



The development of an *in vitro* floral culture transformation system for quinoa

Vladimir Sidorov¹ · Peter J. Maughan² · Peizhen Yang¹

Received: 20 May 2024 / Accepted: 20 July 2024 / Published online: 21 October 2024 / Editor: Thomas Clemente
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Abstract

Because of its high-quality seed protein and ability to thrive in marginal habitats, *Chenopodium quinoa* has been identified as an important emerging grain crop for global food security. However, the lack of an efficient and robust transformation system has been a barrier for conducting the advanced genetic studies needed to better understand and improve the species for agronomic traits. Here we present a novel transformation system based on *Agrobacterium*-mediated transformation of *in vitro* floral culture. Quinoa floral cultures were established from inflorescences that naturally formed on plants grown *in vitro*. When placed on a cytokinin-containing medium, chopped inflorescences rapidly generated highly meristematic floral cultures, primarily composed of floral buds, flowers at various developmental stages, inflorescence shoots, and leafy structures. Transformation of these cultures with *Agrobacterium* carrying selectable and visual markers (NPTII and GUS) produced independent, stably transformed meristematic cultures resistant to paromomycin after an extended selection period (about 3 mo with sub-culture occurring every 15 d). Transformation frequency was about 20% and was calculated as the number of independent transformed events per initial number of floral culture explants used for transformation. *In vitro* flowers and inflorescences from putative transgenic events self-pollinated naturally and produced viable seeds that germinated without dormancy. We also demonstrated that flowering shoots could be successfully grafted onto wild rootstock to increase the number of seeds generated from T₀ floral shoots. Molecular and phenotypic analysis of the progeny confirmed that the transgenes were stably integrated and inherited according to expected Mendelian ratios.

Keywords *In vitro* floral culture · Quinoa · Transformation

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is an important ancient grain crop which originated in the mountains of Bolivia, Chile, and Peru and was originally domesticated by pre-Incan civilizations over 5000 yr ago (Risi and Galwey 1984; Fuentes *et al.* 2009; Bazile *et al.* 2015; Martinez *et al.* 2015). Quinoa is an allotetraploid (2n = 4x = 36 chromosomes), dicotyledonous, short-day annual plant (Jacobsen and Stølen 1993; Tabatabaei *et al.* 2022) that displays unique abiotic stress tolerance, specifically as a halophyte (salt-tolerant crop), and thus has been identified as an important crop

for addressing future food security issues (Jaikishun *et al.* 2019)—especially in agricultural areas with drought- and salt-affected soils. Leaves and other parts of the plant are usually covered with epidermal bladder cells (EBCs) which are modified trichomes and potentially serve as “salt dumps” (Bohm *et al.* 2018; Moog *et al.* 2022). The plant produces large panicle-type inflorescences having hermaphroditic and pistillate flowers. The hermaphrodite flower possesses five imbricate sepals and five antisepalous stamens (Abdelbar 2018).

Because of the high quality of the seed protein, which is also gluten-free, quinoa is commonly referenced as a superfood (Vega-Gálvez *et al.* 2010). Indeed, the United Nations declared 2013 as International Year of Quinoa.

Over the past two decades, several public and private breeding programs have been initiated with the aim to improve quinoa production—specifically for regions outside of the Andes. These efforts have been aided by the development of quinoa-specific genetic tools including genetic

✉ Vladimir Sidorov
vladimirsdrv@gmail.com

¹ Bayer Crop Science AG, 700 Parkway W,
Chesterfield, St. Louis, MO 63017, USA

² Brigham Young University, Provo, UT, USA



markers and linkage maps (Maughan *et al.* 2012; Zhang *et al.* 2017) and most recently with the sequencing of the quinoa genome (Jarvis *et al.* 2017; Rey *et al.* 2023)—all of which have facilitated QTL studies of agronomically important traits in quinoa (Maldonado-Taipe *et al.* 2022). While the number of genetic resources available for studying quinoa has increased, the development of a reliable genetic transformation system has proven elusive. A simple, robust transformation system is vital for future quinoa research. Already specific targets for gene editing have been identified, including anti-nutritional triterpenoid saponins that are deposited in the seed coat that must be removed prior to consumption (El Hazzam *et al.* 2020). Unfortunately, quinoa is recalcitrant to *in vitro* regeneration from callus cultures. Micropropagation protocols have been developed for different quinoa cultivars (Regalado *et al.* 2020) but research on *in vitro* regeneration of quinoa is extremely limited. Protocols for plant regeneration through somatic embryogenesis (Eisa *et al.* 2005) or organogenesis (Bagga *et al.* 1987; Hesami and Daneshvar 2016; Al Gethami and El Sayed 2020) have been described in the literature but have proven inefficient or even non-reproducible outside of the lab of origin. Recently, successful editing of quinoa was demonstrated, but generation of stably inherited transgenic or gene-edited plants was not achieved (Xiao *et al.* 2022).

In this report, the development of a new transformation system for quinoa, independent of *in vitro* plant regeneration, is described based on *Agrobacterium*-mediated transformation of *in vitro* “floral culture.” The floral culture was obtained on cytokinin-containing medium from inflorescences which spontaneously formed *in vitro*. The production of transformed non-chimeric inflorescences, which produced seeds, and stable inheritance of transgenes (*nptII* and *uidA*) in progeny is documented.

Material and Methods

Initial Material Seeds of *Chenopodium quinoa* Willd., line QQ74 (PI 614886), were kindly provided by the US National Plant Germplasm System (<https://npgsweb.ars-grin.gov/gringlobal/query/query>). They were surface sterilized with 20% sodium hypochlorite for 20 min. After thoroughly rinsing with sterile water, seeds were placed into PlantCon™ containers (MP Biomedicals, Solon, OH) and cultivated on Driver and Kuniyuki Walnut (DKW; Driver and Kuniyuki 1984) medium (PhytoTech Labs®, Lenexa, KS, D2470) with 20.0 g L⁻¹ sucrose, pH 5.7 (DKW # 1), at 25 °C, 80 µE m⁻² s⁻¹ light intensity, 16-hr day length. Combination of cool white and warm white fluorescent tubes was used.

For induction and propagation of *in vitro* floral cultures, inflorescences from *in vitro* grown plants were chopped and placed on DKW medium with 20.0 g L⁻¹ sucrose, 1.0 mg

L⁻¹ kinetin (Kin), 0.5 mg L⁻¹ 6-benzylaminopurine (BA), 0.1 mg L⁻¹ gibberellic acid (GA3), and 3.5 g L⁻¹ agarose (Millipore Sigma®, Burlington, MA; A6013), pH 5.7 (DKW # 2). All growth regulators, including α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), zeatin (Zea), and indole-3-butyric acid (IBA), were obtained from PhytoTech Labs.

Transformation *Agrobacterium tumefaciens* strain AB32 was used for all transformation experiments. AB32 has a constitutively active VirG (Ye *et al.* 2016), which eliminates the need for induction with acetosyringone prior to inoculation. AB32 also carries a pVS1-based Ti binary vector (Itoh *et al.* 1984) that has a *nptII/uidA* (GUS) cassette in its T-DNA region. The *nptII* and *uidA* genes were under control of the CaMV 35S and enhanced CaMV 35S promoters, respectively.

For the preparation of *Agrobacterium*, 0.5 mL of glycerol stock was inoculated into 200 mL of LB liquid medium with 50.0 mg L⁻¹ spectinomycin (Spec) and 30.0 mg L⁻¹ gentamycin (Gent) and cultured on a shaker at 200 rpm at 28 °C for 20 to 22 hr, resulting in the bacterium culture with OD₆₆₀ = 1.2 to 1.4. The *Agrobacterium* culture was subsequently centrifuged at 3000 g at 4 °C for 30 min. The pelleted *Agrobacterium* was re-suspended in an inoculation medium, and the density was adjusted to 0.8 to 1.0 at OD₆₆₀. The inoculation medium contained 2.16 g L⁻¹ Murashige and Skoog (MS; Murashige and Skoog 1962) basal salt mixture (PhytoTech Labs, M524), 1.0 mL L⁻¹ MS vitamins 1000× (PhytoTech Labs, M553), 68.5 g L⁻¹ sucrose, 36.0 g L⁻¹ glucose, 0.115 g L⁻¹ L-proline, and 200.0 µM acetosyringone (added immediately prior to use).

For inoculation, approximately 200 explants (approximately 140 g) were transferred to 250-mL Corning® bottles with 150 mL of *Agrobacterium* suspended in the inoculation medium. Bottles were placed in a vacuum desiccator and vacuum infiltrated for 5 min after bubbling was observed. Explants with the *Agrobacterium* suspension were then transferred to 50-mL tubes and centrifuged for 30 min at 320 g-force (Eppendorf™ Centrifuge 5810 R). The centrifuged samples were then poured through 4-in. stainless steel mesh strainer (0.4 to 1 mm mesh size; Oneida, New York, NY) and the separated explants blotted dry on sterile paper towels for 3 to 5 min. Lastly, explants were spread on a sterile 8.2-mm Whatman® filter paper (Cat # 1001–082), pre-wetted with 0.5 mL of inoculation medium in a Petri dish (25 × 85 mm) and co-cultured in a Percival (Percival Scientific, Inc. Perry, IA) for 4 to 5 d under light (20 µE m⁻² s⁻¹ light intensity with cool white fluorescent tubes, 16-hr day length) at 21 °C and 65% relative humidity.

After co-culture, all explants were transferred to semi-solid DKW # 2 selection medium (DKW # 1, supplemented with 50 mg/L paromomycin (Par), 750.0 mg L⁻¹

carbenicillin (Carb) (PhytoTech Labs, C346), and 300.0 mg L⁻¹ cefotaxime (Cef) (PhytoTech Labs, C380) and incubated in the growth chamber set to 25°C, 80 $\mu\text{E m}^{-2} \text{s}^{-1}$ light intensity, and a 16-hr day length. The transformed explants were sub-cultured in Petri plates every 12 to 14 d on the same medium but with reduced concentrations of Carb (500.0 mg L⁻¹) and Cef (200.0 mg L⁻¹).

After 3 to 3.5 mo of selection, explants were transferred to DKW # 1 medium for floral shoot induction and flower maturation. For production of large number of seeds, transgenic floral shoots were grafted to the wild-type plants, sealed with Parafilm M® laboratory film, and secured with 2-mm plastic grafting clips.

Transformation frequency was calculated as the number of transgenic events per initial number of explants used for transformation. The T₀ events were defined as the primary floral shoot lines obtained after selection and T₁ plants as the progeny plants derived from the self-pollinated seeds produced from the T₀ events.

Protoplast Isolation and Culture Protocol for protoplast isolation was the same as described previously (Sidorov *et al.* 2022) except the enzyme composition was modified to contain 2.0% Cellulase RS, 2.0% Cellulysin, 0.5% Driselase, and 0.5% Macerozyme R-10 (all from Millipore Sigma®). Plant material was incubated 3 hr at 28 °C. After protoplast isolation and washing, protoplasts were imbedded into a thin alginate layer and cultured in Kao and Michyluk (KM) modified medium (see Sidorov *et al.* 2022), containing 1.0 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA, and 0.25 mg L⁻¹ 2,4-D.

Analysis of Plant Material and Statistics Plant explants and seedlings were stained with X-Gluc according to the published protocol (Jefferson 1989). After overnight incubation at 37 °C in X-Gluc, the plant material was de-stained in 70% v/v ethanol and examined for GUS expression. The presence of transgenes was also determined by TAQMAN PCR procedure according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

Chi-square tests were used to analyze expected transgene segregation of T₁ seeds.

Results and Discussion

Initiation and Maintenance of Floral Culture Sterilized quinoa seeds were sown on DKW #1 medium without plant growth regulators (PGR-free medium) and incubated, as described above, at long-day (16 hr) illumination. Interestingly, while quinoa is classified as a short-day plant, the quinoa plants started flowering in approximately 30 d under these conditions. This precocious flowering is likely the result of stress induced from the *in vitro* growing conditions. While the plants appeared stressed (elongated, spindly, increased epidermal bladder cell production, and poor root development), they were easily propagated by cutting. DKW-based medium was the best for quinoa; however, MS-based medium was also tested. During culture, plants spontaneously formed typical, highly branched panicle inflorescences (Prusinkiewicz *et al.* 2007), schematically presented in Fig. 1A. Panicle or branching inflorescence likely plays an essential role in the development and rapid production of meristems in floral cultures. The conversion of shoot apical meristems to inflorescence meristems has been described in detail previously (Bertero *et al.* 1996; Hake and Wilt 2003; Gaarslev *et al.* 2021) and is presented schematically on Fig. 1B. A quinoa flower can be considered a modified shoot wherein the shoot apical meristem changes into a floral meristem (Taiz *et al.* 2015). The similar nature of apical meristem and floral buds, which are also highly meristematic, highlights one of the difficulties of working with floral cultures—specifically that transformation of highly organized meristematic tissue requires a prolonged selection period to obtain complete sorting out of non-transformed tissue from transformed tissue.

In vitro inflorescences had normal flowers, which self-pollinated and produced seeds (Fig. 2A, D). All quinoa organs, especially young leaves, typically were covered

Figure 1. Type of inflorescence in *Chenopodium quinoa* Willd. and drawing of possible conversion of vegetative shoot meristem to inflorescence meristem. (A) Diagram showing typical panicle-type architecture of the quinoa inflorescence. Yellow circles indicate flowers. (B) Schematic drawing of shoot apical meristem and its conversion to inflorescence and flower meristems.

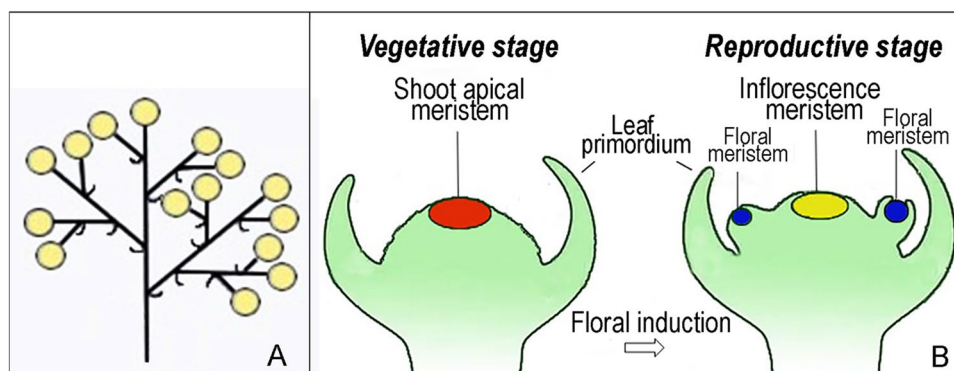




Figure 2. *Chenopodium quinoa* Willd. inflorescences and seed development *in vitro*. (A) Inflorescence on *in vitro* quinoa plant (variety QQ74) grown for approximately 1 mo grown from surfaced sterilized seed. (B), (C) Leaves are covered with epidermal bladder cells (EBC).

(D), (E), (F) Seeds matured and germinated on Driver and Kuniyuki Walnut (DKW) medium (PhytoTech Labs®, D2470) with 20 g L⁻¹ sucrose, pH 5.7 (DKW # 1) medium without plant growth regulators (16-hr light photoperiod, 25 °C).

with epidermal bladder cells (Fig. 2B, C). Seeds showed no dormancy and could germinate immediately on DKW #1 medium (Fig. 2E, F). For induction of floral culture, panicles from *in vitro* plants were collected, chopped, and placed on DKW #2 medium. After three to four sub-cultures (at 12- to 14-d intervals), a highly meristematic floral culture was established which could be used for transformation. The floral culture consisted mainly of flowers at different stages of development, panicle shoots, and leafy structures (Fig. 3A–E).

Transformation of Floral Culture and Selection In each treatment, approximately 200 explants were inoculated with *Agrobacterium* (AB32 carrying pVS1 Ti plasmid containing *nptII* and *uidA* markers). The essential steps for increasing the efficiency of transformation were the vacuum infiltration of explants in *Agrobacterium* solution and centrifugation. For selection of transformants, we used DKW #2 medium supplemented with 50.0 mg L⁻¹ Par. Selection was applied without delay and was stringent enough to cause bleaching

and prevent growth of non-transformed tissue (Fig. 4A). During each sub-culture, meristematic clusters were broken into small pieces and only the green viable tissue was transferred to new selection plates. Approximately 45 d after transformation, clumps of what appeared as non-chimeric tissue were clearly visible on the selection medium (Fig. 4A, B); however, after staining with X-Gluc, several of these clumps were deemed to be still chimeric. Regeneration of chimeric plants is always a concern in meristem explant-based transformation systems (Ye *et al.* 2022, Ye *et al.* 2023). Indeed, in experiments using corn seed embryo explants (SEEs) for transformation, the frequency of plants with a visible chimeric leaf phenotype was as high as 80% (Ye *et al.* 2022). The occurrence of chimerism in the SEE experiments was likely due to very low selection pressure and/or limited time on selection. Because of these concerns, we used significantly higher levels of Par (50.0 mg L⁻¹) for selection with the intent of significantly inhibited growth of non-transformed tissue at the outset of the experiment. We also extended the selection period to more than 3 mo. After the



Figure 3. *In vitro* floral culture. (A) Micropropagation of floral culture on Driver and Kuniyuki Walnut (DKW) medium with 20.0 g L⁻¹ sucrose, 1.0 mg L⁻¹ kinetin (Kin), 0. mg L⁻¹ 6-benzylaminopu-

rine (BA), 0.1 mg L⁻¹ gibberellic acid (GA3), and 3.5 g L⁻¹ agarose (Millipore Sigma®, A6013), pH 5.7 (DKW # 2). (B, C, D, E) Development of flower buds and formation of flowers *in vitro*.

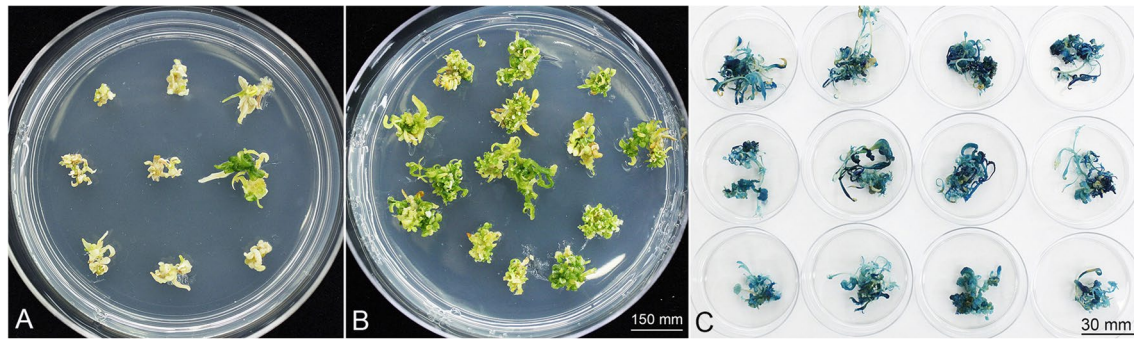


Figure 4. Selection for paromomycin (Par) resistance after transformation. (A) 20-d and (B) 45-d selection on Driver and Kuniyuki Walnut (DKW) medium with 20.0 g L⁻¹ sucrose, 1.0 mg L⁻¹ Kin, 0.5 mg

L⁻¹ BA, 0.1 mg/L GA3, and 3.5 g L⁻¹ agarose, pH 5.7 (DKW # 2) at 50.0 mg L⁻¹ Par. (C) GUS (β-glucuronidase) expression in randomly picked lines.

extended selection period, all of the randomly picked lines were GUS-positive and non-chimeric (Fig. 4C). TAQMAN PCR analysis shows that out of 26 regenerants, 20 were GUS and NPTII positive (GUS⁺ and NPTII⁺), 2 were negative (GUS⁻ and NPTII⁻), 2 were GUS⁺ but NPTII⁻, and 2 were GUS⁻ but NPTII⁺. Data on copy number of transgenes was not obtained. We note that it is likely that the time frame for production of transgenics using the floral culture systems reported here could be considerably shortened with the use of more stringent selection or application of non-destructive visual reporter gene, such as RUBY (see He *et al.* 2020).

Production of Seeds and Analysis of T₁ Progeny After selection, the transformed lines were transferred to DKW #2 medium without Carb/Cef to which only 20 mg L⁻¹ Par was added. Selected lines on this medium start flowering and even form seeds. For shoot induction, the transformed plant material was transferred to DKW #1 PGR-free medium and to ½ DKW, 10.0 g L⁻¹ sucrose 20.0 mg L⁻¹ Par. As shown on the Fig. 5A, B, flowering shoots formed on both media types; however, the shoots failed to produce roots and thus could not be transferred directly to soil. Nonetheless, seeds did form on the *in vitro* flowering shoots (Fig. 5C).

For production of larger quantities of seeds, transformed floral shoots were grafted onto quinoa wild-type rootstock (Fig. 6A, B). The wild-type rootstock was initially grown *in vitro* for 2 to 3 wk before grafting with a transformed scion. Within 2 wk, the plants were ready to transplant to soil. Hundreds of seeds were produced from each of the transplanted plants (maintained at 25 °C with a 13-hr light photoperiod). Positive GUS expression in leaves of grafted plants confirmed that they were transformants (Fig. 6C). The absence of possibility to root transgenic shoots indicates that we are producing floral shoots. At the same time, selected flowering shoots were successfully grafted, which suggest that vegetative shoots were produced. So, we are not excluding that produced meristematic cultures had mixture of floral shoots and some vegetative shoots. It is also possible that we indeed produced floral shoots which were later converted to vegetative shoots. Since apical vegetative buds can convert to floral buds (see Fig. 1B), we expect that floral buds could also be converted into vegetative apical buds and initiate vegetative shoots.

The presence of the transgenes was checked in seeds derived from five shoots that flowered *in vitro* and from seeds obtained from transformed shoots grafted to rootstock



Figure 5. *De novo* formation of flower clusters with floral shoots after selection, and germination of produced *in vitro* seeds. (A) Driver and Kuniyuki Walnut (DKW) medium with 20.0 g L⁻¹ sucrose, 1.0 mg L⁻¹ Kin, 0.5 mg L⁻¹ BA, 0.1 mg L⁻¹ GA3, and 3.5 g L⁻¹ aga-

rose, pH 5.7 with 50.0 mg L⁻¹ Par. (B) ½ DKW, 10.0 g L⁻¹ sucrose, 20.0 mg L⁻¹ Par. (C) Seeds were directly germinated from transformed floral culture on DKW #2 medium with 20.0 mg L⁻¹ Par.

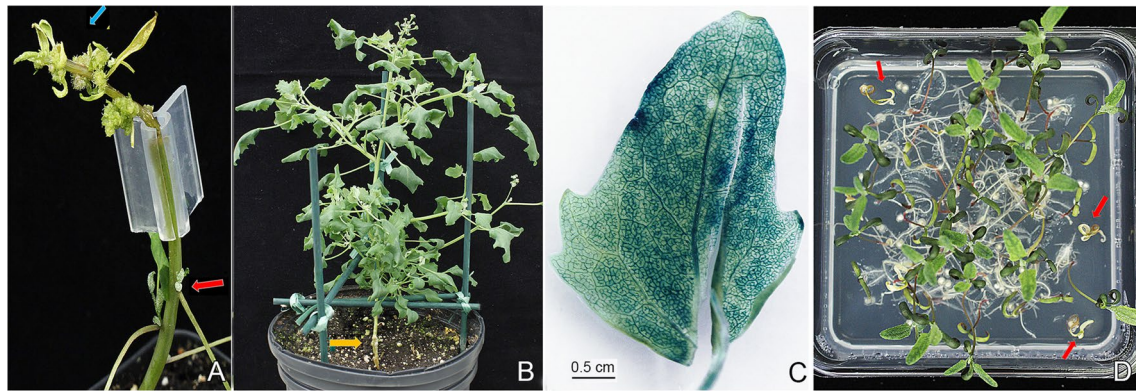


Figure 6. Grafting of transformed quinoa shoots and analysis of progeny. (A) Grafting of transformed shoots to wild rootstock. Red arrow indicates wild-type rootstock; blue arrow indicates transgenic scion. (B) Grafted plant; yellow arrow indicates grafting area. (C) GUS

(β -glucuronidase) expression in leaf of grafted plant. (D) T₁ seedlings of one proof-of-concept (POC) event in PlantCon™ with PGR-free medium supplemented with 100.0 mg L⁻¹ paromomycin (Par); red arrows indicate Par-sensitive seedlings.

Table 1. T₁ transgene transmission of the five proof-of-concept (POC) events from the transformation of *Chenopodium quinoa* Willd. Deviation from the expected Mendelian ratio of 3:1 was tested with a chi-square test

Event number	Number of T ₁ seedlings	NPTII ⁺	NPTII ⁻	χ^2 value	<i>p</i> -value
1	8	8	0	NA	0.1 ^{ns}
2	12	12	0	NA	0.04*
3	72	55	17	0.07	0.78 ^{ns}
4	119	91	28	0.14	0.71 ^{ns}
5	138	121	17	11.84	0.0005**

NA, not applicable; ns, not significant. * $p \leq 0.01$, ** $p \leq 0.01$

plants. These seeds were sown on PGR-free medium supplemented with 100.0 mg L⁻¹ Par and checked for transgene segregation (Fig. 6D). Green seedlings on this medium were classified as resistant (NPTII⁺), while yellow, smaller seedlings were designated as Par-sensitive (NPTII⁻). Segregation of transgenes in the progeny plants was statistically analyzed using a chi-square test for a 3:1 segregation ratio as expected for a single copy insertion event. Likely due to the low number of seed tested, segregation for resistance/sensitivity to Par (NPTII⁺/NPTII⁻) was not observed for events 1 and 2 (Table 1). For both events, all seeds were scored as resistant (NPTII⁺) and likely could be indicated on multi-copy of *nptII*. Analysis of the much larger progeny set for events 3 and 4 fit the expected 3:1 segregation in T₁ progeny—suggesting that these lines likely carried a single copy of the *nptII* transgene. Event #5 also segregated for the transmission of the *nptII* gene to the T₁ progeny. However, the segregation data did not fit the expected Mendelian segregation ratio expected for a single copy insertion (p -value < 0.01), which might be connected with multiple copy insertion of *nptII* gene in this event.

Protoplast Isolation and Culture In *in vitro* floral culture, leaves and hypocotyls were used for protoplast isolation and culture. For protoplast isolation, we used the method described previously by Sidorov *et al.* (2022). Briefly, culture from 4 to 5 plates was homogenized in a blender with 30 mL of W-5 medium for 5 to 8 s at high-speed (approximately 20,000 rpm; Fig. 7A). The homogenized material was then sieved through a commercial strainer, washed with W-5 medium, patted dried on sterile paper towels, and transferred to a Petri plates. The cell wall was removed by incubating the homogenized material at 28 °C for 3 hr with an enzyme solution (see “Material and Methods”). Because of the high concentration of digestive enzymes, care should be taken to avoid over digestion. After 3 hr, protoplasts were released from tissue by gentle rocking, followed by an immediate wash (Fig. 7B). Freshly isolated protoplasts were embedded into a thin alginate layer and cultured in KM modified medium, containing 72.0 g L⁻¹ glucose, 1.0 mg L⁻¹ NAA, 1.0 mg L⁻¹ BA, and 0.25 mg L⁻¹ 2,4-D (Fig. 7C). After dissolving the alginate layer, protoplast-derived colonies were cultured on MS B5 Vit medium (PhytoTech Labs®, M519), 32.0 g L⁻¹ mannitol, 10.0 g L⁻¹ sucrose, 3.0 mg L⁻¹ BA, and 0.01 mg L⁻¹ IAA (Fig. 7D, E). All details and media utilized were as previously described by Sidorov *et al.* (2022). After 1 mo, protoplast culture-derived colonies could be transferred to solid regeneration medium (MS Vit, plus 32.0 g L⁻¹ mannitol, 10.0 g L⁻¹ sucrose and supplemented with 1.0 mg L⁻¹ BA, 1.0 mg L⁻¹ Kin, and 0.2 mg L⁻¹ IBA). Colonies on this medium grew very fast, but no sign of regeneration was found. Callus derived from protoplasts was also tested for regeneration on various media with different PGR compositions (see examples on Fig. 7E, F, G). The most compact and green tissue was obtained on DKW B5 Vit medium with 10.0 g L⁻¹ sucrose, 5.0 mg L⁻¹ Zea, 3.0 mg L⁻¹ Kin, and 0.1 mg

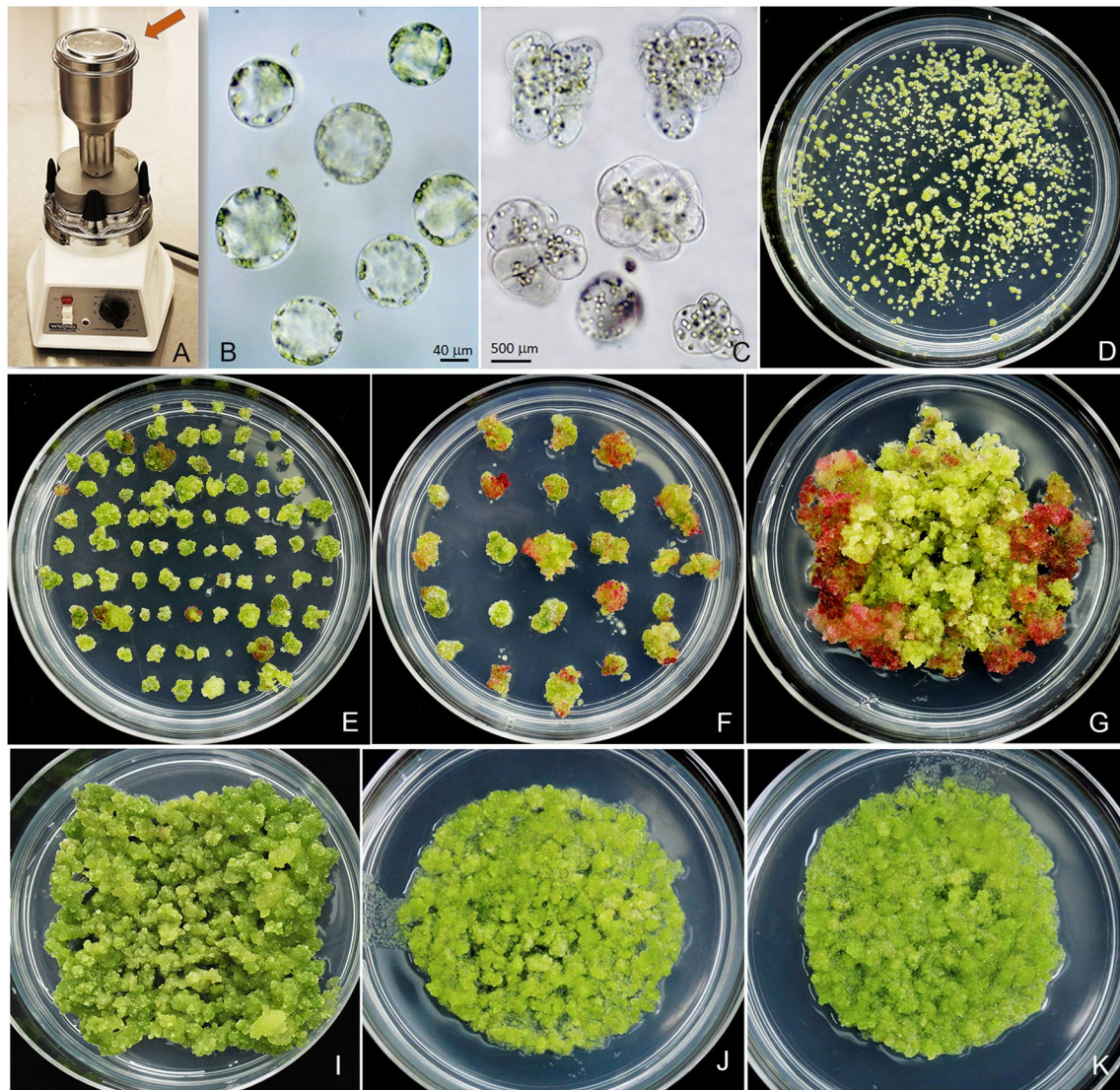


Figure 7. Inflorescence culture of *Chenopodium quinoa* Willd. protoplasts. (A) Blending of inflorescence before incubation in enzyme solution. (B) Freshly isolated protoplasts. (C, D) Dividing protoplasts and colonies formation. (E) Callus from protoplasts, Murashige and Skoog (MS) B5 Vit, 32.0 g L⁻¹ mannitol, 10.0 g L⁻¹ sucrose, 3.0 mg L⁻¹ BA, and 0.01 mg L⁻¹ indole-3-acetic acid (IAA). (F) MS B5 Vit, 30.0 g L⁻¹ sucrose, 3.0 mg L⁻¹ BA, and 0.01 mg L⁻¹ IAA. (G) MS

B5 Vit 32.0 g L⁻¹ mannitol, 10.0 g L⁻¹ sucrose, 1.0 mg L⁻¹ zeatin (Zea), and 0.1 mg L⁻¹ IAA. (I) DKW medium (PhytoTech Labs®, D2470), 10.0 g L⁻¹ sucrose, 5.0 mg L⁻¹ Zea, 3.0 mg L⁻¹ kinetin (Kin), and 0.1 mg L⁻¹ IAA. (J) Quinoa callus on DKW medium (20.0 g L⁻¹ sucrose, 1.0 mg L⁻¹ Kin, 0.5 mg L⁻¹ BA, 0.2 mg L⁻¹ indole-3-butyric acid) without NaCl, and on the same medium with 50.0 mM NaCl (K); 20-d culture.

L⁻¹ IAA (Fig. 7I). Since quinoa is a halophyte, the possibility of regenerating plants on media supplemented with NaCl (50 mM) was also tested. No difference in growth rate of the callus on medium with or without NaCl was observed (no data presented, see comparison on Fig. 7J, K), nor could plants be regenerated in any of the treatments. In summary, it was shown that plant protoplast cultures could be established from quinoa leaves, hypocotyls, and floral explants; however, regeneration could not be established from any of the protoplast-derived callus on the media we tested. Despite the inability to regenerate plants from the protoplast culture, we note that

the protoplast cultures could be used for transient assays, as well as for stable transformation and editing of individual callus lines—ideal for the analysis of potential constructs and expression of transgenes.

Conclusion

The development of a robust transformation protocol is critical for advanced genetic studies and plant improvement. Here, we report the development of a transformation

system based on *Agrobacterium*-mediated transformation of *in vitro* floral culture of quinoa. Floral culture is meristematic culture primarily consisting of flowers at different stages of development that can be used for transformation and plant production without the need for callus generation or plant regeneration. Like transforming apical meristems, transforming floral cultures requires stringent and extended selection to prevent the production of chimeric explants. The production of non-chimeric T₀ transgenic floral shoots, which produce transgenic seeds was demonstrated. It is opening new possibilities for creating novel genetic modification and advanced our understanding of the underlying genetics of valuable agricultural traits in this emerging crop species. These results suggest that floral culture transformation also has a great potential as a transformation system for other species, particularly for other *in vitro* recalcitrant species.

Acknowledgements We thank Jenn To, Nahum Matamoros Castillo, and the Genotyping TAQMAN Team from Bayer Crop Sciences (Ches-terfield, MO) for their support and technical assistance on the project. We gratefully acknowledge David Brenner (USDA-NPGS, Ames, IA) for providing QQ74 seeds.

Declarations

Competing Interests Not applicable.

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