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ORIGINAL PAPER

First insights into transgene-free genome editing in European hazelnut protoplasts: a step forward in hazelnut breeding

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Abstract

New Plant Breeding Techniques (*NPBTs*) are emerging as innovative tools for plant breeding, with the CRISPR/Cas9 system being a valuable method for accelerating genetic improvement. Currently, the combination of the CRISPR/Cas9 system with protoplast technology offers a promising approach to producing transgene-free edited plants. In this study, we established the first protoplast isolation protocol in *Corylus avellana* L., cv 'Tonda Gentile Trilobata'. Friable calli were obtained from *in vitro*-derived leaves cultured on a callus induction medium based on a modified Murashige and Skoog medium with half-strength macroelements, supplemented with 0.2 mg/l benzylaminopurine (BAP) and 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4D). For cell-wall digestion, the solution contained Cellulase Onozuka R-10 (2%), Cellulase from *Aspergillus niger* (2%), Macerozyme R-10 (0.5%), and Pectinase from *Aspergillus aculeatus* (1%) was used; the concentration obtained was 1,111,000 protoplasts/ml with 99% viability. Among the purification methods tested, filtration through nylon sieves with washing steps (P1 method) provided the best balance between purity and yield, resulting in 1,008,800 protoplasts/ml. We also developed a protoplast transfection protocol using the CRISPR/Cas9 ribonucleoprotein system, designed to edit the *phytoene desaturase* (*pds*) gene. Protoplasts were also cultured on semi-solid media, resulting in microcalli formation after 60 days. The microcalli emerged on a modified MS medium with half-strength NH_4NO_3 and KNO_3 (MS1B), supplemented with 0.1 mg/l BAP and 0.01 mg/l 2,4-D. This study represents the first detailed protoplast isolation and transfection protocol from somatic tissues in hazelnut, providing the basis for future breeding approaches in this plant species.

Keywords CRISPR/Cas9 · Protoplasts technology · *Corylus avellana* L. · *In vitro* culture · Gene knockout

Introduction

Among the innovative approaches in plant breeding, the CRISPR/Cas9 system is a precise and cost-effective genome-editing tool, enabling targeted mutations in plant genomes (Lee et al. 2020; Safari et al. 2018). Although its application has been successfully demonstrated in several woody species, including poplar (Zhou et al. 2015), sweet orange (Jia and Wang 2014), apple (Nishitani et al. 2016), cacao (Fister et al. 2018), grapevine (Osakabe et al. 2018), pear (Charrier et al. 2019), chestnut (Pavese et al. 2021a), blueberry (Vaia et al. 2022) and oak (Pavese et al. 2024), its application in European hazelnut (*Corylus avellana* L.) has not yet been reported.

European hazelnut is an economically important species in Italy, valued for its high-quality nuts used in the confectionery industry (Talucci et al. 2022). The 'Tonda

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Gentile Trilobata' (syn. 'TGT'; 'Tonda Gentile delle Langhe'; 'TGL'; 'TGdL') cultivar is particularly appreciated in Piedmont, thanks to its superior flavour and aroma, especially after roasting (Valentini et al. 2025). However, hazelnut cultivation in Italy has recently declined due to a combination of environmental stresses and pathogen pressure. Climate change has reduced yields through drought, late frosts, and temperatures above 35 °C, which affect photosynthesis and trigger photoinhibition (Luciani et al. 2019). These fluctuations also increased susceptibility to diseases. Among them, *Erysiphe corylacearum*, the causal agent of powdery mildew, has become a major threat across Europe, reducing yields and the productive life cycle of hazelnut orchards (Matic et al. 2024). More recently, fungal trunk disease (FTD), caused by *Anthostoma decipiens* and *Diaporthe ereses* has severely affected production, particularly in Piedmont, causing dieback and decay of trunks, branches, and entire plants (Martino et al. 2025). To mitigate these challenges, growers rely on agrochemicals and sustainable orchard management practices. However, techniques such as pruning are labour-intensive, especially given hazelnut's natural tendency to form multi-stemmed shrubs (Silvestri et al. 2021). Furthermore, prolonged agrochemical use raises concerns about the development of pathogen tolerance and resistance (Nicoletti et al. 2022). Therefore, developing new hazelnut cultivars with increased tolerance to abiotic stresses, reduced susceptibility to pathogens, and higher productivity has become a goal for breeding programs and sustainable orchard management. Conventional breeding, however, in woody species is slow and challenging due to their long juvenile phase, self-incompatibility mechanisms (Limera et al. 2017), and high genetic variability (Pavese et al. 2021b). CRISPR/Cas9 technology offers a promising alternative, potentially accelerating the development of improved hazelnut varieties. The editing machinery can be introduced into plant cells using several techniques, such as particle bombardment (Dong et al. 2021) or *Agrobacterium tumefaciens*-mediated transformation (Zhou et al. 2020). Although effective, these methods often lead to integration of the Cas9 endonuclease and single guide RNA (gRNA) into the plant genome, resulting in prolonged nuclease activity and a higher risk of off-target effects (Pavese et al. 2022). To overcome these limitations and address public concerns regarding genetically modified organisms (GMOs) (Yu et al. 2021), CRISPR/Cas9 can be delivered as ribonucleoprotein (RNP) complex (Park and Choe 2019). In this approach, the CRISPR components are assembled in vitro and introduced into plant protoplasts (Woo et al. 2015; Guo et al. 2023; Lin et al. 2018). The RNP system acts directly at the genomic target site and is rapidly degraded by cellular mechanisms, avoiding transgene integration and reducing off-target risks (Kim et al. 2014). The success of CRISPR/Cas9 applications depends on efficient in vitro regeneration protocols. Hazelnut is a recalcitrant species for genetic transformation, shoot organogenesis, and somatic embryogenesis (Silvestri et al. 2016, 2020). Regeneration success depends on several factors such as genotype (Gniech Karasawa et al. 2016; Yahyaoui et al. 2021), explant type, culture medium composition, and phytohormone balance (Thomson and Deering 2011; Corredoira et al. 2013; Ferrucci et al. 2023). Pavese et al. (2023) demonstrated the potential for regenerating hazelnut from somatic tissue, which represents an important step toward biotechnological advancements in this species (Botta et al. 2019). Nevertheless, further optimizations are needed to improve regeneration efficiency.

The *phytoene desaturase* (*pds*) gene, which acts in chlorophyll biosynthesis, is widely used as a marker gene in gene-editing research since its knockout leads to an albino phenotype, providing a clear and rapid visual marker of editing success (Naim et al. 2018). This approach has been applied in several woody species, including chestnut (Pavese et al. 2022), walnut (Walawage et al. 2019), highbush blueberry (Vaia et al. 2022), apple (Malabarba et al. 2020), coffee (Casarin et al. 2022), sweet orange (Jia and Wang 2014), and poplar (Fan et al. 2015).

In this study, we present the first protocol for isolating and transfecting hazelnut protoplasts, using *Green fluorescent protein* (GFP) as a marker to detect transfection success. CRISPR/Cas9 RNPs to target the *pds* gene was also applied. This research provides a foundation for future biotechnological advances in hazelnut breeding.

Materials and methods

Plant material

Micropropagated shoots of hazelnut cv 'TGT' were used as explant source for callus induction. Leaves were dissected into small pieces (0.2-0.3 cm) and placed on three different callus induction media (100 explants per medium). Media were based on a modified MS (Murashige and Skoog 1962) medium with half-strength macroelements (MS1B): **Ca1**: supplemented with 0.2 mg/l BAP (benzylaminopurine) and 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid); **Ca2**: supplemented with 0.5 mg/l kinetin and 2 mg/l 2,4-D and **Ca3**: supplemented with 0.3 mg/l BAP and 0.3 mg/l 2,4-D.

All media contained sucrose (30 g/L) and Plant Agar (Duchefa, NL) (8 g/L). The pH was adjusted to 5.8 with 0.1 N HCl or 0.1 N NaOH. Cultures were maintained for four weeks in a growth chamber under dark conditions at 25 ± 1 °C (Fig. 1A) (Marino et al. 2025). Details of all media compositions and solutions used are provided in Supplementary Material 1.

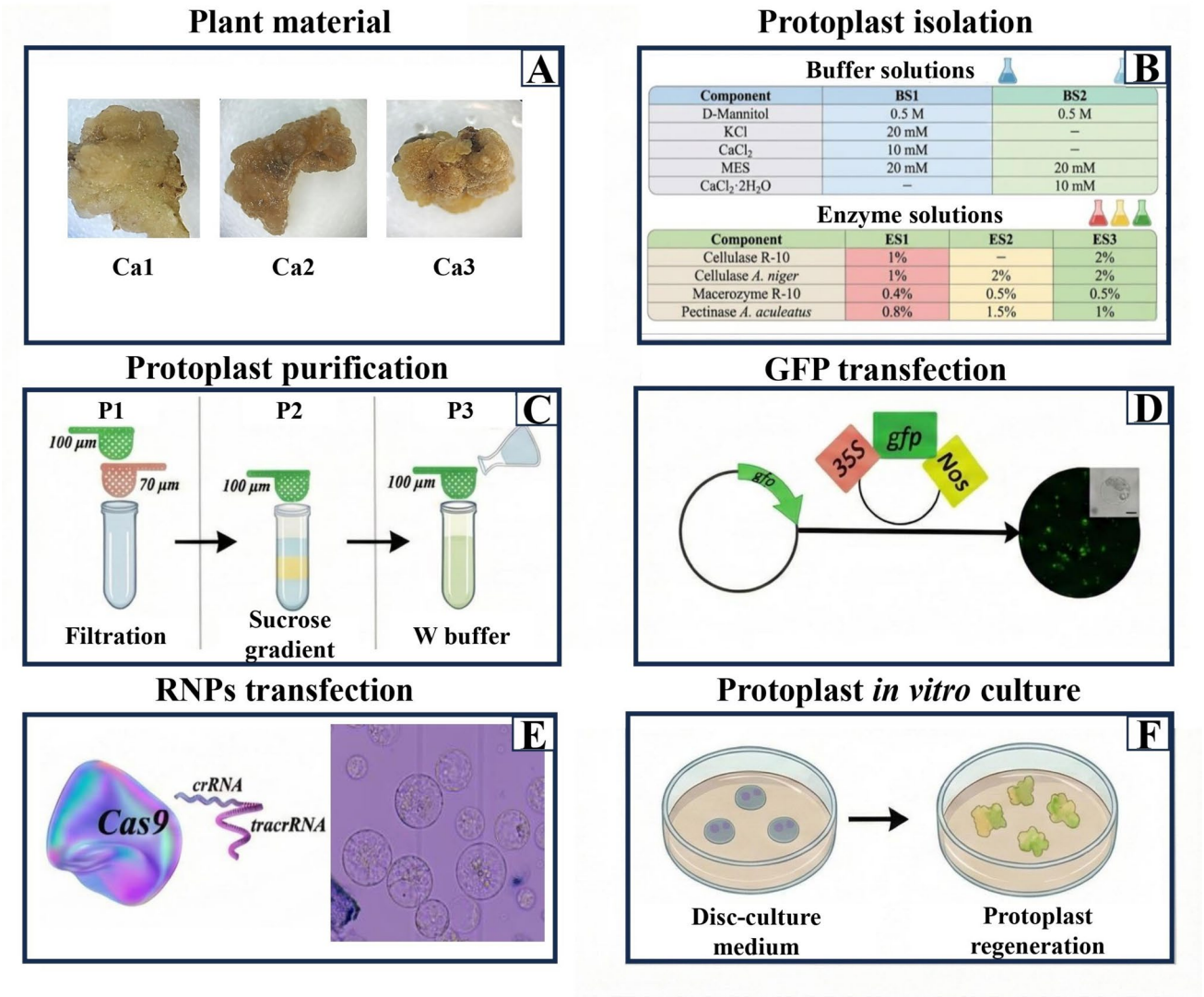

Fig. 1 Workflow of the experimental design for *Corylus avellana* cv. 'TGT' protoplast isolation, transfection, and culture: (**A**) Plant material used in the protoplast isolation process. (**B**) Buffer solutions (BS1 and BS2) and enzyme solutions (ES1, ES2, and ES3) tested for optimal protoplast concentration and viability. Missing components are indicated by a dash (—). (**C**) Protoplast purification strategies: P1) Filtration using 100 and 70 µm nylon sieves, P2) 21% sucrose gradient, P3) W buffer. (**D**) GFP transfection protocol and evaluation of transfection efficiency using fluorescence microscopy. (**E**) RNP-mediated transfection and subsequent mutation analysis of the *pds* gene (**F**) Callus induction on three regeneration media (R1, R2, and R3), monitored for *de novo* organogenesis

Determination of callus biomass and histological analysis

After four weeks, callus induction rate, histology, morphology, and water content were assessed. Callus induction rate (%) was calculated as the number of explants producing callus/total number of cultured explants × 100. Water content (%) was determined based on fresh weight (FW) and dry weight (DW). Ten calli per medium were blotted dry, weighed to obtain FW, dried at 100±2 °C until constant weight, and reweighed to determine DW. Water content was calculated as ((FW−DW)/FW) × 100, following Errabii et al. (2006).

For histological analysis, three calli per medium were sectioned (12 µm thickness) using a rotary microtome (Leica Microsystems, Wetzlar, Germany). Sections were rehydrated, stained with 0.05% Toluidine Blue (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature, rinsed three times with distilled water, dehydrated, and mounted in isoparaffin (Girelli Alcool, Milan, Italy), according to O'Brien et al. (1964) and following the procedure described by Vilela et al. (2021).

Samples were observed under an OPTIKA B-190 Series microscope (OPTIKA, Bergamo, Italy). Image analysis was performed using ImageJ software (version 1.54 g; https://imagej.net/ij/index.html) to measure cell area (µm²). The mean

value was calculated from 10 cells per section and three sections per callus to provide quantitative insights into callus structure (Fig. 1A).

Protoplast isolation

Three enzyme solutions (ES1-ES3) and two cell-wall digestion buffers (BS1, BS2) were tested to optimize protoplast concentration and quality.

Enzyme solutions

ES1 1% Cellulase Onozuka R-10 (Duchefa Biochemie, Netherlands), 1% Cellulase from *Aspergillus niger* (Sigma-Aldrich, St. Louis, MO), 0.4% Macerozyme R-10 (Duchefa Biochemie, Netherlands), and 0.8% Pectinase from *A. aculeatus* (Sigma-Aldrich, St. Louis, MO);

ES2 2% Cellulase from *A. niger* (Sigma-Aldrich, St. Louis, MO), 0.5% Macerozyme R-10 (Duchefa Biochemie, Netherlands), and 1.5% Pectinase from *A. aculeatus* (Sigma-Aldrich, St. Louis, MO);

ES3 2% Cellulase Onozuka R-10 (Duchefa Biochemie, Netherlands), 2% Cellulase from *A. niger* (Sigma-Aldrich, St. Louis, MO), 0.5% Macerozyme R-10 (Duchefa Biochemie, Netherlands), and 1% Pectinase from *A. aculeatus* (Sigma-Aldrich, St. Louis, MO).

Digestion buffers

BS1 0.5 M D-mannitol, 20 mM KCl, 10 mM $CaCl_2$, and 20 mM MES (2-(N morpholino) ethanesulfonic acid) (pH 5.7) (Pavese et al. 2022);

BS2 0.5 M D-mannitol, 20 mM MES, and 10 mM $CaCl_2 \cdot 2\ H_2O$ (pH 5.7).

For each trial, 100 mg of 'TGT' calli were sliced and pretreated with the respective BS solution for 1 h. The solution was then replaced with an equal volume of ES solution, prewarmed at 55 °C for 10 min to activate the enzymes and incubated for 17 h in the dark on a rotary shaker at 25 °C and 25 rpm (Lin et al. 2023) (Fig. 1B).

Protoplast purification

Purification tests were carried out using protoplasts previously isolated from **Ca1**-derived calli with **BS1** and **ES3**, which provided the highest yield and viability. Three purification strategies (P1-P3) were compared (Fig. 1C).

P1: Filtration through 100 µm nylon sieve, wash with WS (Whashing Solution), centrifuge (50 × g, 5 min), repeat filtration through 70 µm mesh, four additional washes with WS, final resuspension in 300 µl MMG solution (Pavese et al. 2021a); **P2**: Filtration through 100 µm mesh, wash and centrifuge, resuspension in WS, sucrose gradient purification (21% w/v), collection of viable ring, final resuspension in 1 ml WS, incubation at 4 °C for 30 min, centrifugation, and final resuspension in 300 µl MMG solution. **P3**: Filtration through 100 µm mesh, centrifugation, resuspension in W buffer (Li et al. 2022), sequential centrifugations (15 × g for 4 min, then 50 × g for 3 min), two washes with W buffer, final resuspension in 300 µl MMG solution.

Protoplast concentration was counted with a hemacytometer under an OPTIKA B-190 microscope. Viability was assessed with 4% Trypan Blue staining, calculating viable protoplasts as the number of observed protoplasts not stained blue/number of total protoplasts observed × 100.

Protoplast transfection using the GFP plasmid

The pAVA393 plasmid was utilized to assess the transfection efficiency of hazelnut protoplasts (Pavese et al. 2022; Ochatt et al. 2005). pAVA393:GFP is a plant expression reporter vector carrying the GFP (*green fluorescent protein*) coding sequence, which produces a green fluorescence signal in successfully transfected cells, under the control of the CaMV 35S promoter and the NOS terminator; it was used here exclusively to quantify PEG-mediated transfection efficiency by fluorescence microscopy. For the transfection assay, 200 µl of protoplast suspension, containing approximately 2×10^5 protoplasts, was gently mixed with 10 µg of the pAVA393:GFP plasmid. To facilitate plasmid uptake, 200 µl of 40% (w/v) PEG (polyethylene glycol) 4000 was added, followed by a gentle mix and incubation for 20 min at room temperature. After incubation, the protoplasts were washed twice with WS, and the resulting pellet was resuspended in 1 ml of WS. The transfected protoplasts were maintained at room temperature in the dark to minimize photooxidative stress. The transfection efficiency was evaluated at three time points: 12, 24, and 48 h post-transfection. Observations were performed using a fluorescence microscope (Nikon Eclipse Ti2, Japan) to detect GFP expression. The transfection efficiency was calculated as the ratio between the GFP-fluorescent cells and the total number of protoplasts observed × 100 (Fig. 1D). As a negative control, protoplasts were transfected with an empty pAVA vector to confirm the specificity of GFP expression (Fig. 1D).

Protoplast transfection via RNPs

The *pds* gene sequence was retrieved from the 'TGT' genome (Pavese et al. 2021b; Zenodo database). Two gRNAs (gRNA1: 5'-GAGGCAAGAGATGTTCTAGG-3'; gRNA2: 5'-GAGATTGGAGAAATTAGTTG-3') were designed using CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/, accessed on February 2nd, 2024) (Fig. 2A). Crispr RNA (crRNA) (2 nmol), transactivating CRISPR RNA (tracrRNA) (2 nmol), and Alt-R-SpCas9 nuclease 3NLS (61 µM) were provided by Integrated DNA Technologies, Inc (IDT, Coralville, IA, USA). To assemble the two guide RNAs (gRNAs) (*pds*_gRNA1 and *pds*_gRNA2), the crRNAs and tracrRNAs were annealed in vitro at 95 °C for 5 min using a duplex buffer. The annealed gRNAs were then combined with Alt-R-SpCas9 nuclease 3NLS in the presence of 10× Phosphate Buffered Saline (PBS) (pH 7.4) to assemble the RNP complexes. For transfection, the two RNP complexes were used in separate reactions: S1 (Cas9 RNP+*pds*_gRNA1) and S2 (Cas9 RNP+*pds*_gRNA2). In each reaction, 10 µl of the RNPs complex (S1: *pds*_gRNA1 and S2: *pds*_gRNA2) were mixed with 2×10^5 protoplasts suspended in 200 µl of solution. These mixtures were combined with 200 µl of 40% (w/v) PEG 4000 and incubated at room temperature for 20 min (Fig. 2B). After incubation, the protoplasts were washed twice in WS, and the resulting pellet was resuspended by adding 1 ml of WS. Two negative controls were also included: (1) untransformed protoplasts and (2) protoplasts transfected with gRNA but lacking the Cas9 nuclease. To analyze mutation outcomes, genomic DNA was extracted from the transfected protoplasts using the EZNA® Plant DNA kit (Omega Bio-tek, Norcross, GA, USA). The *pds* target sequence was amplified by PCR, using primers designed to flank the gRNAs target site: (*pds*_1_F:5'CTGCTCATTACTTGGGATGTGT3'; *pds*_1_R:5'GCAACAACGAAAGAGACCGA3'; *pds*_2_F:5'ACGTGATTGAAGGGGATGCT3'; *pds*_2_R:5'TGTGACTGACGGACATAAAAGG3')

PCR products were purified using the DNA/RNA Clean-Up E.Z.N.A.® kit (Omega Bio-tek, Norcross, GA, USA).

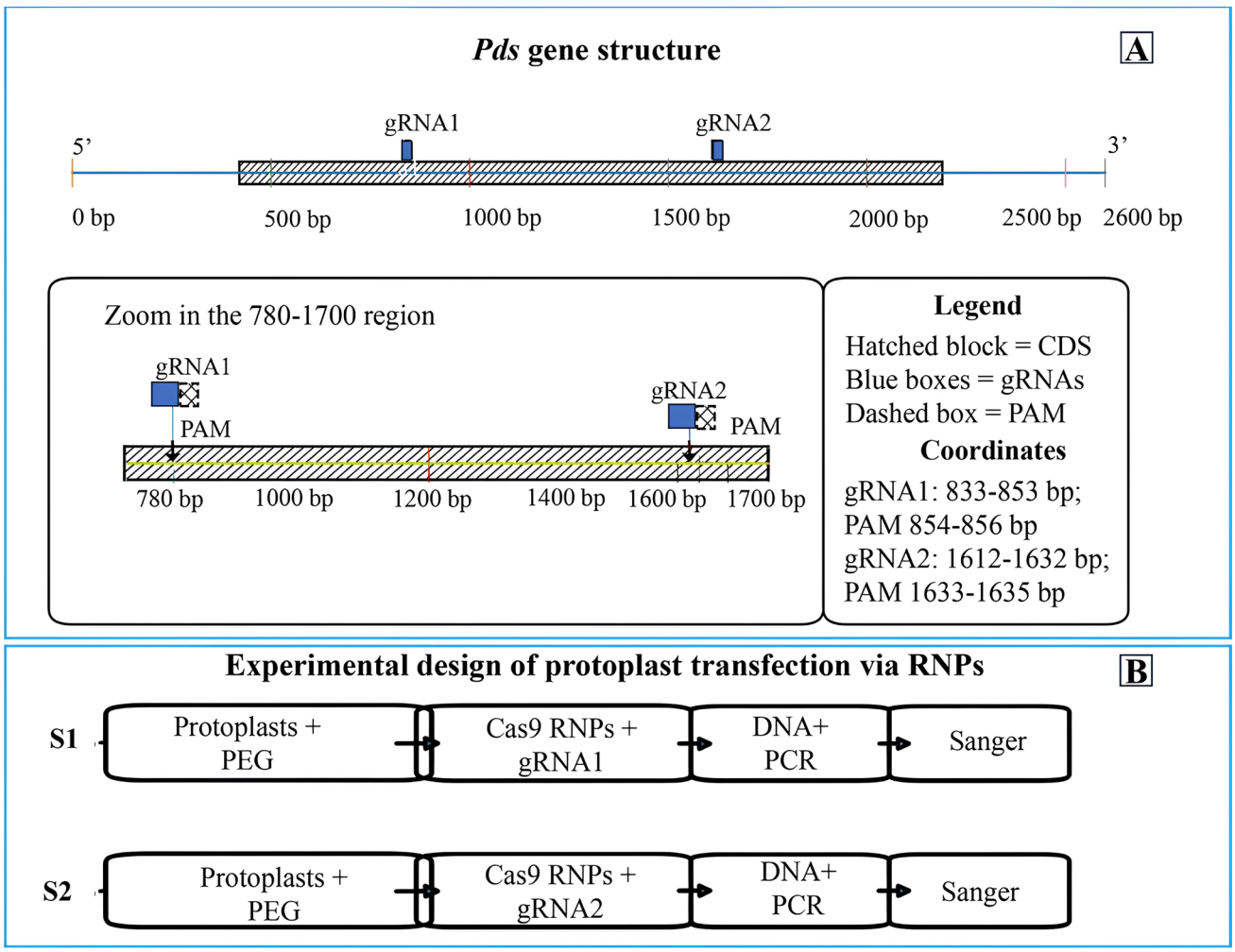

Fig. 2 (**A**) *Pds* gene structure and gRNAs design (**B**) Experimental design of the protoplast transfection via RNPs

Mutation screening in the *pds* target region was conducted using Sanger sequencing. Sanger sequencing offers detailed insights into mutation frequencies and types (Liang et al. 2018), making it an ideal method for targeted mutational analysis in protoplast systems (Brandt et al. 2020; Poovaiah et al. 2021; Pavese et al. 2022; Scintilla et al. 2022; Hoengenaert et al. 2023). The sequencing chromatograms were analyzed using the bioinformatics tool TIDE (Tracking of Indels by Decomposition) (https://tide.nki.nl/; accessed on February 2nd, 2024), which enables the accurate detection of mutation types and frequencies in pooled cell populations (Fig. 1E). Data were also validated using the Synthego CRISPR Analysis Tool (https://www.synthego.com/; accessed on February 2nd, 2024), to confirm the mutation frequencies and provide additional insights into the editing outcomes, ensuring the reliability of the observed indel patterns (Fig. 1E).

Protoplast culture and proto-calli induction

To induce *de novo* organogenesis, three regeneration media (R1, R2, R3) were tested following the disc-culture method (Pavese et al. 2022) (Fig. 1F). **R1**: 0.1 mg/l BAP + 0.1 mg/l 2,4-D; **R2**: 0.1 mg/l BAP + 0.01 mg/l 2,4-D and **R3**: 2 mg/l BAP + 0.02 mg/l NAA.

All media have a MS basal medium with half-strength NH_4NO_3 and KNO_3, supplemented with 90 g/L D-mannitol, 50 g/L glucose, 5 g/L sucrose, and 5 g/L agar. Protoplasts were embedded in semi-solid agar clumps and surrounded by agar-free medium without glucose (Supplementary Material 1). Cultures were incubated at 23 °C in the dark and monitored weekly under a Leica-Wild Heerbrugg M8 stereoscope (Fig. 1F).

Statistical analysis

Data were analyzed by analysis of variance (ANOVA; one-way or three-way, depending on the experiment) using SPSS software for Windows (version 26.0; SPSS Inc., Chicago, IL, USA). Percentage data were arcsine-transformed prior to analysis of variance. Post hoc comparisons were conducted using Tukey's test at a significance level of $p < 0.05$.

Results

Determination of callus biomass and histological analysis

After four weeks of culture, callus growth was evaluated on the three-induction media tested (Ca1, Ca2, and Ca3). The highest callus induction rate, 98% ± 0.4, was observed on Ca1 medium, which was supplemented with 0.2 mg/l BAP and 2.0 mg/l 2,4-D (Fig. 3A). Calli developed under this condition exhibited a yellowish colour with nodular texture. On Ca2 medium (0.5 mg/l kinetin and 2.0 mg/l 2,4-D), the callus induction rate was slightly lower at 97% ± 0.05; calli appeared compact and smaller, with a brownish colour (Fig. 3B). By contrast, Ca3 medium (0.3 mg/l BAP and 0.3 mg/l 2,4-D) produced a lower induction rate of 83% ± 5 with yellowish calli characterized by brownish and compact upper layer (Fig. 3C). Morphological differences among calli were further highlighted by toluidine blue staining, revealing distinct cellular organizations. On Ca1 medium, the calli were composed of small (265.72 ± 47.28 µm² area), globular, and regularly shaped cells (Fig. 3D) while in Ca2 medium, the cells were larger (984.90 ± 91.22 µm²) with a more compact structure (Fig. 3E). Calli grown on Ca3 medium exhibited irregularly shaped cells and a less cohesive organization (1340.48 ± 170.24 µm² area) (Fig. 3F). Additionally, water content analysis revealed that calli grown on Ca1 medium had the lowest water content (91.98% ± 0.5) compared to those on Ca2 and Ca3 media, for which no significant differences were observed (95.85% ± 1.17 and 95.92% ± 1.8, respectively) (Table 1).

Protoplast isolation

Calli grown on Ca1, Ca2, and Ca3 media were used as starting materials for protoplast isolation. To establish a standardized protocol for obtaining high-quality protoplasts, calli were treated with two digestion buffers (BS1 and BS2) in combination with three enzyme solutions (ES1, ES2, and ES3). Three-way ANOVA revealed highly significant effects of callus type ($p < 0.001$) and enzyme solution ($p < 0.001$) on protoplast concentration, whereas the effect of digestion buffer was not significant ($p > 0.05$). Significant interactions were detected for callus type × enzyme solution, digestion buffers × enzyme solution, and the three-way interaction (all $p < 0.001$), confirming that protoplast concentration is strongly dependent on the combination of these factors (Fig. 4A, Supplementary Table 1). Overall, calli grown on Ca1 medium produced the highest protoplast concentration, followed by Ca2 and Ca3. The combination of ES3 (2% Cellulase Onozuka R-10, 2% Cellulase from *Aspergillus niger*, 0.5% Macerozyme R-10, and 1% Pectinase from *A. aculeatus*) with BS1 provided the highest protoplast concentration (1.1×10^6 protoplasts/ml) and 99 ± 1% viability after 17 h of digestion. Similar trends have been reported in other woody species, where the combination of multiple cellulases and pectinase significantly improves protoplast release (Adedeji et al. 2020; Ahmed et al. 2021; Adjei et al. 2023). Ca2-derived calli yielded significantly fewer protoplasts under the same conditions (1.9×10^5 protoplasts/

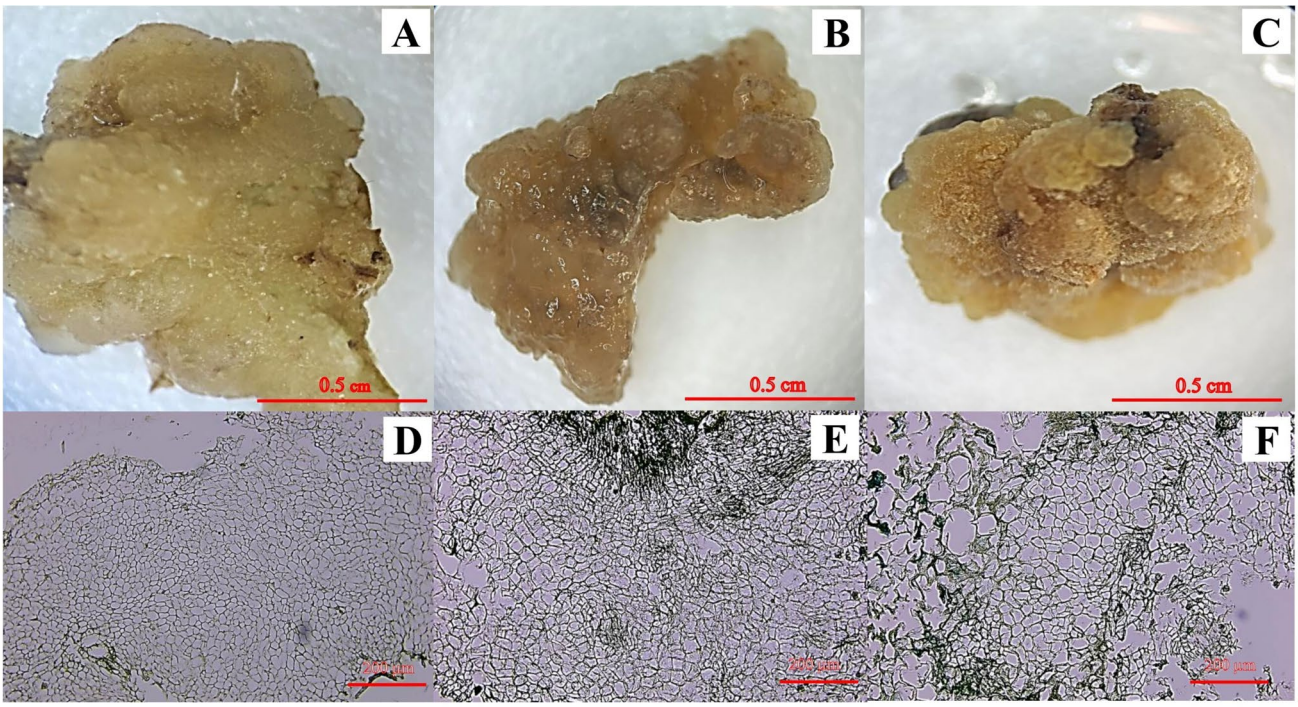

Fig. 3 Plant material used for protoplast extraction. (**A**) Calli on Ca1 medium with 0.2 mg/l BAP and 2.0 mg/l 2,4D. (**B**) Calli on Ca2 medium with 0.5 mg/l kinetin and 2.0 mg/l 2,4D. (**C**) Calli on Ca3 medium with 0.3 mg/l BAP and 0.3 mg/l 2,4D. (**D**) Morphological structure of cells grown on Ca1 medium. (**E**) Morphological structure of cells grown on Ca2 medium. (**F**) Morphological structure of cells grown on Ca3 medium. Magnification 10X

Table 1 Parameters evaluated on calli grown on Ca1, Ca2, and Ca3 media (100 leaf explants per medium). Callus induction rate (%), fresh weight FW (g), dry weight DW (g), and calli water content (%) determined as ratio between (FW-DW/FW) x 100. Cells area (μm^2) and No. Cells/mm^2 were also evaluated. Different letters associated with the means indicate significant differences, as determined by Tukey's HSD test ($p \leq 0.05$)

Callus types	Callus rate (%)	FW (g)	DW (g)	Water content (%)	Cells area (μm^2)	No. Cells/mm^2
Ca1	98 ± 0.04 a	0.095 ± 0.005 a	0.007 ± 0.001 a	91.98 ± 0.5 a	265.72 ± 47.28 a	28.30 ± 1.45 a
Ca2	97 ± 0.05 a	0.201 ± 0.05 a	0.008 ± 0.003 a	95.8 ± 1.2 b	984.90 ± 91.22 b	9.17 ± 4.46 b
Ca3	83 ± 5 b	0.370 ± 0.06 b	0.014 ± 0.004 a	95.9 ± 1.8 b	1340.48 ± 170.24 b	3.70 ± 0.89 b
Sign.	***	**	NS	*	***	**

ml), whereas Ca3-derived calli produced low concentration ($< 4 \times 10^3$ protoplasts/ml), confirming that compact, less friable calli are suboptimal for enzymatic digestion (Terhaag et al. 2023). Although combinations involving BS2 generally resulted in lower protoplast numbers, ES1 + BS2 on Ca1 calli still produced a relatively high concentration (8.1×10^5 protoplasts/ml, 93 ± 1.45% viability), suggesting that callus texture rather than digestion buffers composition was the major determinant of success. This is consistent with reports highlighting the importance of callus friability for efficient cell wall digestion and protoplast release (Carron et al. 1995; Pavese et al. 2022). Figure 4B shows representative Trypan Blue-stained protoplasts isolated from Ca1 and Ca2 calli, highlighting their high viability.

Protoplast purification

The purification step highly influenced protoplast quality and recovery. To ensure comparability, all purification methods (P1-P3) were tested on protoplasts isolated using the best-performing extraction protocol (Ca1 calli with BS1 and ES3), previously identified as optimal for yield and viability (Fig. 5A). In P1 two different nylon sieves were used for filtration followed by two rinses with WS. In P2, protoplasts were purified using the 21% sucrose cushion method, and P3 protoplasts were purified using the W buffer solution as described by Li et al. (2022). The results highlighted that the P2 method allowed to obtain a protoplast solution with lower debris content (Fig. 5B_P2) compared to P1 and P3.

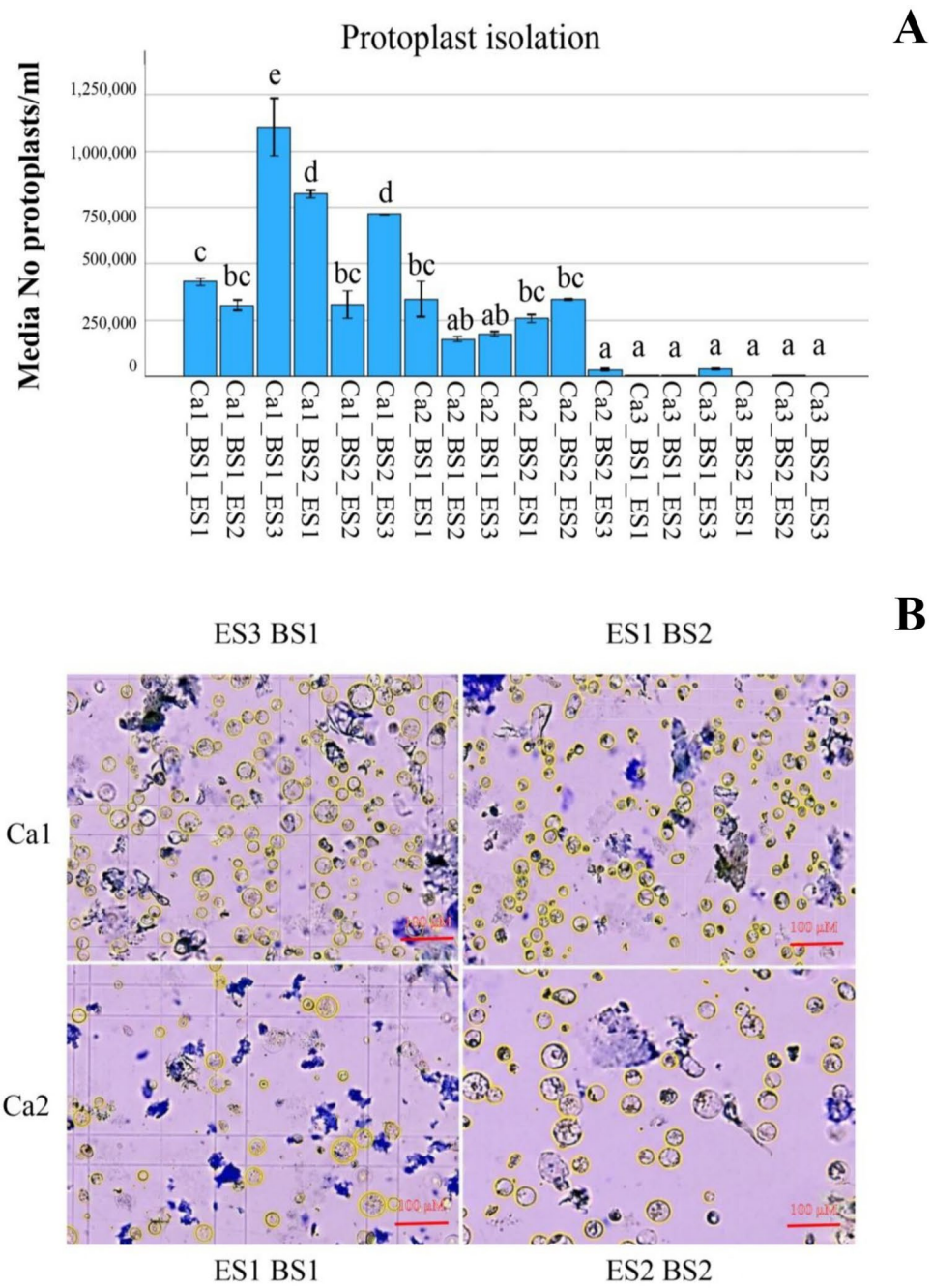

Fig. 4 Protoplast isolation. (A) Protoplast concentration (media No. of protoplasts/ml) obtained from calli grown on Ca1, Ca2, and Ca3 media, using three enzyme solutions (ES1, ES2, ES3) in combination with two digestion buffers (BS1, BS2). Values are expressed as the mean ± SE of three independent replicates. Different letters indicate statistically significant differences according to three-way ANOVA followed by Tukey's HSD test ($p < 0.05$). B) Trypan blue viability assay of protoplasts isolated from Ca1 and Ca2-grown calli. Representative viable protoplasts are highlighted in yellow. Magnification: 10X

However, this method resulted in a lower protoplast concentration (Fig. 5A_P2: 634,000 protoplasts/ml) compared to the other two methods. The nylon sieves filtration with WS rinses yielded the highest protoplast concentration (Fig. 5A_P1: 1,008,800 protoplasts/ml), followed closely by the Li et al. (2022) method using W buffer (Fig. 5A_P3: 958,333 protoplasts/ml).

Protoplast transfection using the GFP plasmid

Calli-derived protoplasts with the highest quality and concentration (isolated from Ca1 calli using BS1 and ES3 solutions) were transfected with 10 µg of pAVA393 plasmid containing the *Green Fluorescent Protein* (GFP) marker gene as in Pavese et al. (2022). No GFP expression was detected in the control samples, confirming the specificity of the transfection procedure (Fig. 6A, B). GFP expression was observed at 12 h (Fig. 6C), 24 h (Fig. 6D), and 48 h

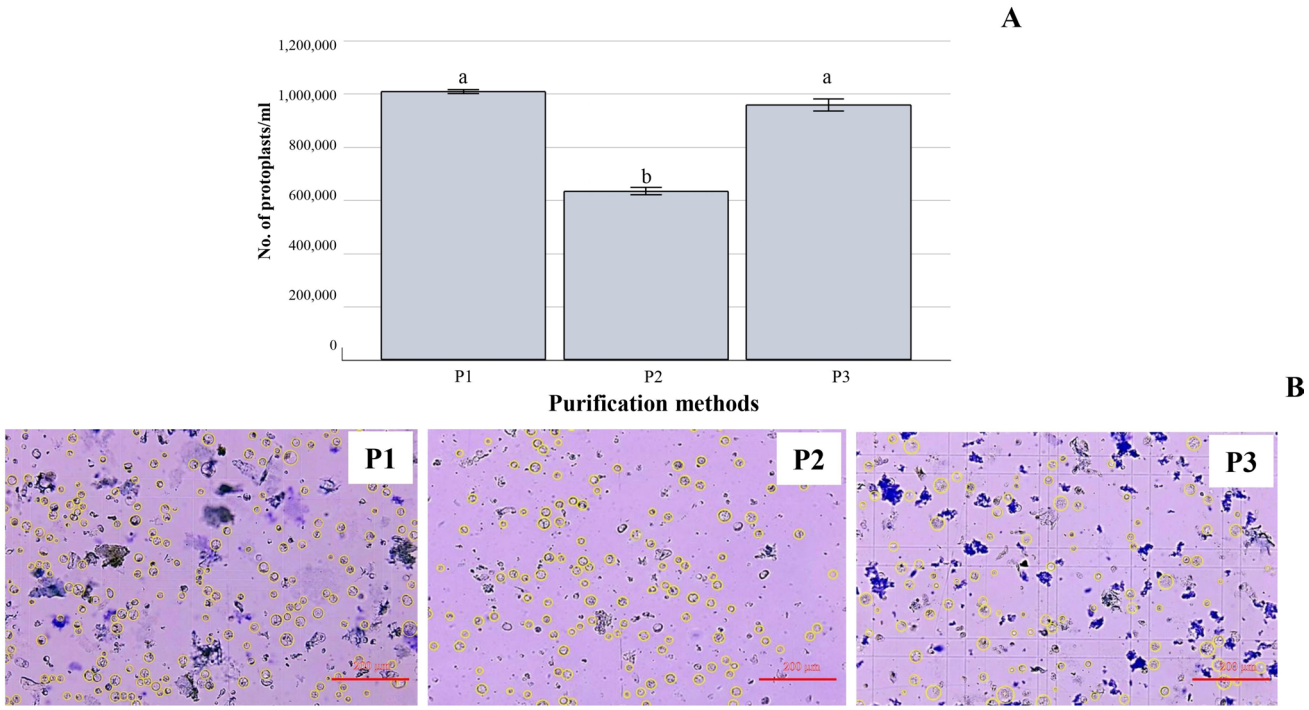

Fig. 5 Protoplast purification methods. (A) The protoplast concentration (No. of protoplasts/ml) achieved after the purification steps. Purified protoplasts were obtained using three different methods: P1) two nylon sieves filtration and WS rinses, P2) 21% sucrose gradient, P3) Li et al. (2022) purification method using W buffer. (B) Protoplast purified with P1, P2 and P3 methods. Representative viable protoplasts are highlighted in yellow. Magnification 10X

(Fig. 6E) post-transfection. The GFP signal was clear and 72% of the protoplasts showing the GFP expression was detected after 24 h, with the signal decreasing after 48 h from the transfection event.

Protoplast transfection via RNPs

Molecular screening revealed different levels of editing efficiency depending on the gRNA used, 14.2% and 5.7%, respectively, for *pds*_gRNA1 (S1) and *pds*_gRNA2 (S2) (Table 2). No mutations were detected in the control sample, in which protoplasts were transfected with sgRNA only, without the Cas9 enzyme. The predominant mutations detected for both sgRNAs consisted of single-nucleotide insertions, occurring at frequencies of 6.8% for sgRNA1 and 4.6% for sgRNA2. In addition, the sample transfected with *pds*_gRNA1 also exhibited deletions of two (4.5%) and five nucleotides (2%). Sequence chromatogram alignments of wild-type (WT) and edited samples (S1, S2) generated using *pds*_gRNA1 and *pds*_gRNA2 (target sites highlighted in red) show that, following the PAM sequence (highlighted in blue), Sanger sequencing traces of PCR-amplified target regions display the mutations introduced in the edited samples (Fig. 7A, B).

Protoplast culture and micro-calli induction

The pAVA393:GFP protoplasts (Pavese et al. 2022) were cultured in three regeneration media: R1, R2, and R3. Media R1 and R3 proved unsuitable for protoplast divisions and callus growth. In contrast, the R2 medium, supplemented with 0.1 mg/l BAP and 0.01 mg/l 2,4-D, was the only medium capable of supporting protoplast regeneration. Protoplasts were introduced into culture (Fig. 8A), with the first division occurring after 5 days in R2 medium (Fig. 8B, C). Microcolonies were visible after 15 days (Fig. 8D), and microcalli were observed after 60 days in culture (Fig. 8E).

Discussion

Biotechnological techniques, like genetic transformation and genome editing, provide innovative approaches to accelerate crop improvement particularly in woody species where long juvenility and complex reproductive biology hamper conventional breeding. Among these tools, CRISPR/Cas9 has emerged as a highly effective technology for targeted mutagenesis in many crops (Ali et al. 2023). Delivering CRISPR/Cas9 as RNPs avoids stable transgene integration and supportstransient nuclease activity (Pavese et al. 2022). However, practical application of RNP editing

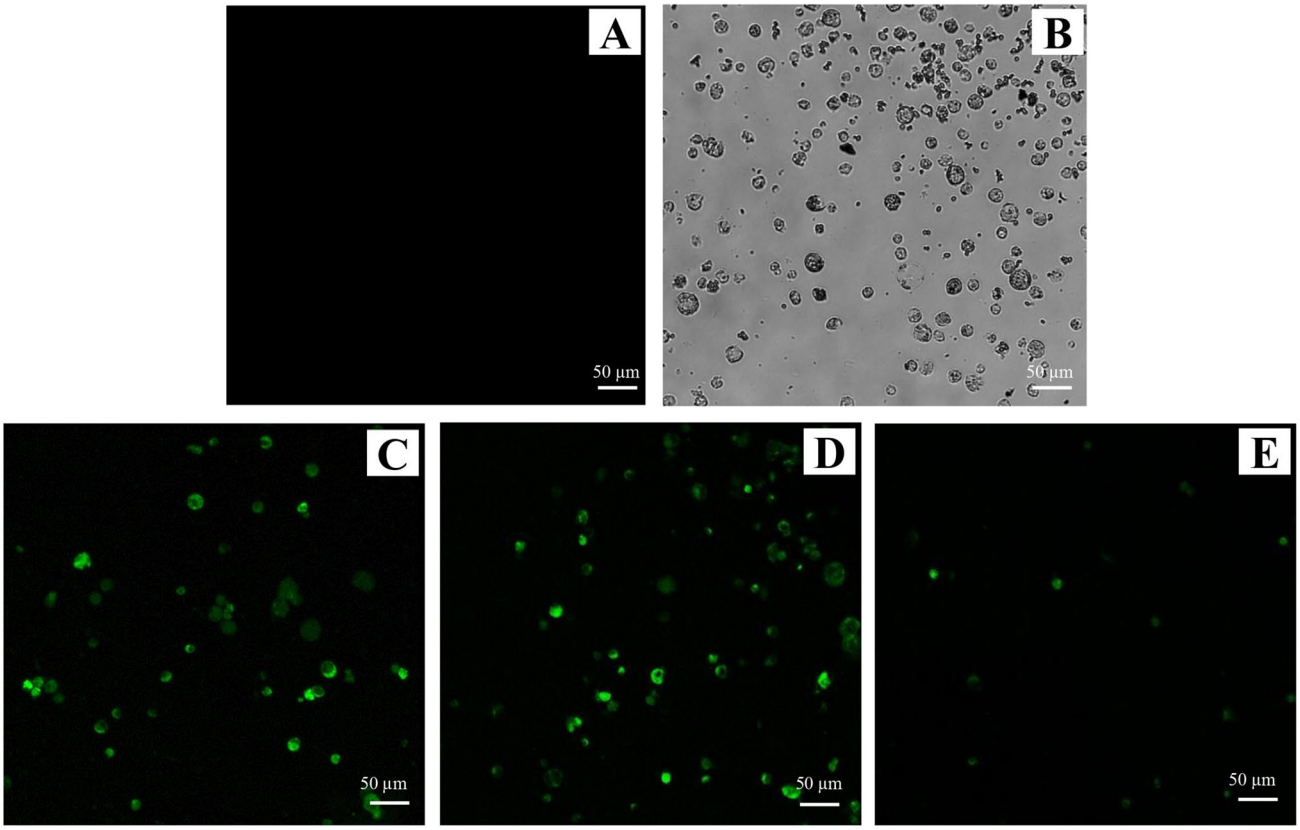

Fig. 6 GFP expression in calli-derived protoplasts was analyzed following transfection with the pAVA393 plasmid. (**A**) Negative control showing no GFP expression. (**B**) Brightfield image of isolated protoplasts. (**C**) GFP fluorescence detected 12 h post-transfection. (**D**) Maximum GFP fluorescence observed 24 h post-transfection. (**E**) Decline in GFP fluorescence at 48 h post-transfection. Scale bar: 50 µm

Table 2 Analysis of mutations in S1 and S2 protoplasts transfected with two distinct gRNAs as RNPs, focusing on editing efficiency (%), goodness-of-fit (R^2), and mutation types.

Samples	Efficiency (%)	R^2	Mutation
S1-*pds*_gRNA1	14.2	0.98	-5;-2;+1
S2-*pds*_gRNA2	5.7	0.98	+1

in recalcitrant woody species depends on three technically linked steps: protoplast isolation, RNP delivery, and regeneration (Reed and Bargmann 2021).Our results provide an integrated workflow in *Corylus avellana* cv. 'TGT' that benchmarks each of these steps and clarifies where the current bottleneck remains.

Leaf-derived callus is a suitable starting material for hazelnut protoplast workflows

The choice of starting material significantly impacts on the quality and quantity of protoplasts obtained, as different tissues or calli exhibit distinct morphological and physiological features that influence their suitability for isolation and regeneration. In many herbaceous and horticultural crops, in vitro leaves are often preferred for protoplast isolation because they can yield large numbers of cells with good viability (Liu et al. 2022; Moon et al. 2021; Yue et al. 2021). In woody species, however, leaves tissues are often unsuccessful due to the high content of cellulose, lignin, and phenolics, that reduce enzyme efficiency and compromise cell integrity (Kuzminsky et al. 2016; Bottone et al. 2019). In this context, embryogenic or leaf-derived calli are commonly usedbecause they are more friable andeasier to disaggregate, improving isolation outcomes (Pavese et al. 2022). For hazelnut specifically, highly recalcitrant to in vitro morphogenesis, the acquisition of embryogenic material has been reported mainly from seed-derived tissue (Ferrucci et

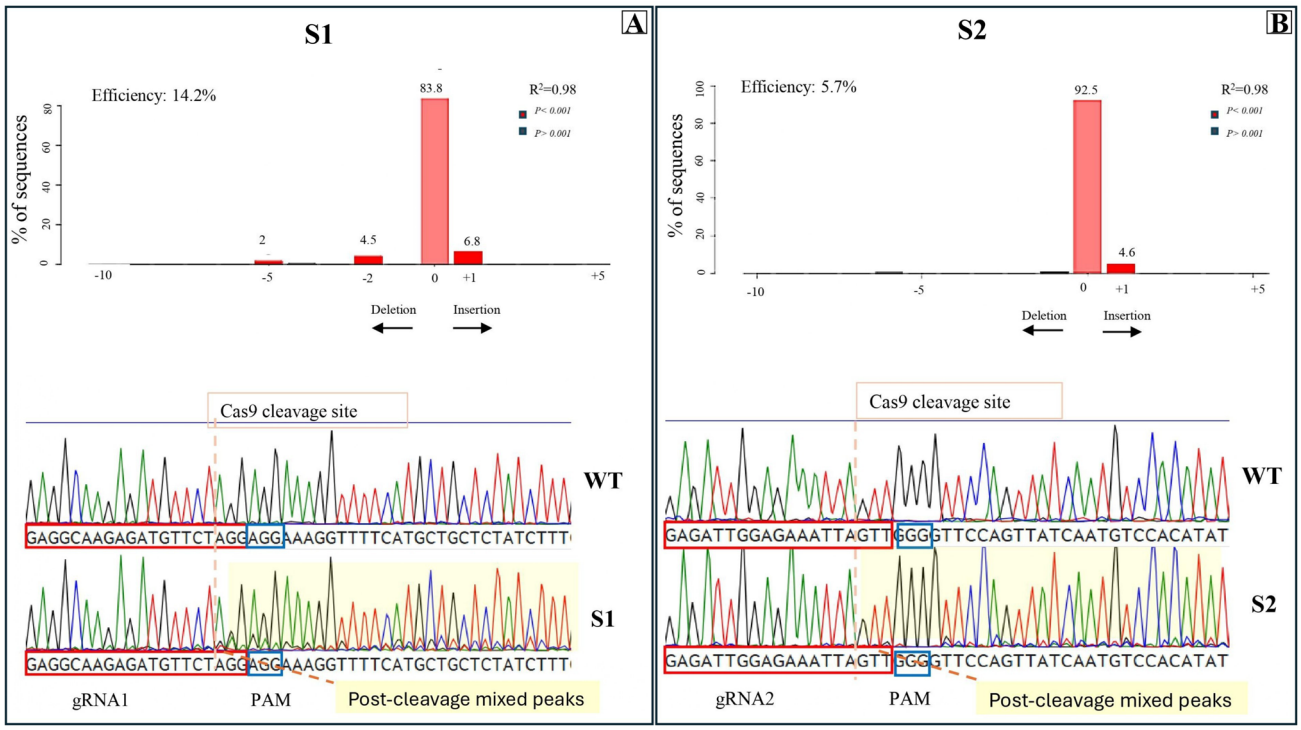

Fig. 7 TIDE mutation profiling and Sanger validation of CRISPR/Cas9 RNP editing at the *pds* target site in hazelnut protoplast pools. (**A**) Sample transfected with gRNA1 (S1). (**B**) Sample transfected with gRNA2 (S2). For each sample, the upper plot reports editing efficiency, indel spectrum, and R^2 from TIDE analysis. Below, WT and edited chromatograms are aligned across the target region (gRNA target sequence boxed in red; PAM boxed in blue), with the Cas9 cleavage site indicated. The shaded region highlights post-cleavage mixed peaks consistent with a mixture of indel alleles in pooled DNA samples

al. 2023). However, seed-derived tissues are unsuitable for clonal propagation of elite genotypes because seedsare the product of cross-pollination and are not genetically true-to-type with the original cultivar. Therefore, selecting somatic calli derived from leaves represents a practical compromise: it preserves cultivar identity while enabling a starting material that is compatible with high-yield protoplast isolation (Adedeji et al. 2020). Our result confirmed that a high auxin/cytokinin ratio is essential for callus induction (Su et al. 2011; Nosov and Fomenkov 2023), with 2,4-D being particularly effective in hazelnut morphogenesis (Silvestri et al. 2016). Indeed, 2,4-D combined with BAP produced the highest callus induction rate as reported in *Ficus religiosa* (Hesami et al. 2018) and *Pyrus armeniacaefolia* (Wang et al. 2021a). The auxin-to-cytokinin ratio strongly influenced callus quality and, consequently, its suitability as a starting material for downstream protoplast workflows, resulting in a yellowish and friable callus that is generally considered more amenable to enzymatic digestion and cell release, as reported in kefir lime (Damayanti et al. 2020). Toluidine blue staining revealed that calli obtained from Ca1 medium are characterized by small, isodiametric cells with dense cytoplasm, like those observed in pineapple (Kessel-Domini et al. 2022). These traits are consistent with cellular features typically associated with higher morphogenic competence since embryogenic cultures, the best starting material for protoplast isolation, typically show isodiametric cells with thin walls, small vacuoles, and dense cytoplasm (Sangwan et al. 1992; Ribas et al. 2011) and easily disaggregate under enzymatic treatment (Carron et al. 1995). Although embryogenic tissues can achieve very high yields in some woody systems (Pavese et al. 2022; Malnoy et al. 2016; Bertini et al. 2019), callus derived from in vitro leaves represents a realistic and cultivar-faithful route for hazelnut, where clonal material is required for genome editing pipelines. In contrast, calli formed under lower concentrations of 2,4-D combined with BAP and kinetin, as studied in *Citrus* species (Mumtaz et al. 2015), are generally characterized by compact texture. Histological analysis of compact calli in *Araucaria angustifolia* using Toluidine blue staining revealed larger, irregularly shaped isodiametric cells, a feature also observed in hazelnut callus grown on Ca3 medium (Terhaag et al. 2023). Overall, these observations support the broader implication that callus induction conditions are not only important for biomass production, but can be used as a suitable strategy to obtain competent, friable tissues that facilitate reproducible protoplast isolation in recalcitrant hazelnuts.

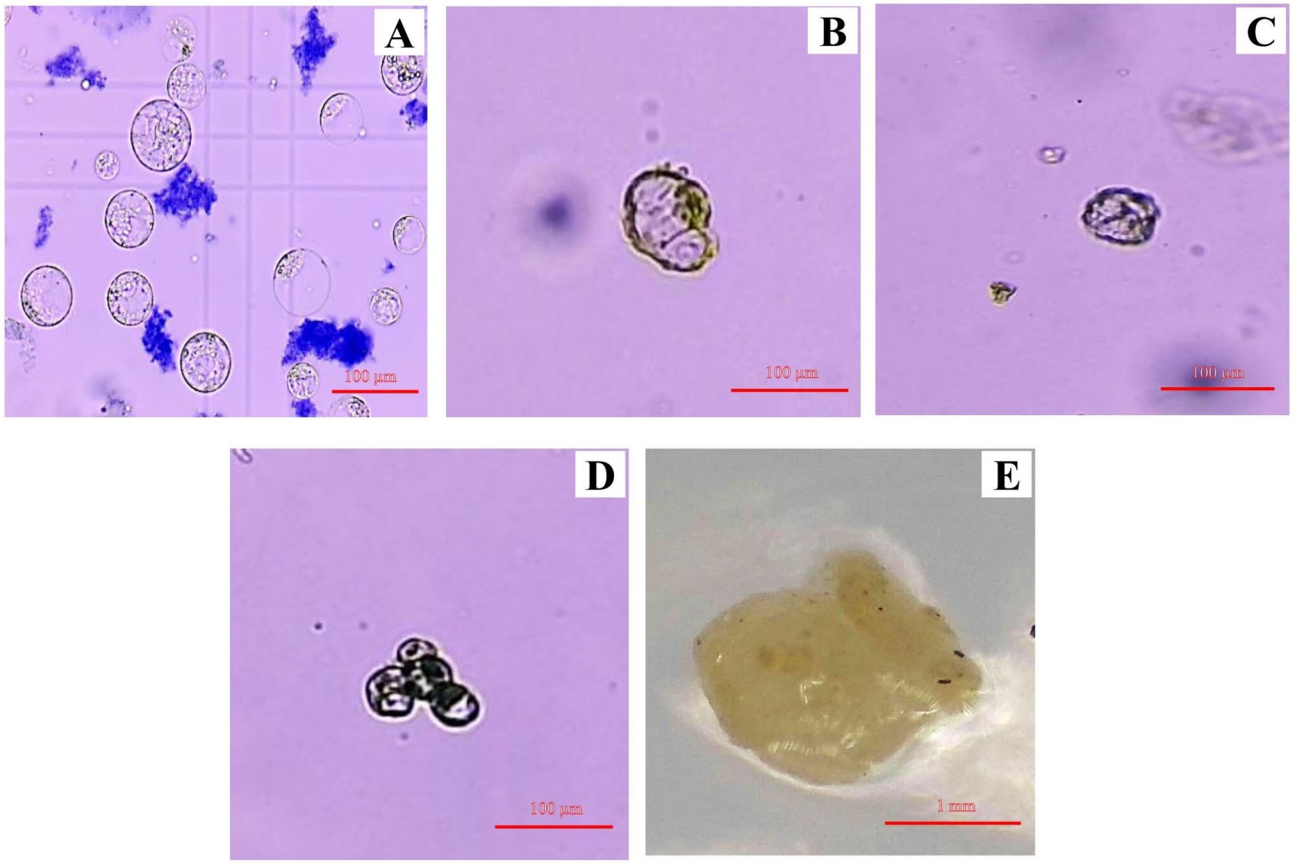

Fig. 8 Protoplast culture and micro-calli induction. (**A**) protoplast in culture, (**B**, **C**) protoplast divisions, (**D**) microcolony formation, (**E**) micro-calli development

Isolation and purification: practical choices that affect protoplast quality

Protoplast isolation and regeneration protocols for woody plants, including hazelnuts, remain still challenging (Nerva et al. 2023). Protoplast yield and quality depend on starting material, enzyme conditions and purification steps (Cimen 2020; Xu et al. 2021).

In this study, enzymatic solution optimizationresulted in a decisivestepto obtain both high recovery and good viability. The synergistic action of cellulases on the cellulose and hemicellulose network, together with macerozyme and pectinase targeting the pectin matrix, enabled efficient cell-wall degradation and protoplast release. Increasing Cellulase Onozuka R-10 from 1% to 2% improved viable concentration, consistent with findings in *Chrysanthemum* (Adedeji et al. 2020) and mango (Adjei et al. 2023). Similar evidence comes from *Jasminium* spp., where high yields were obtained from callus using Cellulases Onozuka R-10 (1.5%), Macerozyme R-10 (0.4%), and Pectinase (0.8%), highlighting again the importance of enzyme synergy (Ahmed et al. 2021). From a practical perspective, this means that small changes in cellulase level and enzyme balance can make a major difference in recalcitrant woody systems.

The digestion buffer solution also plays an important role because it supports enzyme activity and helps maintain osmotic balance (Reed and Bargmann 2021). In hazelnut, BS1 yielded the highest numbers of viable protoplasts, suggesting that its ionic strength and osmotic potential helped to prevent stress during digestion. This is consistent with evidence that the addition of KCl and $CaCl_2$ can stabilize plasma membranes and improve viability (Yang et al. 2024), as highlighted also in *Quercus ilex* where a MES-mannitol buffer supplemented with KCl and $CaCl_2$ increased recovery from embryogenic calli (Pavese et al. 2024). Overall, these comparisons suggest that buffer composition is not a secondary detail in woody protoplast work, but a factor that directly affects viability (Reed and Bargmann 2021; Yang et al. 2024; Pavese et al. 2024).

Purification protocols also affect protoplast culture performance. Among the three methods tested in hazelnut, P1 provided the highest recovery using a simple workflow, as reported in apple and grapevine (Osakabe et al. 2018) and chestnut (Pavese et al. 2022), but it retained more debris, which may reduce transfection performance and culture

progression. The P3 method proposed by Li et al. (2022) in *Camellia oleifera* achieved similarly high recovery with slightly improved purity. By contrast, the sucrose-cushion interface (P2) reduced recovery, likely due to higher osmotic stress (Poddar et al. 2020) but effectively removed debris and non-viable cells, as shown in other woody species such as mango (Rezazadeh et al. 2011) and sweet cherry (Ochatt 1991). Since cell-wall debris and dead cells can inhibit division, purification should be chosen based on the next step: higher recovery can be useful for rapid delivery or editing screening, while less debris protoplast may better support culture progression and regeneration attempts (Stajic 2023).

GFP transfection provides a quick benchmark to optimize delivery conditions

To evaluate transfection efficiency, a GFP reporter system was employed, as previously described for woody species such as chestnut (Pavese et al. 2022), quercus (Pavese et al. 2024), citrus (Yang et al. 2023), grapevine (Najafi et al. 2023), mango (Adjei et al. 2023), and passion fruit (Wang et al. 2023). Several factors affect transfection efficiency, including the method used, such as PEG-mediated transfection, electroporation (Niedz et al. 2003), or particle bombardment (Kim et al. 2021). In this study we adopted PEG-mediated transfection, whose efficacy depends on PEG concentration (Wu et al. 2020; Lin et al. 2023). Because ≥50% PEG can reduce viability (Wang et al. 2021b), we used 40% PEG, in line with protocols reporting optimal performance (Yao et al. 2016; Wang et al. 2022; Pavese et al. 2022; Lin et al. 2023). Our GFP-positive rates were higher than those reported for chestnut (Pavese et al. 2022), rice, and wheat (Zong et al. 2017), and comparable to those observed in mango (Adjei et al. 2023) and passion fruit (Wang et al. 2023). This is important because GFP-based transient expression enables rapid optimization of transfection conditions before CRISPR testing. It provides a practical benchmark for delivery efficiency that is not dependent on later regeneration outcomes.

DNA-free RNP editing is feasible in hazelnut, but regeneration remains the main bottleneck

Advances in *New Plant Breeding Techniques* (NPBTs), particularly CRISPR/Cas9 technology, offer a strong opportunity to speed up improvement in woody crops, where classical breeding is slow and labor-intensive (Son and Park 2022; Min et al. 2022). Here, we developed a transgene-free CRISPR workflow in hazelnut protoplasts using RNP delivery by targeting the *pds* gene, a widely used gene marker for proof-of-concept editing (Pavese et al. 2024). Editing was confirmed using both TIDE (Tracking of Indels by Decomposition) and Sinthego CRISPR analysis tools providing a practical quantitative benchmark for this system. The highest editing efficiency was 14.2%, within the range reported for other woody species, including chestnut and citrus (Pavese et al. 2022; Nerva et al. 2023; Su et al. 2023), and higher than values reported in some apple, grapevine, and oak protoplast workflows (Malnoy et al. 2016; Pavese et al. 2024). As expected, editing outcomes depend on multiple factors, including gRNA choice, PEG concentration, Cas9:gRNA ratio, incubation time, and protoplast viability (Sandhya et al. 2020; Shao et al. 2023). From an applied perspective, these results support that delivery and DNA-free editing are achievable in hazelnut protoplasts and can be used for rapid screening of RNPs activity before investing effort in regeneration.

At the same time, regeneration remains the major challenge in woody species. We tested three culture mediaand observed that balanced BAP and 2,4-D levels supported cell division and microcolony formation, while auxin-cytokinin imbalance reduced culture progression, consistent with observations in *Quercus acutissima* and other plants (Wakita et al. 1992; Kang et al. 2023; Sija et al. 2016; Sarkar et al. 2018; Osakabe et al. 2018; Mukami et al. 2022). Although whole-plant regeneration was not achieved, this study represents the first successful application of NPBTs in hazelnut and a promising step toward developing an efficient regeneration system, as it delivers a robust, transgene-free CRISPR platform and identifies hormone combinations that sustain protoplast division and microcolony formation. Importantly, reaching microcolonies and microcalli is a meaningful milestone, since it shows that isolation, delivery, and early culture conditions can produce viable, dividing cells, and it points to the post-division stage as the main limitation. Therefore, future work should focus on improving the transition from microcalli to organized tissues, aiming at organogenesis or somatic embryogenesis, while using this protoplast platform for rapid screening of gRNAs and delivery parameters before starting regeneration trials. Together, these advances provide a solid experimental and conceptual framework for future efforts aimed at improving regeneration and implementing functional genomics and precision breeding in hazelnut and other recalcitrant woody species.

Conclusion

In this study, we report the first efficient protocol for protoplast isolation in *Corylus avellana* cv. 'TGT', a cultivar highly valued in Italy and Europe for nut quality in the confectionery industry. We also established transfection protocols using both a GFP reporter and CRISPR/Cas9 RNPs targeting *pds*. To our knowledge, no gene-editing

protocols were previously available for hazelnut, making these results a substantial step forward. By standardizing isolation and transfection, we provide the basis for improving regeneration with the goal of generating transgene-free, genome-edited hazelnut plants, with regeneration remaining the major step to be optimized. Future work could explore protoplast transfection with lipofection, which may better preserve protoplast viability and organogenetic capacity, potentially enhancing overall efficiency (Gambino et al. 2024). Among the potential applications of gene editing in hazelnut, targets include modification of the self-incompatibility system (controlled at a single locus) and enhanced pathogens resistance such as powdery mildew (*Erysiphe corylacearum*) by editing susceptibility genes involved in host-pathogen interactions. These advances could accelerate the development of improved hazelnut varieties.

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Data availability This article does not contain any studies with human or animal subjects.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval This article does not contain any studies with human or animal subjects.

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