



# Towards a genetic model organism: an efficient method for stable genetic transformation of *Eschscholzia californica* (Ranunculales)

Dominik Lotz<sup>1</sup> · Jafargholi Imani<sup>2</sup> · Katrin Ehlers<sup>1</sup> · Annette Becker<sup>1</sup>

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## Abstract

California poppy (*Eschscholzia californica*) is a member of the Ranunculales, the sister order to all other eudicots and as such in a phylogenetically highly informative position. Ranunculales are known for their diverse floral morphologies and biosynthesis of many pharmaceutically relevant alkaloids. *E. californica* is widely used as model system to study the conservation of flower developmental control genes. However, within the Ranunculales, options for stable genetic manipulations are rare and genetic model systems are thus difficult to establish. Here, we present a method for the efficient and stable genetic transformation via *Agrobacterium tumefaciens*-mediated transformation, somatic embryo induction, and regeneration of *E. californica*. Further, we provide a rapid method for protoplast isolation and transformation. This allows the study of gene functions in a single-cell and full plant context to enable gene function analysis and modification of alkaloid biosynthesis pathways by e.g., genome editing techniques providing important resources for the genetic model organism *E. californica*.

## Key Message

This work describes an efficient and reproducible in vitro method for stable genetic transformation and regeneration of California poppy (*Eschscholzia californica*), member of the sister lineage of eudicots.

**Keywords** *Eschscholzia californica* · Ranunculales · Papaveraceae · Transformation · Protoplasts

## Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
35S	35 Svedberg units DNA fragment of the cauliflower mosaic virus promoter region
BAP	6-Benzylaminopurine
BIA	Benzylisoquinoline alkaloids
GFP	Green fluorescent protein
NAA	1-Naphthaleneacetic acid
pUBQ10	Promoter of the UBIQUITIN 10 gene of <i>Arabidopsis thaliana</i>
VIGS	Virus-induced-gene-silencing

## Introduction

The order Ranunculales holds a key position in the phylogenetic tree of angiosperms, because it is the sister lineage to all extant eudicots (APG IV 2016). Ranunculales emerged approx. 130 Million years ago (Magallón et al. 2015) and are characterized by a large diversity in life-history traits, growth habit, leaf shape, flower and fruit forms, etc. and the flowers of Ranunculales in particular are extremely diverse (Damerval and Becker 2017). Besides their stunning floral morphology, they are also extremely diverse in the production of economically valuable benzylisoquinoline alkaloids (BIAs). Around 2500 BIAs have been identified, with the major BIAs being morphine (analgesic), noscapine (anti-cancer drug), papaverine (spasmolytic), and sanguinarine (antibacterial), and they are predominantly found in Ranunculales (Dastmalchi et al. 2018).

*E. californica* was developed as a model organism for the analysis of BIA metabolism and its regulation and to study the evolution and functional conservation of key regulators of flower development. Several flower developmental regulators were analyzed already by Virus-Induced Gene Silencing

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✉ Annette Becker  
annette.becker@bot1.bio.uni-giessen.de

<sup>1</sup> Plant Development Group, Institute of Botany, Justus-Liebig-University, Heinrich-Buff-Ring 38, 35392 Giessen, Germany

<sup>2</sup> Institute of Phytopathology, Justus-Liebig-University, Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany

(Wege et al. 2007) providing a comprehensive view on the regulation of floral morphogenesis in *E. californica* and the conservation of the flower developmental program in dicots (Orashakova et al. 2009; Lange et al. 2013; Wreath et al. 2013; Zhao et al. 2018; Zhou et al. 2018). Although, VIGS was adopted in many Ranunculales (Damerval and Becker 2017), it is a transient method and produces sectors with differing gene expression, that is often not heritable (Dommes et al. 2019). Therefore a protocol to generate stable transgenic lines of *E. californica* is required for many researchers working in this field.

*E. californica* has provoked additional scientific interest because it is a highly invasive species. Native to mainly California, it has extended its range in regions of South America, Europe, and Australia with a Mediterranean climate (Stebbins 1965; Peña-Gómez et al. 2014; Bustamante et al. 2017). Previous studies have shown that the fecundity of invasive populations of *E. californica* is higher than that of native populations and the seeds can stay viable in seed banks for years (Peña-Gómez et al. 2014; Hickman 1993). These invasive populations are locally adapted and show a superior colonization ability (Leger and Rice 2007).

Within the Ranunculales, a high-quality genome is available for opium poppy (*Papaver somniferum*) (Guo et al. 2018). In addition, draft genomes are available for several Ranunculales members such as *Eschscholzia californica* and *Macleaya cordata* of the Papaveraceae family and *Aquilegia coerulea* of the Ranunculaceae family (Liu et al. 2017; Hori et al. 2018; Filiault et al. 2018). These provided the base for the reconstruction of the evolutionary history of enzymes involved in BIA biosynthesis (Li et al. 2020). *E. californica*'s major BIAs are (S)-reticuline, berberine, and sanguinarine, however, it lacks the enzymes necessary to synthesize noscapine and morphine (Li et al. 2020; Balažová et al. 2020). Several regulators of the BIA biosynthesis (Yamada et al. 2017) or parts of the biosynthesis pathway of isoquinoline alkaloids area have been described, but insights in BIA and isoquinoline alkaloid biosynthesis and their regulation were mostly obtained by analyses utilizing suspension cultures (Yamada et al. 2017; Balažová et al. 2020) and lack information on regulation of alkaloid biosynthesis obtained from fully grown transgenic plants of *E. californica*. A detailed protocol for suspension cultures of *E. californica* was established previously (Park und Facchini 2000a), where culture conditions were optimized by investigating the effect of different phytohormones and component. Park und Facchini (2000a, b, c) has reported a protocol to generate stable transgenics in this plant.

In Ranunculales species, reliable protocols for stable genetic transformation and subsequent regeneration of mature plants do not exist, with the exception of *Papaver somniferum* (Yoshimatsu und Shimomura 1992; Chitty et al. 2003; Park und Facchini 2000a). Here, we present a simple

and efficient protocol for the isolation and transformation of *E. californica* protoplasts and the optimization of methods for plant regeneration, transformation, and generation of stable transgenic *E. californica* plants with better efficiency than the protocol of Park and Facchini (2000b). This provides an important step towards gene function analysis in *E. californica*, allowing the experiments especially in the field of evo-devo and BIA metabolism and its regulation.

## Materials and methods

### Plant culture, protoplast isolation and transformation

All methods described here are documented in a detailed step-by-step protocol on protoplast isolation, stable genetic transformation and in vitro regeneration, of *E. californica* and includes a trouble shooting guide (Online Resource 3). Our step by step protocol provides the reader with a detailed protocol on the sterile cultivation of *E. californica*. Furthermore, we provide the materials, the compositions of media and enzyme solutions.

To test vectors used for stable genetic transformation, a protoplast system can be very useful because it provides preliminary data fast. For example, different CRISPR-Cas9 constructs can be tested in the protoplast system for their genome editing efficiency. Firstly, protoplasts were isolated from cotyledons and hypocotyl of seedlings grown in sterile conditions. *E. californica* seeds (cv. Aurantiaca Orange King, B&T World Seeds, Aigues-Vive, France) were surface sterilised by using 2.8% sodium hypochlorite solution supplemented with a drop of Tween-20 or Triton-X for 5 min and subsequently rinsed in sterile water (see Supplemental Protocol for a detailed step-to-step protocol) and sown on solid full strength Gamborg B5 medium (Gamborg et al. 1968) including vitamins (B5, for a detailed composition of all media, see Supplemental Material 3) supplemented with 1% sucrose and 1% Gelrite (Duchefa Biochemie, Haarlem, The Netherlands) and maintained in controlled and sterile conditions for the entire procedure. The seeds were germinated at 25 °C under long day conditions (16 h of light, light intensity  $\sim 95 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). The cotyledons and hypocotyls of 10-days-old seedling were used for protoplast isolation based on a procedure described by Shan et al. (2014) with the following modifications: about 2 g of tissue was sliced into thin strips and incubated in 0.6 M mannitol solution for plasmolysis at RT for 10 min and transferred to 50 ml enzyme solution, and vacuum infiltrated (350 mmHg) for 30 min in the dark respectively. After 4 h of incubations in the dark with 70 rpm shaking at RT, the mixture was filtered through a 100  $\mu\text{m}$  nylon mesh and pelleted by centrifugation at 300 g for 3 min, and resuspended in 10 ml of W5 solution

including 2 mM MES (pH 5.7) containing 154 mM NaCl, 125 mM CaCl<sub>2</sub> and 5 mM KCl. Then protoplasts were pelleted at 300 g for 3 min and resuspended in 4 ml of mannitol MgCl<sub>2</sub> (MMG, 0.4 M mannitol, 15 mM MgCl<sub>2</sub>, pH 5.7) solution. Protoplast density was counted by hemocytometry.

For protoplast transformation, the working density of the protoplasts was adjusted to  $5 \times 10^5$  protoplasts per ml with MMG solution. PEG-mediated transformation with the vector pGGZ003 pro35S:Omega:GFP-NLS:Dummy:TerRBCS;pMAS:BASTA:tMAS carrying 35S:GFP was performed as described in Liang et al. (2018) with the following modifications: After the protoplast-PEG-vector mixture was incubated for 20 min in the dark, the protoplasts were centrifuged at 300×g for 3 min. Following transformation, the protoplasts were incubated for about 64 h at 23 °C in the dark. Fluorescence was examined with a Leica DM5500B microscope, a Leica DFC450 camera and the Leica L5 FL Cold Light Fluorescence System Leica Filter Cube: L5. Photos were arranged by the Inkscape 1.0 software (Inkscape Developers, 2020).

### Callus regeneration and stable genetic transformation

Plant regeneration and transformation was optimized by analyzing several variables in the previously published protocol (Park and Facchini 2000a, b, c), for example hormone type and concentration, carbohydrate composition, or the use of activated charcoal based on literature. The optimized protocol commences with plant germination, where surface sterilised *E. californica* seeds were used. Hypocotyls of 10 days old plants were used to induce callus growth on solid callus induction medium containing full strength MS + vitamins (Supplemental Material 3), 6% sucrose, 1.6% Phytoagar (Duchefa Biochemie, Haarlem, Netherlands), 1 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D), 5 replicates were analysed and consisted of three seedlings each, of which the hypocotyl was sliced into 1–2 cm parts. The calli formed after 4–6 weeks were then transferred to somatic embryo induction medium including full strength B5 medium, 6% sucrose, 1.6% Phytoagar, 1 mg/l 1-Naphthaleneacetic acid (NAA), 0.5 mg/l 6-Benzylaminopurine (BAP), to generate somatic embryos after six to eight weeks. Somatic embryos were transferred to maturation medium including full strength B5 medium, 6% maltose, 1.6% Phytoagar, for 21 days. Subsequently, the somatic embryos were transferred to plant regeneration medium including full strength B5 medium, 6% sucrose and 1% Gelrite. Regenerated plantlets were transferred to soil and grown under long day conditions (16 h of light, light intensity  $\sim 95 \mu\text{mol m}^{-2} \text{s}^{-1}$  at 22 °C).

During the optimization process of the plant regeneration protocol, different concentrations of media components

were compared to find the optimal levels. We analyzed the influence of different auxins of different concentrations: 2,4-D with a concentration of 0.5 mg/l up to 3 mg/l were compared with 2 mg/l NAA in combination with 0.1 mg/l BAP. The capacity of cotyledons, hypocotyl, unripe seeds, somatic embryos and root tissues for suitability as explant were analyzed by incubation for four weeks on callus induction medium. To get rid of the huge number of secondary metabolites within *E. californica* plants, different combinations of activated charcoal was tested. For maturation of somatic embryos formed, they were transferred to medium supplemented with 3 g/l Maltose + 2.5 mg/l ABA + 4 g/l PEG.

Susceptibility to antibiotics was analysed with a kill curve: *E. californica* calli were transferred to selection medium supplemented with various concentrations of Hygromycin B (5 mg/l, 10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l) for four weeks and visually analysed. For removal of remaining *Agrobacteria* and other potential infectious agents, callus induction medium and somatic embryo induction medium included 200 mg/l Cefotaxime.

To optimize the efficiency of plant transformation, different tissues were analysed with *Agrobacterium tumefaciens* mediated transformation, with callus tissue being most susceptible. Callus tissues was immersed in *A. tumefaciens* strain GV3101 (carrying a p35S:GFP cassette included in the vector pGGZ003\_p35S:GFP\_HygR) suspension (with an OD<sub>600</sub> of 1.0) for 20 min with mild shaking. Then, callus tissue was transferred to callus induction medium for co-cultivation with *A. tumefaciens* for two days in the dark, and then moved to callus induction medium with 200 mg/l Cefotaxime added. Subsequently after 4 weeks, the calli were transferred to callus induction medium supplemented with 200 mg/l Cefotaxime and 40 mg/l Hygromycin and grown for four weeks on selective medium. Resistant calli were transferred to somatic embryo induction medium, and treated similarly as described above for non-transformed plants. Somatic embryo induction medium was supplemented with 200 mg/l Cefotaxime and 40 mg/l Hygromycin.

### Vector cloning

The GFP reporter vectors pGGZ003pro35S:GFP:HygR and pGGZ003 pro35S:Omega:GFP-NLS:Dummy:TerRBCS;pMAS:BASTA:tMAS were constructed using the Greengate system based on the vector pGGZ003 (Lampropoulos et al. 2013). A reaction containing 150 ng of each module (pro35S:Ω:GFP:Dummy:TerRBCS;pUBQ10::HygromycinR::tOCS)(pro35S:Ω:GFP-NLS:Dummy:TerRBCS;pMAS:BASTA:tMAS) and 100 ng of pGGZ003 were set up according to Lampropoulos et al. (2013). Chemocompetent *E. coli* DH5α cells were transformed with 4 μl reaction mix to amplify the construct. The

correct assembly was monitored by sequencing and the vector was isolated and introduced in *A. tumefaciens* strain GV3101 by electroporation following standard protocols.

## Western blot analysis

Tissue from putatively transgenic *E. californica* plants was collected from different developmental stages (callus, somatic embryos, young regenerated plants) during plant regeneration. Material was frozen in liquid nitrogen and ground using a tissue-lyser. The sample was then mixed with pre-heated 4×Laemmli buffer (100 µl/200 mg sample containing 30 mg/ml DDT). After 5 min at 100 °C, the samples were centrifuged at 14,000 rpm for one minute and 30 µl of the supernatant was loaded on a 12% SDS gel. PageRuler Prestained Protein Ladder (Thermo Fischer, Langensfeld, Germany) was used as a marker. After 30 min at 80 V followed by 2 h at 100 V) the gel was transferred to a PVDF membrane. The samples were blocked with 1% BSA in TBS buffer + Tween for 2 h and incubated with an Anti-Green Fluorescent Protein – Horseradish Peroxidase (Miltenyi Biotec, Bergisch Gladbach, Germany) (GFP-HRP) antibody 1:8000 in 2.5% BSA overnight at 4 °C with shaking. After the incubation, the gel was washed three times for 10 min each in TBS-T (0.1% TWEEN20) solution. To detect the proteins, 500 µl of Amersham ECL Western Blotting Detection Reagent (1:1 solution A and solution B, Amersham, Freiburg, Germany) was used. Images were created using a ChemiDOC MP Imaging System (Biorad, Neuberg, Germany).

## Results and discussion

### Protoplast isolation and transformation

Many experimental approaches, such as biochemical, protein–protein or protein–DNA interaction analyses do not require fully-grown plants and protoplasts are suitable experimental systems. We thus developed a protocol for rapid protoplast isolation and transformation (Online Resource 3, step-by-step protocol). Protoplasts were isolated from the leaf and hypocotyl tissue of 10 days old *in vitro* grown plants using macerozyme and cellulase R-10 to digest cell walls. The protoplast isolation yielded between 0.45 and  $0.85 \times 10^6$  protoplasts/g plant tissue. These protoplasts were round, only occasionally was the cell wall still attached, and they included many chloroplasts (Fig. 1A, B).

Next, we transformed the freshly isolated protoplasts with a nuclear localized, ubiquitously expressed GFP protein as fluorescent marker driven by UBQ10 promoter (pUBQ10:GFP:NLS). Employing a PEG-mediated transformation protocol, the transformation efficiency was between

13.5 and 24.8% (Fig. 1C). The transformation efficiency is dependent on the incubation time of the enzyme solution, such that an incubation time of 1.5 h yielded 11.8% transformed protoplasts and 4 h 24.8% transformed protoplasts (Fig. 1D). Incompletely digested cell walls may thus inhibit the PEG-mediated transformation. Nuclear localized GFP signal was detected using fluorescent microscopy 64 h post transformation (Fig. E–G). Protoplasts were visually unchanged (Fig. 1E) when compared to freshly isolated protoplasts (Fig. 1A) suggesting that the transformation and subsequent incubation time had no obvious negative effect on protoplast viability. The protoplasts showed a very low level of auto fluorescence (Fig. 1F). Online Resource 1, Fig. 1 provides a flowchart summarizing the experimental steps and expected time line for protoplast transformation and all other approaches described here.

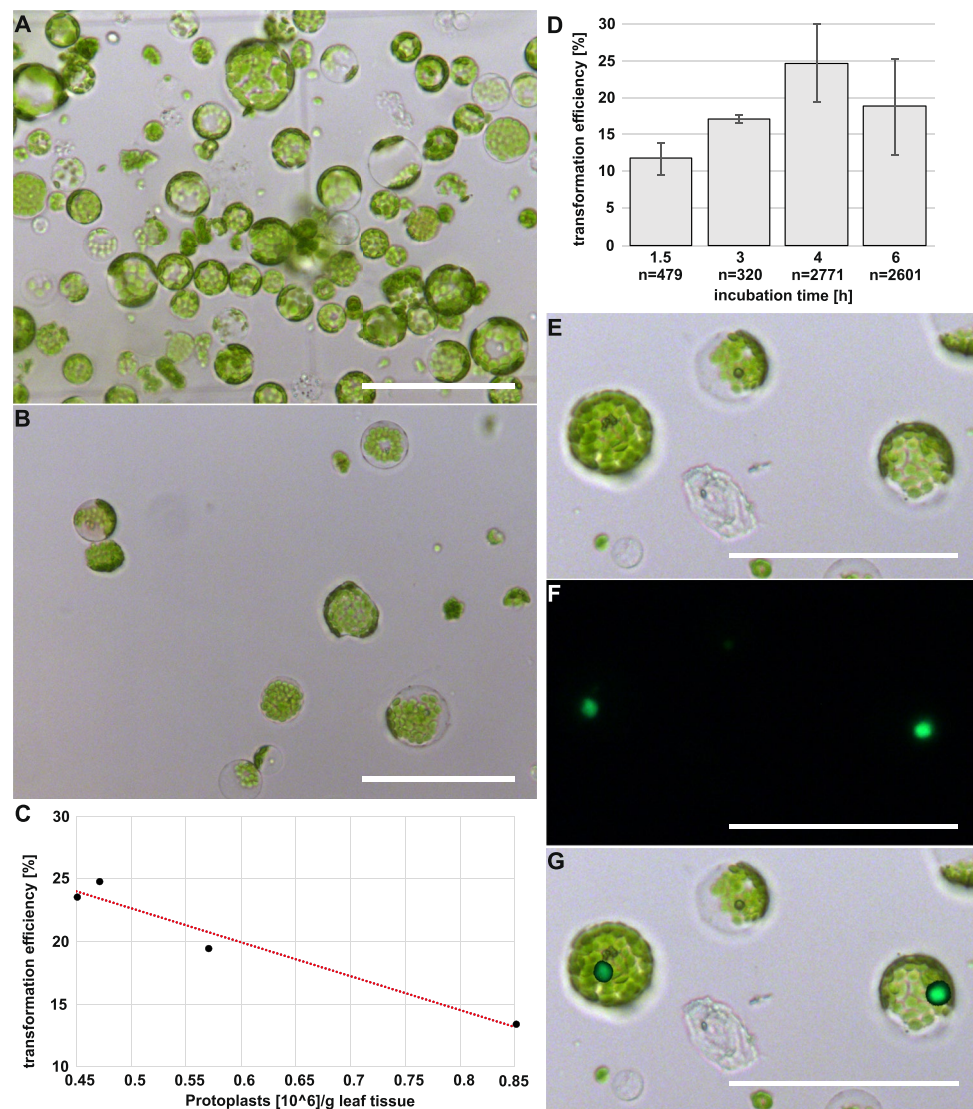
### Plant regeneration from explants

Establishing *E. californica* as a genetic model plant requires the possibility for stable transformation and thus we developed a reliable, tissue-culture based method for plant regeneration as a first step towards a transformation protocol.

We first analyzed the type of explant, and type and concentration of auxin best suitable for callus regeneration of *E. californica* (Table 1). Using 2,4-D as auxin source resulted in callus regeneration in all the tested concentrations, with hypocotyl being more amenable to callus regeneration. A concentration of 1 mg/l 2,4-D was sufficient for regeneration of callus tissue from every explant. After four weeks, almost all hypocotyl explants regenerated callus (97.8%) that were subsequently induced to develop somatic embryos. The details of the following experiments can be found in Online Resource 2, Table 1 to 6). After seven weeks, 44% of the calli produced somatic embryos and after this time, only marginally more calli generated somatic embryos. The somatic embryos were removed from the calli and matured for three weeks on a specific maturation medium including maltose instead of sucrose and subsequently, they were kept in the light until they turned green and commenced organogenesis. From 58% of the somatic embryos, plantlets could be regenerated, and 60% of these plantlets developed into fully grown plants in soil. The regenerated plantlets were transferred to soil after 4 to 6 weeks. The surviving plants formed morphologies similar to plants grown from seeds and were fully fertile. Generally, the regeneration of *E. californica* plants was carried out in at least five repetitions.

Taken together, we established an efficient *E. californica in vitro* plant regeneration protocol, from hypocotyl explants to mature plants via callus induction and somatic embryogenesis. Figure 2 shows an overview of the time scale and morphology of *E. californica* regeneration intermediates.

**Fig. 1** Protoplast isolation and transformation. Morphology of freshly isolated *E. californica* leaf and hypocotyl protoplasts (**A**, **B**). **C** Protoplast isolation efficiency in relation to the percentage of *pUBQ10:GFP:NLS* carrying, fluorescing protoplasts. **D** Incubation time in cell-wall dissolving enzyme solution affects transformation efficiency. Bars represent standard deviation, based on at least five independent repetitions. **E** Protoplasts transformed with *pUBQ10:GFP:NLS* in brightfield, fluorescence (**F**), and overlay (**G**). Size bars are 100  $\mu\text{m}$



### Optimization of the plant regeneration protocol

To obtain an efficient and reproducible protocol for *E. californica* transformation, several modifications to the transformation protocol reported earlier (Park and Facchini 2000b) were tested. For example, the addition of 2.0 mg/l NAA and 0.1 mg/l BAP to the callus induction medium was replaced by adding only 1 mg/l 2,4-D, which raised the number of explants producing calli to nearly 100% (Table 1). Further, we analyzed if cotyledons or hypocotyl produced callus material more reliably and found that with the optimal concentration of 1 mg/l 2,4-D, all hypocotyl explants produced callus while only max. 80% of the cotyledon explants produced calli (with an optimal 2,4-D concentration of 1.5 mg/l, Table 1). Next we analysed if unripe seeds (21 days after pollination) have the potential to regenerate callus. We compared 1 mg/l 2,4-D with 2 mg/l NAA and 0.1 mg/l BAP and our results show that unripe seeds have a lower callus

regeneration rate than cotyledons and hypocotyl, independent of the auxin source (Table 1). We also analyzed if reducing cell culture inhibitory secondary metabolites by adding 0.5 g/l activated charcoal to the medium improves callus formation (Möller et al. 2006; Thomas 2008). However, the callus regeneration from hypocotyl dropped from 100 to 33% when charcoal was added (Table 1), suggesting that charcoal may block phytohormone activity or availability. Additionally, we analysed if somatic embryos can dedifferentiate to callus by adding 1 mg/l 2,4-D or 2 mg/l NAA, 0.1 mg/l BAP and activated charcoal. 2,4-D leads to new callus regeneration in more than 90%. Interestingly, the combination of NAA, BAP and activated charcoal leads to the regeneration of new somatic embryos within six weeks in nearly 100% of all samples (Table 1). Finally, a somatic embryo maturation phase was introduced to the protocol to improve greening and proper morphogenesis of the embryos. We analyzed several combinations of maltose, PEG and abscisic acid (ABA),

**Table 1** Callus regeneration rate dependent on different *E. californica* organ explants and varying concentrations of 2,4-D, NAA, BAP and 0.5 g/l activated charcoal

Cotyledons as explants for callus production					Cotyledons as explants for callus production					
mg/l 2,4-D	Total number	Callus	Dead tissue	Callus regeneration (%)	mg/l 2,4-D + activated charcoal	Total number	Callus	Dead tissue	Callus regeneration (%)	
0.5	8	4	4	50.00	0.5	8	1	7	12.50	
1	13	3	10	23.08	1	8	2	6	25.00	
1.5	5	4	1	80.00	1.5	5	0	5	0.00	
2	5	1	4	20.00	2	4	0	4	0.00	
3	6	4	2	66.67	3	4	1	3	25.00	
Hypocotyls as explants for callus production					Hypocotyls as explants for callus production					
mg/l 2,4-D	Total number	Callus	Dead tissue	Callus regeneration (%)	mg/l 2,4-D + activated charcoal	Total number	Callus	Dead tissue	Callus regeneration (%)	
0.5	7	5	2	71.43	0.5	9	0	9	0.00	
1	4	4	0	100.00	1	3	1	2	33.33	
1.5	5	3	2	60.00	1.5	5	1	4	20.00	
2	4	4	0	100.00	2	2	0	2	0.00	
3	7	7	0	100.00	3	4	0	4	0.00	
Unripe seeds as explants for callus production					Somatic embryos as source for callus production					
	Total number	Callus	Dead tissue	Callus regeneration (%)		Total number	Callus	New SEs	Root hair	Callus regeneration (%)
1 mg/l 2,4-D	310	70	240	22.58	1 mg/l 2,4-D	26	24	7	–	92.31
2 mg/l NAA + 0.1 mg/l BAP	204	81	123	39.71	2 mg/l NAA + 0.1 mg/l BAP + activated charcoal	24	7	23	9	29.17

**Fig. 2** Time scale of plant regeneration from *E. californica* hypocotyl explants. Hypocotyl of 10 days old *E. californica* plants was used to induce callus growth on callus medium. After 6–8 weeks in the dark at 25 °C, regenerated callus cells were transferred to somatic embryo induction medium. For embryo maturation, regenerated embryos

were transferred for three weeks to maturation medium. After maturation, all embryos were incubated in long day conditions (16 h light 8 h dark) on plant regeneration medium without hormones. Regenerated plants were transferred to soil, covered with a cap for two weeks, to maintain humidity

and found that exchanging sucrose as carbon source with 3% maltose alone performed best, with around 84% of the embryos started photosynthesis in contrast to a combination of 3% maltose with 2.5 mg/l ABA which killed around 97%

of the calli (Online Resource 2, Table 3). In addition, the regenerated plants grew better in plain standard soil; around 80% survived the transfer from *in vitro* culture to soil when

compared to vermiculite as substrate, which only around 54% of the plants survived (Online Resource 2, Table 6).

### Agrobacterium-mediated transformation of *E. californica*

Based on the tissue culture and regeneration method described earlier, we developed an efficient transformation protocol using *E. californica* hypocotyl as explant and employing *Agrobacterium tumefaciens* based transformation to generate transgenic lines carrying the reporter construct *p35S::GFP* using hygromycin as selectable marker. A killing-curve experiment showed that a concentration of 40 mg/l hygromycin is sufficient to select transgenic plants (Online Resource 1, Fig. 2; Online Resource 2, Table 7). The transgenic calli produced somatic embryos, which were then regenerated into fully grown, fertile plants employing the plant regeneration protocol. Figure 3A–P shows mock-treated and 35S:GFP treated calli and somatic embryos in bright field and fluorescence microscopy. Only a small fraction of callus cells is transformed and has already started to divide (Fig. 3A–H). Those will later provide the transgenic calli shown in Fig. 3L and P, while the untransformed cells of the calli discontinue division and die eventually. Healthy calli under selection are then removed and incubated on somatic embryo induction medium. Wild type calli regenerating somatic embryos are shown in Fig. 3I, J, M, N). Figure 3K, L, O, and P show two fully fluorescent calli that are derived from transformation events. Those generate genetically identical somatic embryos that are also completely fluorescent, showing that the 35S promoter is active in callus tissue and somatic embryos. The presence of transgenic GFP protein in the transformed tissues was corroborated by Western blot analysis (Fig. 3Q). In total, 23 independently transformed transgenic calli were derived that, when cultured on somatic embryo induction medium, all continuously produced genetically identical somatic embryos. A strong GFP signal was detected in 65% of the regenerated somatic embryos (Fig. 3 and Online Resource 2, Table 8).

Unexpectedly, the mock-treated calli and somatic embryos also show a faint fluorescence (Fig. 3B, F, J, N), but this is much lower in intensity than the GFP fluorescence in Fig. 3C, D, G, H, L, and P, and suggestive of fluorescing secondary metabolites. Interestingly, it was shown previously, that *E. californica* suspension cultures produce benzophenanthridine alkaloids with an excitation of 270–550 nm and emissions at 310–590 nm (Hisiger and Jolicœur 2005). Those lie within the range of the GFP excitation and emission spectrum (max. 488 nm and 507 nm, respectively) and could explain the low intensity fluorescence observed in wild type calli and somatic embryos. *p35S::GFP* transformed plants show a morphology similar to that of wild type plants that underwent tissue culture based regeneration, except for

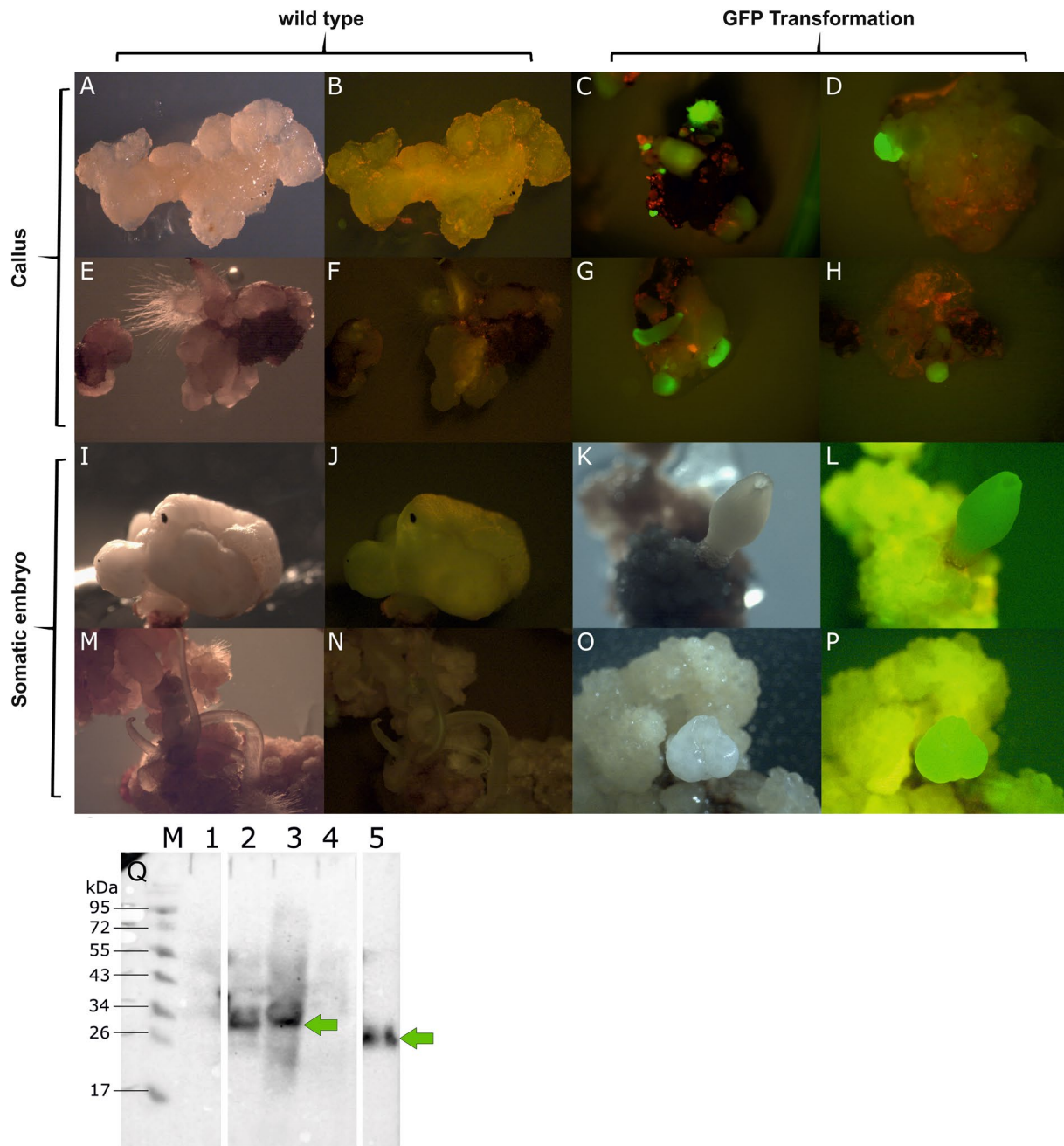
the flowers, which show folded mature petals in the transgenics (Online Resource 1, Fig. 3).

### Other *E. californica* transformation methods

There are other means to generate transgenic *E. californica* tissue, for example by using biolistic bombardment of callus tissue (Popelka et al. 2003; Viehweger et al. 2006; Angelova et al. 2010), but this requires an expensive biolistic particle delivery system. Using the regeneration protocol reported here, this method can be extended to generate transgenic lines. Park and Facchini (2000b) has reported another protocol, but with different media/phytohormone compositions and less efficient in terms of callus induction frequency (80%) than the method standardized in this study (98%). Also the somatic embryo formation from callus was improved (45%) than in the previous study (30%). Further, the somatic embryo induction medium we used could induced continuous production of somatic embryos from transgenic calli, such that up to 37 transgenic lines could be subcultured from a single callus (Online Resource 2, Table 8). Unfortunately, we cannot compare our work with how the method by Park and Facchini (2000b) performs in other labs, as no further work based on this protocol was published.

### Conclusions

The possibility to create stable transgenics is the prerequisite to establish *E. californica* as a genetic model system for the Ranunculales, the sister order to the core eudicots. Other practical aspects such as a short generation cycle of only two months from seed to flower, undemanding growing conditions, and a comparatively small genome six times that of *Arabidopsis thaliana* add to the value of the model species *E. californica* (Becker et al. 2005; Wege et al. 2007; Yamada et al. 2021). Our work provides efficient means for gene function analysis in *E. californica* using stable transgenic lines to generate reproducible results. Approaches like CRISPR-Cas9 mediated genome modifications, reporter lines for phytohormones detection, or stable and localized modifications of alkaloid biosynthesis (Alagoz et al. 2016; Isoda et al. 2021; Hayashi et al. 2020) could be studied with the help of the protocol developed in this study. Previous work (Park and Facchini 2000b) provided a protocol with lower efficiency regarding the regeneration of *E. californica* plants and did not consider protoplast isolation and transformation. Researchers interested in alkaloid biosynthesis and regulation may work with *E. californica* without specific permits as they are required for *P. somniferum*. For example, *E. californica* could be employed as a promising model species to study the mutual influence of development



**Fig. 3** Documentation of transformed *E. californica* plants during regeneration. **A–H** shows examples of growing calli. **A** and **E**, mock-treated calli in bright field. **B** and **F**, same calli under fluorescent light. **C**, **D**, **G** and **H**, calli transformed with *35S:GFP* under fluorescent light. **I** and **M**, mock-treated somatic embryos in bright field, **J** and **N** same embryos in fluorescent light. **K** and **O** calli and somatic embryos carrying *35S:GFP* in bright field, **L** and **P** same tissues in

fluorescent light. **Q** Western blot analysis of transgenic *E. californica* tissue (1) wild type callus, (2) callus carrying *35S:GFP*, (3) somatic embryos carrying *35S:GFP*, (4) mixed sample from young regenerated plants and somatic embryos, (5) GFP antibody positive control: tobacco leaves *Agrobacterium*-infiltrated with HA-GFP tag that were previously analyzed to express GFP. Green arrows point to the GFP-HA bands

and alkaloid biosynthesis (Yamada et al. 2020) or to analyse flower evolution on the molecular level (Zhao et al. 2018). Taken together, our work provides an important step towards the establishment of *E. californica* as a genetic model system to study a large suite of diverse research questions.

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**Author contributions** DL, KE and JI carried out experiments and analysed the data; JI and KE gave input in experimental design and helped with microscopy; AB supervised experiments and wrote the manuscript. All authors read and approved the final version of the manuscript.

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**Data availability** Vectors and seeds of transgenic lines may be obtained from the corresponding author.

**Code availability** n.a.

**Consent for publication** n.a.

## Declarations

**Conflict of interest** There are no conflicts of interest or competing interests.

**Ethical approval** n.a.

**Informed consent** n.a.

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