



The Establishment of Two Efficient Transformation Systems to Manipulate and Analyze Gene Functions in Quinoa (*Chenopodium quinoa* Willd.)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors YW and YZ performed most of experiments and wrote the manuscript. Author JM helped with experimental design and data analysis; Authors CD, SJ, YW and ZY helped with transformation procedure and plasmid preparation. Author YZ provided valuable suggestions for transformation strategy. Authors SS and TX initiated and designed this project and wrote the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Quinoa (*C. quinoa*) is considered a gluten-free food with abundant nutrients and high tolerance to multiple abiotic stresses, which is the potential to become a major crop in future. Genetic manipulation will provide powerful tools to investigate the function and mechanism of those

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important genes in the regulation of quinoa development and stress responses, and further improve the quinoa in the field. However, the efficient plant transformation system for quinoa has not been well developed yet. Here, we established two rapid and efficient transformation systems for quinoa by using hairy roots and agroinfiltration of leaves, which provide useful tools for quick analysis of gene function. Hairy roots were obtained from three types of explants: cotyledon-nod with hypocotyl, cotyledon itself, and hypocotyl pieces. Interestingly, explants of cotyledon-nod with hypocotyl showed the highest transformation efficiency at 67.9%, and cotyledon displayed medium efficiency at 42.2%, while hypocotyl explants with the lowest at 31.6%. We also obtained transgenic quinoa roots successfully *in-vivo*, which showed low efficiency but provides a potential method to test gene functions in live plants. By using young leaves for agroinfiltration, direct injection showed a better transgenic effect compared with vacuum penetration. In juxtaposition, the transformation systems using both hairy root and leaf infiltration establish an efficient and convenient way to manipulate and analyze gene functions in quinoa, and a potential strategy for transgenic quinoa.

Keywords: Agroinfiltration; cotyledon-nod; genes; hairy root; quinoa; transformation.

1. INTRODUCTION

Quinoa, which belongs to the *Chenopodiaceae* family, contains abundant nutrients and a staple diet for the indigenous inhabitants of South America, including the Andean regions for more than 7000 years [1,2]. Quinoa, considered a potential future major crop, has attracted intense public attention since the Food and Agriculture Organization of the United Nations announced the “International Year of Quinoa” in 2013 [3,4]. Previous analyses showed that quinoa constitutes all of the essential amino acids required for human health, and contains more protein content than rice, barley, corn, rye and sorghum [5-11]. Moreover, quinoa can sustainably endure well in extreme growth conditions, including drought, salinity and frost [12,13]. For instance, some varieties of quinoa can tolerate salinity around 700 mM NaCl, which concentration is higher than seawater [14-16]. Due to climate change paradigm shifts, these unique properties of quinoa, including stress tolerance and high nutrition, result in its considerable role in global food security. Recently, there is a surge in the functional analysis of quinoa genes due to the availability of the complete genome sequence [17]. However, all the functional verifications were only through the comparison of gene expression profiles based on the relative phenotypes. Moreover, it is crucial to develop a method to study the gene function through genetic manipulation.

At present, *Agrobacterium tumefaciens*-mediated stable transformation is a prominent method that was used to analyze gene function regulating plant development or environmental adaptation. To date, however, it still has not yet been reported for the successful stable transformation in quinoa. The function analyses were efficiently

done in model plant systems such as *Arabidopsis thaliana* or *Nicotiana benthamiana* [18]. However, there was still a significant barrier referring to unique genes and pathways of quinoa. Thus, both transient and stable transformation systems were critically established to investigate the function of valuable genes in quinoa.

Agroinfiltration is the foremost *A. tumefaciens*-mediated instant transformation mechanism for gene function related studies, which is fast, real-time, and economical. Agroinfiltration is a convenient tool in plant gene function research without resorting to stable transformation [19]. Through the injection of *A. tumefaciens*, extraneous genes are transiently expressed in leaves, fruits, flowers, petals, roots, needles and other tissues [20]. Compared with a stable transformation that produces minimal transgenic lines and needed to consider the effect of T-DNA insert position, agroinfiltration infects a large number of cells with high efficiency in a short time, and T-DNA would not be integrated into the genome [21,22].

Agrobacterium rhizogenes-mediated transformation system has many advantages as compared with *A. tumefaciens*. *A. tumefaciens* is time-consuming, genotype-dependent and laborious [23], *A. rhizogenes* can potentially infect numerous varieties of dicotyledonous plants and subsequently induce transgenic hairy root lines [24]. The hairy root system can excellently characterize root development and related gene functions, which has been extensively applied in metabolic engineering, rhizosphere physiology, environmental stress response, etc. However, when the target genes or biological pathways have a functional expression in the plant roots, the hairy root

system of transformation will not be applicable. The composite plant consisting of the wild-type shoots and transgenic hairy roots can be used to study root-shoot interactions and resistance against the various environmental and abiotic stresses, especially when roots were in stress conditions, such as salinity, drought or heavy metal [25-30]. Besides, composite plants were employed in previous studies to elucidate positive responses in hormone transport, symbiosis, secondary metabolites, and root nutrient uptake. Up to date, various plant species have been established *ex vitro* composite plant transformation system, such as pea, soybean, tea and peanut [31,32]. Both hairy roots *in-vitro* and composite plants could be derived in a cost-effective, fast, and efficient way, which were considered as a practical protocol, especially for gene function study in roots. In this study, we developed the hairy roots system and agroinfiltration of leave system, which were used to analyze gene function rapidly in quinoa.

2 MATERIALS AND METHODS

2.1 Plant Materials Preparation

Four quinoa cultivars: MS509/N, QN108B, 141R, 20ALC, were obtained from the United States Department of Agriculture, all the seeds were stored at 4-6 °C with 15% humidity to maintain viability. Healthy seeds were carefully selected and then surface sterilized with chlorine gas (produced by mixing sodium hypochlorite and HCl) in a desiccator for 4-6 h. Sterilized seeds were then moved to a clean bench for air blowing for one day and germinated in half strength MS medium at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, exposure 16 h light/8 h dark at 25 °C for 5-6 days to obtain suitable explants from seedlings for hairy roots induction [33].

For *in-vivo* seedlings, healthy seeds were carefully selected and sowed in a mixture of soil: vermiculite: perlite (5:3:2) in the greenhouse at 540 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light exposure of 16 h light/8 h dark, two-week-old plants with only primary leaves spread out were used for agroinfiltration, two-month-old plants were used for *in-vivo* hairy roots induction.

2.2 Preparation of the *Agrobacterium* Strains for Infection

A. rhizogenes K599 harbouring the binary vector *PTF102* comprising a *GUS* gene was used in the transgenic hairy root induction. K599 without the vector *PTF102* was used to induce hairy roots,

which were negative control. K599 which was streaked on solidified YEP medium (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl, 15 g L⁻¹ agar, pH 6.8) with antibiotics, after being incubated at 28 °C for two days. Half loop of the cultures was collected and restreaked on a new YEP medium with a disposable plastic inoculation loop then incubated for another one day. The *A. rhizogenes* cultures were collected and suspended to OD₆₆₀ of 0.8 with infection medium (MS basal medium, 19.6 mg L⁻¹ AS, 500 mg L⁻¹ MES, 3% sucrose, pH 5.4). *A. tumefaciens* EHA105 harbouring the binary vector *pBI121* comprising a *GUS* gene was used for agroinfiltration of leaves. EHA105 was prepared in the same way as K599. The cultures were collected and suspended to OD₆₆₀ of 1.0 with infection medium (1/10 MS basal medium, 19.6 mg L⁻¹ AS, 100 mg L⁻¹ MES, 2% sucrose, pH 5.6).

For testing and verifying the hairy roots system, the vector *pX11* (kindly donated by Dr Asaph Aharoni, Israel) comprising *CYP76AD6*, *CYP76AD1*, *BvDODA1* and *cDOPA5GT*, was used to produce betacyanins (kindly donated by Dr Asaph Aharoni, Israel) comprising *CYP76AD6*, *CYP76AD1*, *BvDODA1* and *cDOPA5GT*, which was used to produce betacyanins [34].

2.3 Hairy Roots Induction from Three Types of Explants

Three types of explants were obtained from five-day-old aseptic seedlings (Fig.1-A) (MS509/N, QN108B, 141R, 20ALC), including the cotyledons with hypocotyl segments, tagged as CHE, were excised approximately 1cm below the node of the cotyledons; the cotyledon explants excised from the seedlings, tagged as CE; the hypocotyl explants were cut into a length of 1-1.5 cm, tagged as HE.

The explants of CHE were gently pierced near the cotyledonary node with a syringe tip for two to three times, CEs were pierced in the middle of the abaxial side (Fig. 1B), while the HEs were obtained by just cutting off the hypocotyl into small pieces. The explants were immediately incubated in the infection medium for 30 min at 25°C (Fig. 1C), then transferred to the co-culture medium at 22 °C in the dark (Fig. 1D). Two days later, the explants were transferred to hairy roots induction medium (B5 basal medium, 500 mg L⁻¹ MES, 3% sucrose, pH 5.6) at 25 °C in the dark for two weeks.

2.4 Hairy Roots Induction *in-vivo*

In-vivo hairy roots were induced by injecting the *Agrobacterium* culture into the taproots of two-month-old seedlings (MS509/N) (Fig. 3A). Part of the soil was moved away to expose the taproot. The *A. rhizogenes* K599 infection culture of OD₆₆₀ of 1.0 was slowly injected into the taproots with a 1 mL syringe (Fig. 3B). After injection, the taproot was covered with wet vermiculite and a plastic film to maintain humidity (Fig. 3C). The seedlings were grown at 540 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light exposure of 16 h light/8 h dark at 25°C until the hairy roots growing out.

2.5 Agroinfiltration of Quinoa Leaves

Two-week-old seedlings (MS509/N) with only primary leaves spread out were chosen for agroinfiltration (Fig. 4A). Two methods were developed for agroinfiltration of quinoa leaves, one was syringe injection, and the other was vacuum penetration. The cotyledons and one of the primary leaves were removed. The *A. tumefaciens* EHA105 infection culture was injected into the other primary leaf remaining on the seedlings with a needleless 2.5 mL syringe

(Fig. 4B). The leaf infiltrated with EHA105 harbouring *pBI121* without *GUS* gene was used as the negative control. After injection, the treated seedlings were covered with a plastic jar and grew at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity with the photoperiod of 16 h light/8 h dark at 22 °C for 4 days (Fig. 4C).

In vacuum penetration, the remaining primary leaf was gently damaged by pressing a wire brush on the abaxial surface while placing a rubber cork wood on the adaxial surface (Fig. 4D). The damaged leaf was immediately incubated in *Agrobacterium* culture and vacuumed at 0.7 MPa for 1 min in a tightly sealed desiccator (Fig. 4E). After infiltration, the seedlings were treated in the same way as were injected for recovery.

2.6 Histochemical Staining Assay of GUS Activity

The hairy roots produced from three types of explants and *in-vivo* injection were collected for GUS staining assay. The leaves after agroinfiltration were subjected to GUS assay then soaked in 100% ethanol to dissolve and remove the chlorophyll.

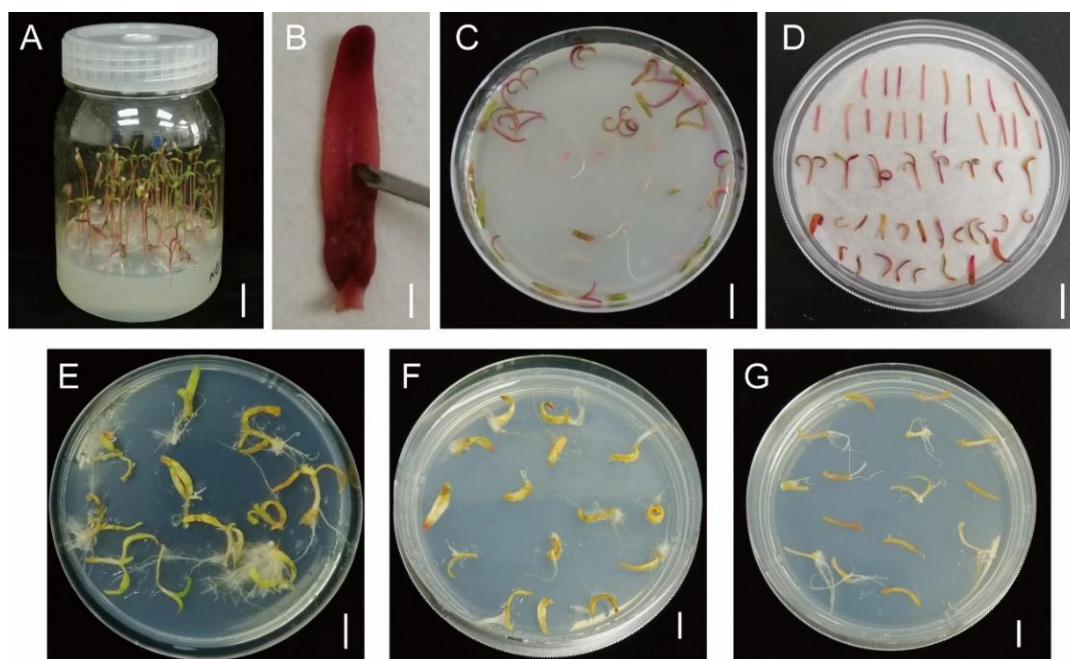


Fig. 1. (A) Hairy roots induction *in-vitro*. 5-day-old seedlings were obtained from half-strength MS medium, (B) cotyledon was pierced with a syringe tip at the abaxial side, (C) three types of explants were immersed into K599 culture, (D) explants were transferred to co-culture medium covered with sterile filter paper, (E) hairy roots from CHE in hairy root induction medium after two weeks, (F) hairy roots from CE, (G) hairy roots from HE. Scale bar A, C-G:1 cm; B: 0.15 cm

2.7 Polymerase Chain Reaction (PCR) Verification of *GUS* Gene

When hairy roots grew out from the explants, they were collected for PCR analysis of *GUS* gene. The plasmid of *PTF102* was used as a positive control. To confirm the insertion of *GUS* gene, PCR was performed with a pair of primers specific to *GUS* gene (5'-GCAGGTCAGTGGATTTTGGT-3';5'-ACGGCAGAGAAGGTACTGGA-3').

The amplification conditions were a 3-min melting step at 95°C, followed by 30 cycles of a 30-s melting step at 95°C, a 30-s annealing step at 55°C, and a 25-s elongation step at 72°C and a 5-min elongation step at 72°C after cycles. PCR products were analyzed by electrophoretic separation on 1% agarose gels.

3. RESULTS

3.1 Hairy Roots Induction *In-vitro*

Hairy roots could be induced from all four cultivars, and MS509/N was mainly used in this study. All three types of explants, including cotyledonary node (CHE), cotyledon (CE) and hypocotyl (HE) produced hairy roots after two weeks (Fig. 1E, F, G). *GUS* staining assay showed that CHE got the highest transformation efficiency of 67.9%, followed by CH of 42.2%, and the lowest was HE of 31.6% (Fig. 2A, B, C). Hairy roots from the negative control showed no blue colour in *GUS* staining assay (Fig. 2D). PCR analysis also confirmed the *GUS* gene expressed successfully in hairy roots (Fig. 2I). The transformation efficiency was calculated by the ratio of the number of explants that produced positive hairy roots to the total explants number (Table 1). The transformation efficiency differed among these explants, CHE and CE showed relatively higher transformation efficiency, which suggests they may be suitable explant type for transformation. The red colour of hairy roots produced by the transformation of *pX11* confirmed that the four genes were expressed in plant cells in this system (Fig. 2E, F, G, H).

3.2 Hairy Roots Induction *In-vivo*

Many hairy roots initiated from the injection sites grew out from soil two weeks after the injection (Fig. 3D). However, with the *GUS* assessment, almost all the roots were negative, and occasionally we can get positive ones (Fig. 3E). Taproot injection was a tedious, time-consuming method with lower transformation efficiency compared with *in-vitro* system.

3.3 Leaf Agroinfiltration

GUS staining assay showed the negative control had no blue colour and almost whole leaf turned blue by the injection of *Agrobacterium*. At the same time, only partial wounded spots appeared blue from wire brush and vacuum penetration system (Fig. 4F, G, H). Since the leaf age, and the concentration of *Agrobacterium* were the same (OD₆₆₀ of 1.0), the result indicated that vacuum penetration has much less throughput compared with syringe injection. For our cultivar, we found that the leaves from two-week-old seedlings were suitable for agroinfiltration. If quinoa seedlings were younger than two weeks, leaves would be too small and delicate to carry out the syringe technique and thus result in being seriously damaged from the injection leading to necrosis. While seedlings older than two weeks had lower transformation efficiency.

4. DISCUSSION

4.1 The Hairy Roots Induction *In-vitro* and *In-vivo*.

Hairy roots could be induced from different organs of quinoa, such as cotyledonary node, cotyledon, hypocotyl, and *in-vivo* taproot in soil. Both CHE and CE had cotyledons and higher transformation efficiency. It was inferred that cotyledons provide more nutrition for hairy roots induction and further quick elongation, and CHE is the optimal explant type for quinoa hairy roots *in-vitro* according to our experiments.

Table 1. The efficiency of three types of explants

Explant type	Total number	Number explants of hairy roots	Infection efficiency
Cotyledon node	56	38	67.9%
Cotyledon	64	27	42.2%
Hypocotyl	57	18	31.6%

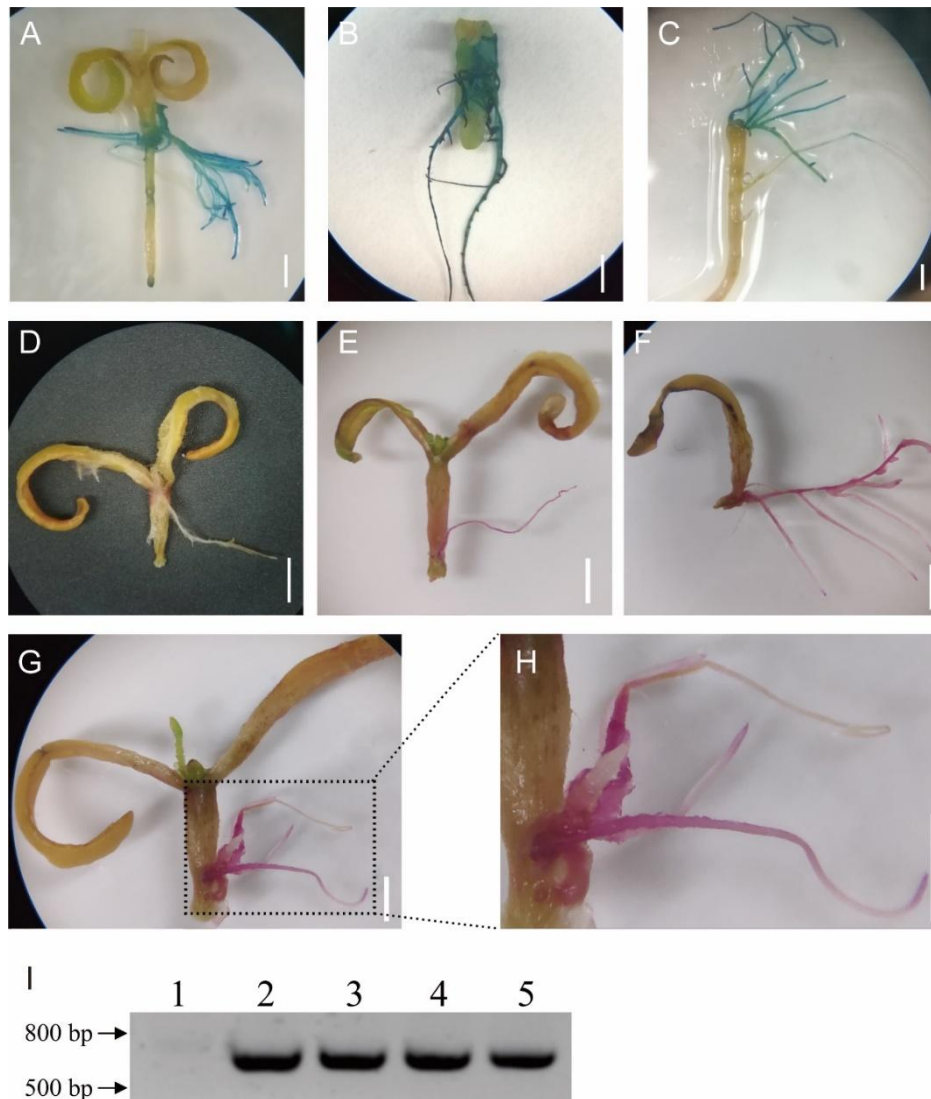


Fig. 2. (A) GUS assay with hairy hoots from three types of explants: CHE, (B) CE, (C) HE, (D) GUS assay with hairy roots from the negative control; (E) red pigmentation in hairy roots: CHE, (F) CE, (G) CHE, (H) and details of hairy roots with enlarged scale; PCR analysis of GUS gene: 1 DNA from original roots as a negative control, 2 plasmid of *PTF102* as a positive control, 3-5 DNA from hairy roots of CHE, CE, HE (I). Scale bar A-C: 0.2 cm, D-H: 0.25 cm.

For the *in-vivo* system, it was quite difficult to get positive hairy roots by injecting the taproot. However, the composite plant with transgenic roots is an excellent research tool when some genes are expressed in the root and the transcriptions regulate the upper part of the plant. It is irreplaceable for studying root-shoot interaction, especially for those plants which stable transformation has been unsuccessful. It is necessary to optimize this transformation system to obtain more composite plants. The age of seedlings used for injection may be a crucial factor in the success of the transformation mechanism with high levels of efficiency. In other

plants, different ages were chosen for producing composite plants for high efficiency such as one-day-old soybean [35], 6-week-old peanut [36], two-month-old tea tree [32]. For herbaceous plant quinoa, we infer that seedlings younger than two months old may be more suitable for infection of the explant. Environmental factors, including humidity, the temperature should also be studied further in this system.

Hairy roots grow fast even without hormonal supplements, which can be considered as bioreactors to produce bioactive substances more efficiently and economically. Quinoa's

abundant bioactive substances also attract people's attention, such as saponins, polyphenols and flavonoids [37]. These secondary metabolites usually accumulated in its roots [38]. In our research, hairy roots produced betacyanins which will be a new suggestion for studying of these bioactive substances in quinoa.

The transformation of the hairy root was considered as a powerful tool to detect gene function *in-vitro*. Hairy root originates from a single cell and is non-chimeric, which is an ideal

material for producing transgenic plants. Up to date, many plants regenerate successfully from hairy root cultures, such as *Nicotiana tabacum*, *Medicago sativa*, *Zea mays*, and *Solanum tuberosum* [39-41]. Especially for *Spinacia oleracea* (spinach), which is closely related to quinoa, the shoots can be regenerated from the hairy roots [42]. Since *A. tumefaciens*-mediated stable transformation has been unsuccessful yet, the hairy root system, this experiment without any doubt provides an alternative way to explore quinoa genetic transformation.

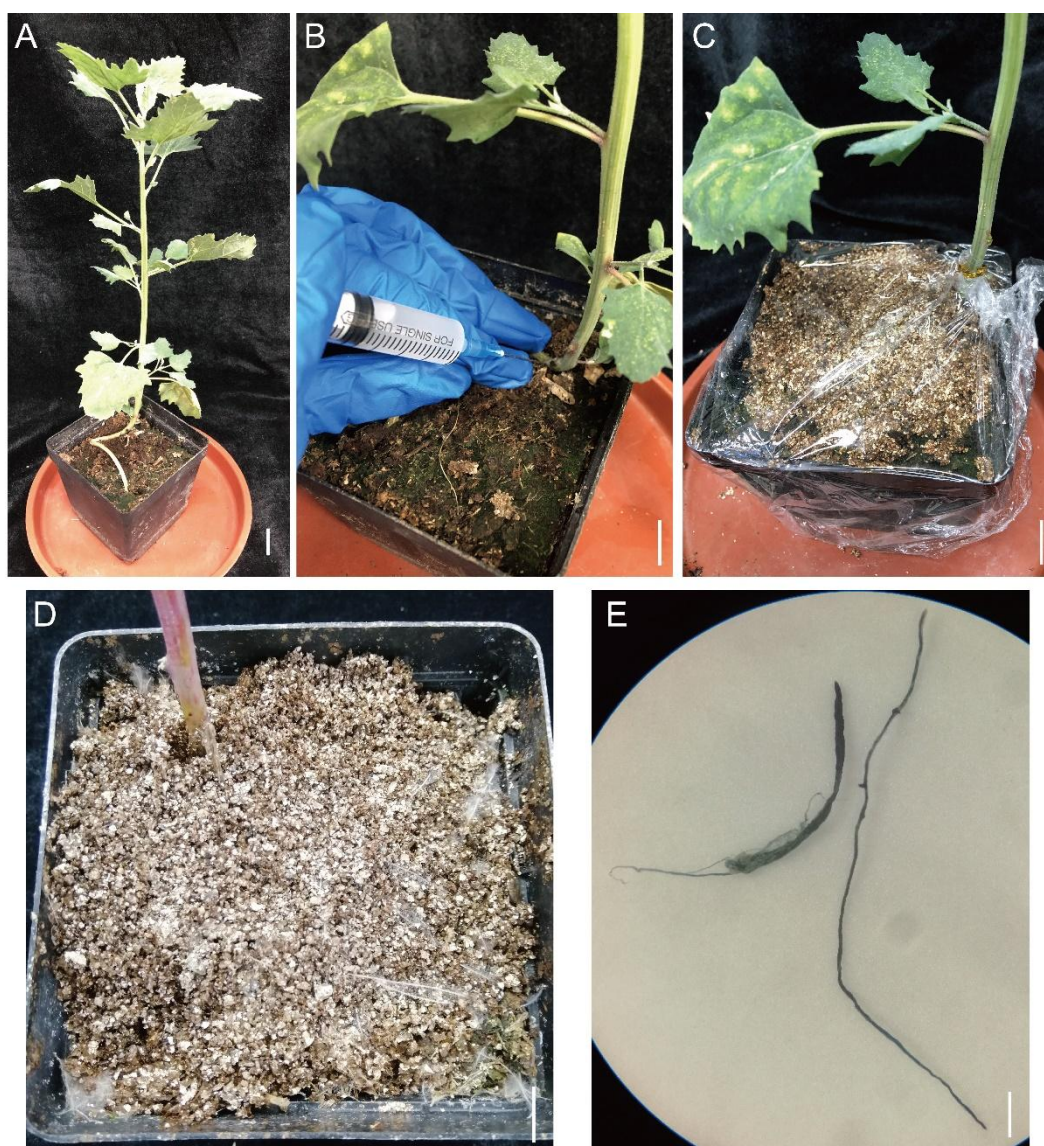


Fig. 3. (A) Hairy roots induction *in-vivo*. 2-month-old seedling was used for injection, (B) the taproot was injected with a syringe needle, (C) covered with wet vermiculite and plastic film to keep humid, (D) hairy roots of quinoa injection hairy roots grew out from quinoa's tap root after two weeks, (E) hairy roots cut from the tap root and assay for β -glucuronidase activity. Scale bar A:0.5 cm; B-C:1 cm; D-E:0.25 cm

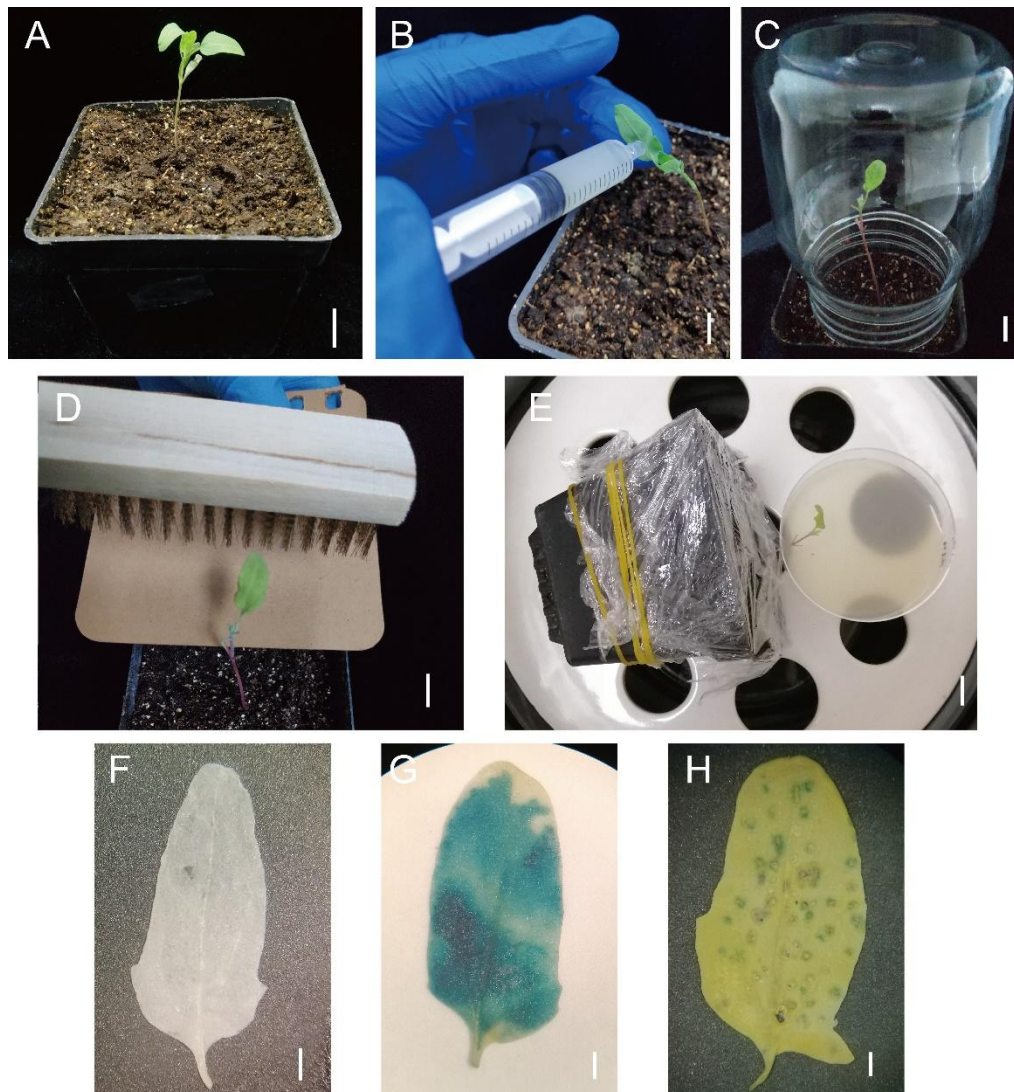


Fig. 4. (A) Agroinfiltration of quinoa leaves. Leaf was used for agroinfiltration from 5-day-old seedling, (B) leaf was injected at the abaxial side with a needleless syringe, (C) the treated seedlings were covered with a plastic jar for recovery for 4 days, (D) leaf was pressed at the abaxial side by the brush, (E) leaf was incubated in *Agrobacterium* culture and vacuumed at 0.7 MPa for 1 min, (F) the GUS staining of leaf injected with 1/10 MS culture (CK), (G) GUS assay of leaf injection, (H) GUS assay of leaf vacuum penetration. Scale bar A-E: 1cm; F-H: 0.2 cm

4.2 Agroinfiltration of Quinoa Leaves

Agroinfiltration is an efficient system in many analyses, such as gene expression, promoter activities, metabolism, and protein-protein interaction [43,44]. To our knowledge, this is the first study in the research of agroinfiltration of quinoa. Some aspects need to be improved, especially for vacuum penetration. Based on our research, we assert that the seedlings stage is one of the crucial factors influencing agroinfiltration efficiency. Similarly, tobacco in

which the age of leaves influenced agroinfiltration efficiency significantly. The younger leaves had higher efficiency and much better result in agroinfiltration [22]. Keeping high humidity was another key factor for the plant to survive from agroinfiltration damage. If quinoa seedlings were not covered with jars to keep humidity, the treated leaves were easy to wither or even to die.

The ingredients of the medium should also be considered. In previous studies, 1/2 MS, or 1/4 MS strength were used as basal infection medium

[45-47]. In our protocol, 1/10 MS strength worked better than full strength MS, presumably due to the sudden increase of high ion concentration, which may be deleterious for cell growth.

The recovery length after agroinfiltration also played an important role in influencing transient expression assay. It was inferred that genes began to express a few hours after transformation [48-50]. And many reports showed that the expression of genes usually reaches a peak in plant tissues 2-4 days after agroinfiltration; hence, the evaluation should be performed during this period [50,51]. In our study, leaves can be stained in dark blue colour when GUS assay was performed on the fourth day, but a much lighter colour on the second day. So, we considered that four days after agroinfiltration was suitable for the recovery of quinoa seedlings.

In the agroinfiltration system, the whole process could be finished within one month and independent of regeneration. Comparably, stable transformation always needs a mature regeneration system and several months of tedious work to obtain the positive T1 plants for gene function analysis. Worse still, there are some plants whose stable transformation is inefficient or has not even been established (such as quinoa). Overall, agroinfiltration will be an effective replacer mechanism to achieve gene transformation under these circumstances.

5. CONCLUSION

Two rapid and efficient systems for gene function analysis were established in this report: hairy roots system and agroinfiltration of leaves system. Hairy roots can be obtained from the three types of explants in two weeks with different efficiency. When cotyledon-nod with hypocotyl (CHE) were used as explants, the transformation efficiency was 67.9%, followed by cotyledon (CE) of 42.2% and hypocotyl (HE) of 31.6%. The injection of quinoa taproot can be successfully completed with very low efficiency. The first pair of spread leaves of two-week-old seedlings were the most suitable material for agroinfiltration. The leaf injection displayed a much better result than vacuum penetration in transient expression assay.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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