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

Abstract

Quinoa (*C. quinoa*) is considered a gluten-free food with abundant nutrition 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 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 obtainedtransgenic quinoa roots successfully*in-vivo*, which showedlowefficiency 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

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1. INTRODUCTION

Quinoa, which belongs to the *Chenopodiaceae* family, containsabundantnutrition and a staple diet for theindigenous 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 thatquinoa constitutes all of the essential amino acids required for human health, and containsmore 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 inthe functional analysis of quinoa genes due to the availability of the complete genome sequence [17]. However, all thefunctional 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, *Agrobacteriumtumefaciens*-mediated stable transformation is a prominent method that was used to analyzegene function regulating plant development or environmental adaptation. To date, however, it still has notyet been reportedfor 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 genesin quinoa.

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Agroinfiltration is the foremost*A. tumefaciens*-mediated instant transformation mechanism for gene function related studies, which is fast, real-time,andeconomical. Agroinfiltration is a convenient tool in plant gene function research without resorting to stable transformation [19]. Through the injection of *A.tumefacien*, extraneous genes are transiently expressed in leaves, fruits, flowers, petals, roots, needles and other tissues[20]. Compared with a stable transformation that producesminimal 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 hasbeen 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 rootswere in stress conditions, such as salinity, drought or heavy metal [25-30].Besides, composite plants wereemployed 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 *exvitro* 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 efficientway, whichwere considered as a practical protocol, especially for genefunction study in roots.In this study, we developed the hairy roots systemand agroinfiltration of leavesystem, which were used to analyze gene function rapidly in quinoa.

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2 MATERIALS AND METHODS

2.1 Plant materials preparation

Four quinoa cultivars: MS509/N, QN108B, 141R, 20ALC, were obtained from the UnitedStates Department of Agriculture, all the seeds were stored at 4-6°C with 15% humidityto 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 4-6h. Sterilized seeds were then moved to a clean bench for air blowing for one day and germinated in haft strength MS medium at 50μmol m^{-2} s^{-1} light, exposure16 h light/8 h dark at 25°Cfor 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 540μmol m^{-2} s^{-1} light exposureof 16 h light/8 h dark, two-week-old plants with only primary leaves spreadout 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. rhizogenesK599 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 gL^{-1}tryptone, 5 gL^{-1} yeast extract, 5 gL^{-1} NaCl, 15 gL^{-1}agar, pH 6.8) with antibiotics, after being incubated at 28℃ for two days. Half loop of the cultureswas 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 OD_{660} of 0.8 with infection medium(MS basal medium, 19.6 mgL^{-1} AS, 500 mgL^{-1} MES, 3% sucrose, pH5.4).*A. tumefaciensEHA105* 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 OD_{660} of 1.0 with infection medium （1/10MS basal medium, 19.6mgL^{-1}AS, 100 mgL^{-1} MES, 2% sucrose, pH5.6).

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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 fromthree types of explants

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

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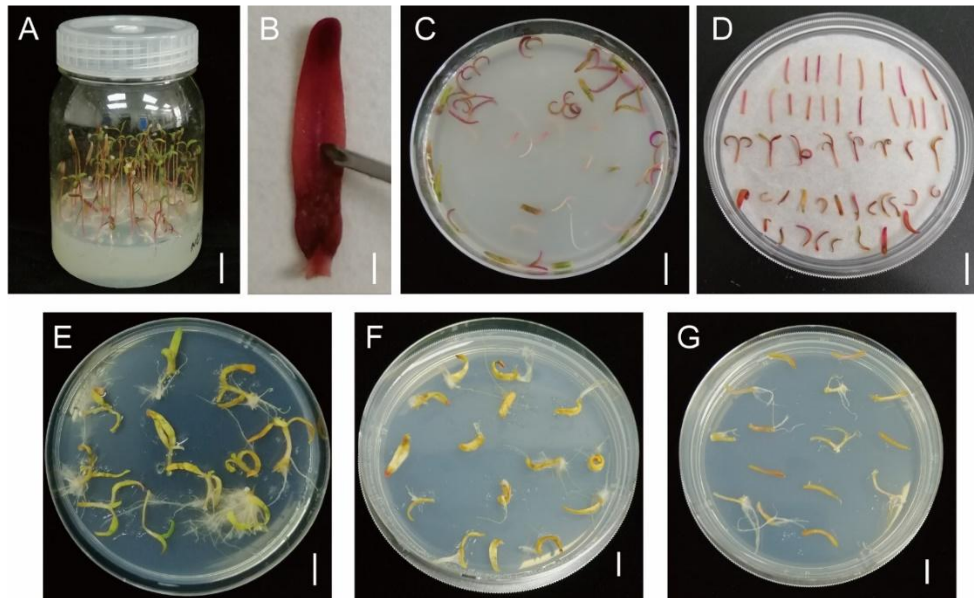

Figure 1(A) Hairy roots induction *in-vitro*. 5-day-old seedlings were obtained from half-strength MS medium, (B) cotyledon was pierced with a syringe tip at the abaxial side , (C) three types of explants were immersed into *K599* culture, (D) explants were transferred to co-culture medium covered with 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.

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.1-B), 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℃ (Fig.1-C),then transferred to the co-culture medium at 22℃in the dark (Fig.1-D). Two days later, the explants were transferred to hairy roots induction medium(B5basal medium, 500 mgL^{-1} MES, 3% sucrose, pH5.6) at 25℃in the dark for two weeks.

2.4 Hairy roots induction*in-vivo*

In-vivo hairy roots were induced by injecting the *Agrobacterium* cultureinto the taproots of two-month-old seedlings (MS509/N) (Fig.3-A). Part of the soil was moved away to expose the taproot.The *A. rhizogenesK599* infection culture of OD_{660} of 1.0 was slowly injected into the taproots with a 1 mL syringe (Fig.3-B). After injection, the taproot was covered with wet vermiculite and a plastic film to maintain humidity (Fig.3-C). The seedlings were grown at 540μmol m^{-2} s^{-1} light exposureof 16 h light/8 h dark at 25℃until the hairy roots growing out.

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2.5 Agroinfiltration of quinoa leaves

Two-week-old seedlings (MS509/N) with only primary leaves spread out were chosen for agroinfiltration (Fig.4-A). 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. tumefaciensEHA105* infection culture was injected into the other primary leaf remaining on the seedlingswith a needleless 2.5 mL syringe (Fig. 4-B). 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 μmol m^{-2} s^{-1}light intensity with the photoperiod of 16 h light/8 h dark at 22℃for 4 days(Fig. 4-C).

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.4-D). The damaged leaf wasimmediately incubated in *Agrobacterium*culture and vacuumed at 0.7 MPa for 1 min in a tightly sealed desiccator (Fig.4-E). 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 forGUS staining assay.The leaves after agroinfiltration were subjected toGUSassaythen soaked in 100% ethanol to dissolve and remove the chlorophyll.

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 insertionof *GUS* gene, PCR was performed with a pair of primers specific to *GUS*gene(5’-GCAGGTCACTGGATTTTGGT-3’;5’-ACGGCAGAGAAGGTACTG GA-3’). The amplification conditions were a 3-min melting step at 95 ℃, followed by 30 cycles of a 30-s melting step at 95 ℃, a 30-s annealing step at 55 ℃, and a 25-s

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elongation step at 72 ℃ and a 5-min elongation step at 72 ℃ 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.1-E,F, G). GUS staining assay showed that CHE got the highest transformation efficiencyof 67.9%, followed by CH of 42.2%, and the lowest was HE of 31.6% (Fig.2-A, B, C).Hairy roots from the negative control showed no blue colour in GUS staining assay (Fig.2-D). PCR analysis also confirmed the *GUS*gene expressed successfullyin hairy roots(Fig.2-I). The transformation efficiency was calculated by the ratio of the number of explants thatproduced positive hairyroots to the total explants number (Table1). 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 geneswere expressed in plant cells in this system (Fig.2-E, F,G,H).

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%

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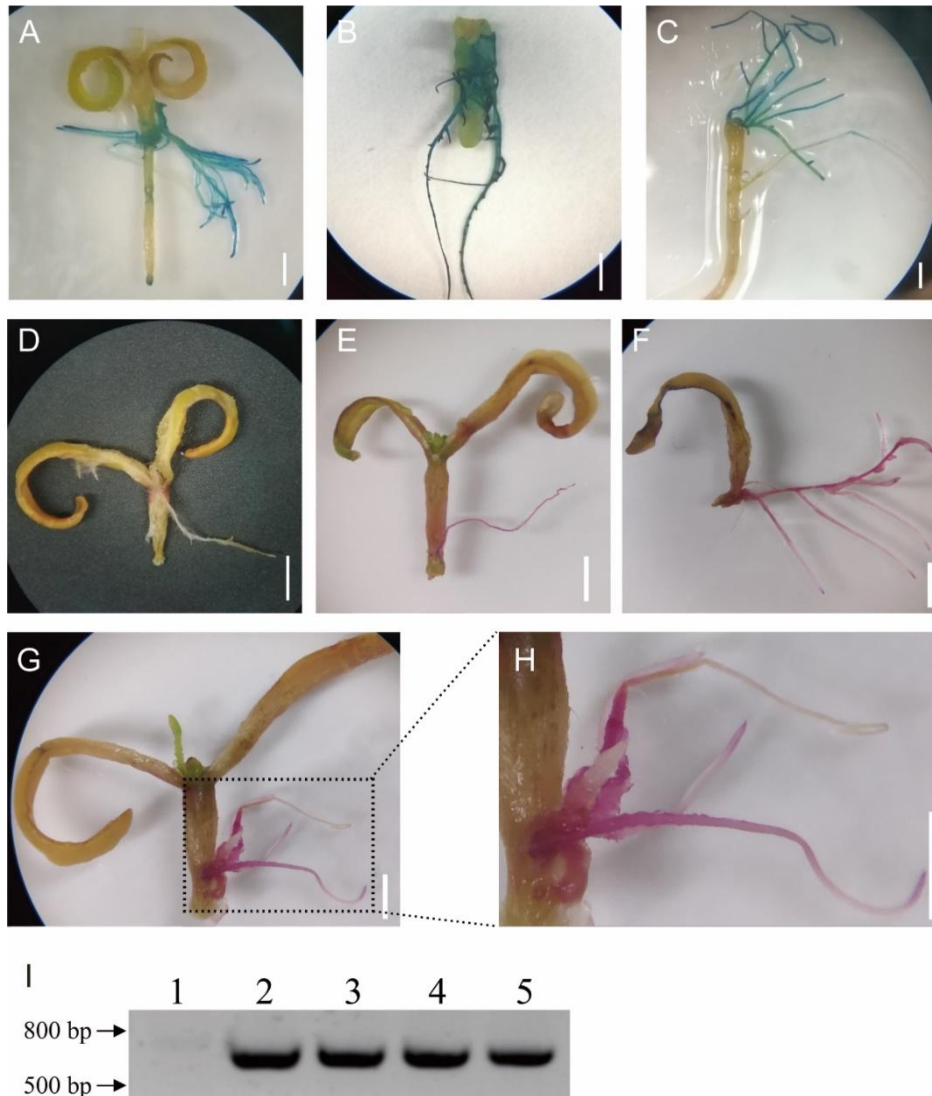

Figure 2 (A) GUS assay with hairy hoots from three types of explants: CHE, (B) CE, (C) HE, (D) GUS assay with hairy roots from the negative control; (E) red pigmentation in hairy roots: CHE, (F) CE, (G) CHE, (H) and details of hairy roots with enlarged scale; PCR analysis of GUS gene: 1 DNA from original 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.

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.3-D). However, with the GUS assessment, almost allthe roots were

negative, and occasionally we can get positive ones (Fig.3-E).Taproot injection was a tedious, time-consuming method with lower transformation efficiency compared with*in-vitro* system.

Figure 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.

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.4-F, G, H). Since the leafage,and the concentration of *Agrobacterium*were the same (OD_{660} 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 inbeingseriously damaged from the injection leading to necrosis. While seedlings older than two weeks had lower transformation efficiency.

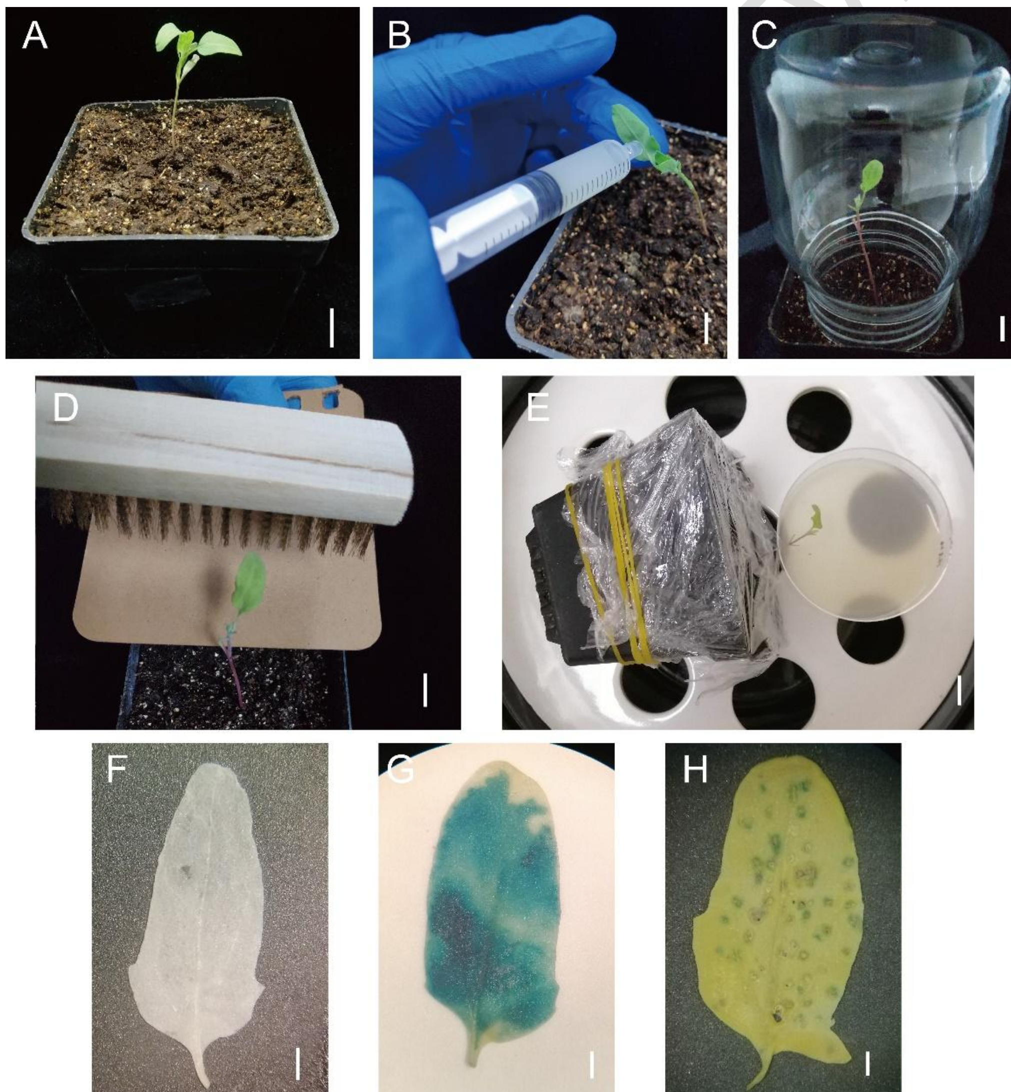

Figure 4 (A) Agroinfiltration of quinoa leaves. Leaf was used for agroinfiltration from 5-day-old seedling, (B) leaf was injected at the abaxial side with a needless syringe, (C) the treated seedlings were covered with a plastic jar for recovery for 4 days, (D) leaf was pressed at the abaxial side by 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. 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.BothCHE and CE had cotyledons and higher transformation efficiency. It was inferred that cotyledons provide more nutrition for hairy roots induction and further quick elongation, andCHE is the optimal explant type for quinoa hairy roots *in-vitro*according to our experiments.

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 toolwhen 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 thatseedlings younger than two months oldmaybe more suitablefor 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.

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Quinoa'sabundantbioactive substancesalso 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 ofthe 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, whichis an ideal material for producing transgenicplants. 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 regeneratedfrom 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 quinoagenetic transformation.

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 firststudyin 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 influencingagroinfiltration efficiency.Similarly, tobacco in which the age of leavesinfluenced agroinfiltration efficiencysignificantly. Theyounger leaveshad 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 treatedleaves were easy to wither or even to die.

The ingredients of the medium should also be considered.In previous studies,½ MS,or 1/4 MS strength were used as basal infection medium [45-47]. In our protocol, 1/10 MSstrength worked better than full strength MS, presumably due to the sudden increase of high ion concentration, which may be deleterious for cell growth.

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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 genesusuallyreaches 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 thefourth day, but a much lighter colouron 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 finishedwithinone monthand independentofregeneration.Comparably,stable transformation always needs a mature regeneration system and several months of tedious work to obtain the positive T1 plantsfor 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 besuccessfully completed with very low efficiency. The firstpair of spreadleaves of two-week-old seedlings were the most suitable material for agroinfiltration. Theleaf injection displayed a much better result than vacuum penetration in transient expression assay.

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Declarations

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Authors' Contributions

Wang YF and Zhang YZ performed most of experiments and wrote the manuscript; Ma J helped with experimental design and data analysis; Dai CJ, S. Jaikishun, Zhou YW and Yan JH helped with transformation procedure and plasmid preparation. Yang ZB provided valuable suggestions for transformation strategy. Song SK and Xu TD initiated and designed this project and wrote the manuscript.

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REFERENCES

1. Wilson HD. Quinua and relatives (Chenopodium sect. Chenopodium subsect. Celluloid). Economic Botany. 1990;44(Suppl 3):92-110.
2. Jacobsen SE, Mujica A, Jensen CR. The Resistance of Quinoa (*Chenopodium quinoa* Willd.) to Adverse Abiotic Factors. Food Rev Int. 2003;19(1-2):99-109.
3. Bazile D, Jacobsen SE, Verniau A. The Global Expansion of Quinoa: Trends and Limits. Front Plant Sci. 2016;7:622.
4. Jaikishun S, Li W, Yang Z, Song S. Quinoa: In perspective of global challenges. Agronomy. 2019;9(4):176.
5. Repo-Carrasco R, Espinoza C, Jacobsen S-E. Nutritional value and use of the Andean crops quinoa (*Chenopodium quinoa*) and kañiwa (*Chenopodium pallidicaule*). Food Rev Int. 2003;19(1-2):179-89.
6. Bodner-Montville J, Ahuja JK, Ingwersen LA, Haggerty ES, Enns CW, Perloff BP. USDA food and nutrient database for dietary studies: released on the web. Journal of Food Composition and Analysis. 2006;19:S100-S7.
7. Abugoch L, Castro E, Tapia C, Añón MC, Gajardo P, Villarroel A. Stability of quinoa flour proteins (Chenopodium quinoa Willd.) during storage. International journal of food science & technology. 2009;44(10):2013-20.
8. Gonzalez JA, Konishi Y, Bruno M, Valoy M, Prado FE. Interrelationships among seed yield, total protein and amino acid composition of ten quinoa (Chenopodium quinoa) cultivars from two different agroecological regions. Journal of the Science of Food and Agriculture. 2012;92(6):1222-9.
9. Escuredo O, Martín MIG, Moncada GW, Fischer S, Hierro JMH. Amino acid profile of the quinoa (Chenopodium quinoa Willd.) using near infrared spectroscopy and chemometric techniques. Journal of Cereal Science. 2014;60(1):67-74.
10. Aloisi I, Parrotta L, Ruiz KB, Landi C, Bini L, Cai G, et al. New insight into quinoa seed quality under salinity: changes in proteomic and amino acid profiles, phenolic content, and antioxidant activity of protein extracts. Frontiers in plant science. 2016;7:656.
11. Filho AM, Pirozi MR, Borges JT, Pinheiro Sant'Ana HM, Chaves JB, Coimbra JS. Quinoa: Nutritional, functional, and antinutritional aspects. Crit Rev Food Sci Nutr. 2017;57(8):1618-30.
12. Ruiz KB, Biondi S, Oses R, Acuna-Rodriguez IS, Antognoni F, Martinez-Mosqueira EA, et al. Quinoa biodiversity and sustainability for food security under climate change. A review. Agronomy for Sustainable Development. 2014;34(2):349-59.
13. Ruiz K, Biondi S, Martínez E, Orsini F, Antognoni F, Jacobsen S-E. Quinoa–a model crop for understanding salt-tolerance mechanisms in halophytes. Plant Biosystems. 2016;150(2):357-71.
14. Hariadi Y, Marandon K, Tian Y, Jacobsen S-E, Shabala S. Ionic and osmotic relations in quinoa (*Chenopodium quinoa* Willd.) plants grown at various salinity levels. Journal of experimental botany. 2010;62(1):185-93.

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15. Adolf VI, Shabala S, Andersen MN, Razzaghi F, Jacobsen S-E. Varietal differences of quinoa's tolerance to saline conditions. Plant and Soil. 2012;357(1-2):117-29.
16. Jaikishun S, Song S, Yang Z. Biochemical Characterization and Responses of Two Contrasting Genotypes of Chenopodium quinoa Willd. to Salinity in a Hydroponic System. Asian Research Journal of Agriculture. 2023;16(1):41-54.
17. Jarvis DE, Ho YS, Lightfoot DJ, Schmöckel SM, Li B, Borm TJ, et al. The genome of *Chenopodium quinoa*. Nature. 2017;542(7641):307.
18. Imamura T, Isozumi N, Higashimura Y, Miyazato A, Mizukoshi H, Ohki S, et al. Isolation of amaranthin synthetase from Chenopodium quinoa and construction of an amaranthin production system using suspension‐cultured tobacco BY‐2 cells. Plant Biotechnology Journal. 2019;17(5):969-81.
19. Del Toro F, Tenllado F, Chung BN, Canto T. A procedure for the transient expression of genes by agroinfiltration above the permissive threshold to study temperature‐sensitive processes in plant–pathogen interactions. Molecular plant pathology. 2014;15(8):848-57.
20. Ma Z, Liu J-J, Zamany A, Williams H. Transient gene expression in western white pine using agroinfiltration. Journal of Forestry Research. 2020;31:1823-32.
21. Gelvin SB. Plant proteins involved in Agrobacterium-mediated genetic transformation. Annual review of phytopathology. 2010;48:45-68.
22. Yang Y, Li R, Qi M. In vivo analysis of plant promoters and transcription factors by agroinfiltration of tobacco leaves. The Plant Journal. 2000;22(6):543-51.
23. Gomes C, Dupas A, Pagano A, Grima-Pettenati J, Paiva JAP. Hairy root transformation: a useful tool to explore gene function and expression in Salix spp. recalcitrant to transformation. Frontiers in plant science. 2019;10:1427.
24. Lam S, Lam B, Harrison L, Strobel G. Genetic information on the Ri plasmid of Agrobacterium rhizogenes determines host specificity. Plant science letters. 1984;34(3):345-52.
25. Mellor KE, Hoffman AM, Timko MP. Use of ex vitro composite plants to study the interaction of cowpea (Vigna unguiculata L.) with the root parasitic angiosperm Striga gesnerioides. Plant methods. 2012;8:1-12.
26. Xue R, Wu X, Wang Y, Zhuang Y, Chen J, Wu J, et al. Hairy root transgene expression analysis of a secretory peroxidase (PvPOX1) from common bean infected by Fusarium wilt. Plant Sci. 2017;260:1-7.
27. Kajikawa M, Morikawa K, Abe Y, Yokota A, Akashi K. Establishment of a transgenic hairy root system in wild and domesticated watermelon (Citrullus lanatus) for studying root vigor under drought. Plant cell reports. 2010;29:771-8.
28. Cho H-J, Farrand SK, Noel GR, Widholm JM. High-efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. Planta. 2000;210:195-204.
29. Cai D, Kleine M, Kifle S, Harloff H-J, Sandal NN, Marcker KA, et al. Positional cloning of a gene for nematode resistance in sugar beet. Science. 1997;275(5301):832-4.

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30. Chen L, Cai Y, Liu X, Guo C, Sun S, Wu C, et al. Soybean hairy roots produced in vitro by Agrobacterium rhizogenes-mediated transformation. The crop journal. 2018;6(2):162-71.
31. An J, Cheng C, Hu Z, Chen H, Cai W, Yu B. The Panax ginseng PgTIP1 gene confers enhanced salt and drought tolerance to transgenic soybean plants by maintaining homeostasis of water, salt ions and ROS. Environmental and experimental botany. 2018;155:45-55.
32. Alagarsamy K, Shamala LF, Wei S. Protocol: high-efficiency in-planta Agrobacterium-mediated transgenic hairy root induction of Camellia sinensis var. sinensis. Plant Methods. 2018;14:1-8.
33. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia plantarum. 1962;15(3):473-97.
34. Polturak G, Breitel D, Grossman N, Sarrion‐Perdigones A, Weithorn E, Pliner M, et al. Elucidation of the first committed step in betalain biosynthesis enables the heterologous engineering of betalain pigments in plants. New Phytologist. 2016;210(1):269-83.
35. Cao D, Hou W, Song S, Sun H, Wu C, Gao Y, et al. Assessment of conditions affecting Agrobacterium rhizogenes-mediated transformation of soybean. Plant Cell, Tissue and Organ Culture. 2009;96:45-52.
36. Guimaraes LA, Pereira BM, Araujo ACG, Guimaraes PM, Brasileiro ACM. Ex vitro hairy root induction in detached peanut leaves for plant–nematode interaction studies. Plant Methods. 2017;13:1-10.
37. Alvarez-Jubete L, Wijngaard H, Arendt EK, Gallagher E. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food chemistry. 2010;119(2):770-8.
38. Lim JG, Park HM, Yoon KS. Analysis of saponin composition and comparison of the antioxidant activity of various parts of the quinoa plant (Chenopodium quinoa Willd.). Food science & nutrition. 2020;8(1):694-702.
39. Gurusamy PD, Schäfer H, Ramamoorthy S, Wink M. Biologically active recombinant human erythropoietin expressed in hairy root cultures and regenerated plantlets of Nicotiana tabacum L. PloS one. 2017;12(8):e0182367.
40. Jin H, Jia J-F, Hao J-G. Protoplasts from Agrobacterium rhizogenes-transformed cell line of Medicago sativa L. regenerated to hairy roots. In Vitro Cellular & Developmental Biology-Plant. 2003;39:208-11.
41. Butler NM, Jansky SH, Jiang J. First‐generation genome editing in potato using hairy root transformation. Plant Biotechnology Journal. 2020;18(11):2201-9.
42. Ishizaki T, Hoshino Y, Masuda K, Oosawa K. Explants of Ri-transformed hairy roots of spinach can develop embryogenic calli in the absence of gibberellic acid, an essential growth regulator for induction of embryogenesis from non-transformed roots. Plant Sci. 2002;163(2):223-31.
43. Levy M, Rachmilevitch S, Abel S. Transient Agrobacterium-mediated gene expression in the Arabidopsis hydroponics root system for subcellular localization studies. Plant Molecular Biology Reporter. 2005;23:179-84.

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44. Mooney BC, Graciet E. A simple and efficient Agrobacterium-mediated transient expression system to dissect molecular processes in Brassica rapa and Brassica napus. Plant Direct. 2020;4(7):e00237.
45. Lu J, Bai M, Ren H, Liu J, Wang C. An efficient transient expression system for gene function analysis in rose. Plant Methods. 2017;13(1):1-12.
46. Zhang Y, Chen M, Siemiatkowska B, Toleco MR, Jing Y, Strotmann V, et al. A highly efficient agrobacterium-mediated method for transient gene expression and functional studies in multiple plant species. Plant communications. 2020;1(5).
47. Ma S, Li J, Chen J-Y, Mei R-M, Cui K, Lan L. Research Progress and a Prospect Analysis of Asexual Bamboo Reproduction. Horticulturae. 2023;9(6):685.
48. Jones H, Ooms G, Jones MG. Transient gene expression in electroporated Solanum protoplasts. Plant molecular biology. 1989;13:503-11.
49. Zhong S, Dong B, Zhou J, Miao Y, Yang L, Wang Y, et al. Highly efficient transient gene expression of three tissues in Osmanthus fragrans mediated by Agrobacterium tumefaciens. Scientia Horticulturae. 2023;310:111725.
50. Zhang K, Liu S, Fu Y, Wang Z, Yang X, Li W, et al. Establishment of an efficient cotton root protoplast isolation protocol suitable for single-cell RNA sequencing and transient gene expression analysis. Plant Methods. 2023;19(1):5.
51. Lacroix B, Citovsky V. The roles of bacterial and host plant factors in Agrobacterium-mediated genetic transformation. Int J Dev Biol. 2013;57(6-8):467-81.

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