation, cloning, and sequencing of *DcPDS* genes; generated knockout transformants via genome editing; and drafted the manuscript. YU designed the experiments, analyzed the data, and revised the manuscript. MY and RK analyzed the data and revised the manuscript. All the authors have read and approved the final manuscript.

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Data availability Sequence information of *DcPDSs* can be obtained as Dca36978.1 (*DcPDS1*), Dca48369.1 (*DcPDS2*), and Dca52111.1 (*DcPDS3*) from the carnation genome database (<http://carnation.kazus.a.or.jp/>; Yagi et al. 2014).

Declarations

Conflict of interest The authors declare no conflicts of interest.

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