Journal of Visualized Experiments

CcCIPK14 gene function analysis to illuminate the efficient root transgenic system

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62304R3
Full Title:	CcCIPK14 gene function analysis to illuminate the efficient root transgenic system
Corresponding Author:	Meng Dong Beijing Forestry University Beijing, Beijing CHINA
Corresponding Author's Institution:	Beijing Forestry University
Corresponding Author E-Mail:	mengdongjlf@163.com
Order of Authors:	Tengyue Liu
	Zhihua Song
	Tingting Du
	Wanlong Yang
	Ting Chen
	Ying Han
	Biying Dong
	Hongyan Cao
	Lili Niu
	Rohul Amin
	Haojie jin
	Qing Yang
	Meng Dong
	Yujie Fu
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Genetics
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Beijing/China
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be	Standard Access ($1400)

Standard Access or Open Access.	

TITLE:

CcCIPK14 Gene Function Analysis to Illuminate the Efficient Root Transgenic System

AUTHORS AND AFFILIATIONS:

Tengyue Liu[1,#], Zhihua Song[1,#], Tingting Du[1], Wanlong Yang[1], Ting Chen[1], Ying Han[1], Biying Dong[1], Hongyan Cao[1], Lili Niu[1,2], Rohul Amin[1], Haojie Jin[1], Qing Yang[1,2], Dong Meng[1,2], Yujie Fu[1,2]

[1]College of Forestry, Beijing Forestry University, Beijing, China

[2]Beijing Advanced Innovation Center for Tree Breeding by Molecular Design, Beijing Forestry University, Beijing, China

[#]These authors contributed equally to the work.

Email Addresses of co-authors:

Liu Tengyue	(786475751@qq.com)
Zhihua Song	(szhxf0801@163.com)
Tingting Du	(2365754692@qq.com)
Wanlong Yang	(664216309@qq.com)
Ting Chen	(1753851316@qq.com)
Ying Han	(13811300787@163.com)
Biying Dong	(dongbiying1029@163.com)
Hongyan Cao	(hongyan89945@163.com)
Lili Niu	(niulili0613@126.com)
Rohul Amin	(roohulamin245@163.com)
Haojie jin	(haojie@bjfu.edu.cn)
Qing Yang	(yang.qing1020@163.com)
Dong Meng	(mengdongjlf@163.com)
Yujie Fu	(yujie_fu@163.com)

Correspondence to:

Dong Meng	(mengdongjlf@163.com)
Yujie Fu	(yujie_fu@163.com)

KEYWORDS:

Flavonoids; gene function; transgenic root system; hairy root; *CcCIPK14* (Calcineurin B-like protein-interacting protein kinases)

SUMMARY:

Here we present an efficient and stable transformation system for the functional analysis of the CcCIPK14 gene as an example, providing a technical basis for studying the metabolism of non-model plants.

ABSTRACT:

An efficient and stable transformation system is fundamental for gene function study and

molecular breeding of plants. Here, we describe the use of an *Agrobacterium rhizogenes* mediated transformation system on pigeon pea. The stem is infected with *A. rhizogenes* carrying a binary vector, which induced callus after 7 days and adventitious roots 14 days later. The generated transgenic hairy root was identified by morphological analysis and a GFP reporter gene. To further illustrate the application range of this system, *CcCIPK14* (Calcineurin B-like protein-interacting protein kinases) was transformed into pigeon pea using this transformation method. The transgenic plants were treated with jasmonic acid (JA) and abscisic acid (ABA), respectively, for the purpose of testing whether *CcCIPK14* responds to those hormones. The results demonstrated that (1) exogenous hormones could significantly upregulate the expression level of *CcCIPK14*, especially in *CcCIPK14* over-expression (OE) plants; (2) the content of Genistein in *CcCIPK14*-OE lines was significantly higher than the control; (3) the expression level of two downstream key flavonoid synthase genes, *CcHIDH1* and *CcHIDH2*, were up-regulated in the *CcCIPK14*-OE lines; and (4) the hairy root transgenic system can be used to study metabolically functional genes in non-model plants.

INTRODUCTION:

Transformation is a basic tool to evaluate the expression of exogenous genes[1,2]. Many biological aspects of resource plants are common to all plants; therefore, functional studies of certain genes can be carried out in model plants (such as *Arabidopsis*)[3]. Yet, many genes in plants are unique in their function and expression patterns, requiring studies in their own or closely related species, especially for resource plants[3,4]. Plant cells can sense various signals that enable plants to show specific changes in gene expression, metabolism, and physiology in response to different environmental stress conditions[5–7]. Flavonoids are crucial players in the signaling process of plants that is responsive to environmental stresses[5,8,9]. In addition, the flavonoid content in horticultural and medicinal plants is also an important indicator for quality evaluation[10]. Identification of genes involved in the regulation of flavonoid synthesis in response to external signals is crucial for understanding the mechanism of flavonoid synthesis in plants. Several studies have revealed that the application of exogenous hormones can promote the accumulation of flavonoids[6,11]. A stable transformation system and gene function validation method are essential to demonstrate the function of genes and to understand secondary metabolism in plants.

Agrobacterium-mediated transformation is widely used in DNA insertion[5,8,9]. *Agrobacterium tumefacient* can transfer ring genes into the chromosomes of plant cells, and exogenous phytohormones induce single or a few host cells that can regenerate plants to obtain stable transformants[12–14]. *Agrobacterium tumefacient*-mediated transformation methods are more applicable to plant species suitable for *in vitro* manipulation, while most perennial woody plants limit the application of this method because of their regeneration difficulty[4,15]. *A. rhizogenes* is also able to modify the genome of host cells[16]. In the present study, we have developed an efficient and stable *A. rhizogenes*-mediated transformation procedure. *A. rhizogen*es contains a second binary plasmid carrying non-natural gene T-DNA in addition to the Ri plasmid. The host plant is infected, and a composite plant can be obtained with transgenic hairy roots emerging from the wild-type shoot[16,17]. The *A. rhizogenes*-mediated transformation systems are suitable for application in woody plant research due to their fast, low cost and non-required plant

regeneration. More than 160 kinds of plants have successfully induced hairy roots, and most of which are in *Solanaceae*, *Compositae*, *Cruciferae*, *Convolvulaceae*, *Umbelliferae*, *Leguminosae*, *Caryophyllaceae*, and *Polygonaceae*[18,19]. Compared with *A. tumefaciens*, *A. rhizogenes* showed higher efficiency in the mediated transformation of pigeon pea[17,20].

In this study, pigeon pea was used as an example to introduce the transformation process mediated by *A. rhizogenes*. From inoculation to rooting, the experiments lasted for 5 weeks. We identified the transformation of the adventitious root through morphology and the *GFP* reporter gene, and the transformation efficiency was as high as 75%. Also, we treated the composite plant with JA and ABA, as well as detected transcripts and secondary metabolites by quantitative real-PCR and HPLC (high performance liquid chromatography). It is confirmed that the expression level of *CcCIPK14* responds not only to JA and ABA but also affects the biosynthesis of flavonoids. This system is adequate for studying function genes associated with secondary metabolism. It also provides a new approach to studying non-model plants in lack of a sufficient stable transformation system[17,21,22].

PROTOCOL:

NOTE: Pigeon pea is a diploid legume crop that belongs to the family *Fabaceae*. The pigeon pea seeds used in this experiment are from the Northeast Forestry University of China and are coded 87119. The primary steps of this protocol are illustrated in **Figure 1A**. The seedling incubation was performed in a high humidity environment at 25 °C under fluorescent lights at 50 µmol photons per $m^{-2}s^{-1}$ in a 16 h photoperiod. *A. rhizogenes* strains K599 (NCPPB2659) were preserved in the laboratory. They were stored in yeast mannitol medium (YEP) with 15% glycerol at -80 °C. The protocol described in this work was based on the protocol Meng et al.[21].

CAUTION: Deposit all the genetically modified bacteria and plants into the appropriate waste container. Use all hazardous chemicals in a fume hood and dispose of them in the hazardous waste container.

1. Preparation of pea seedlings

1.1. Choose plump and undamaged pigeon pea seeds (**Figure 1A**) that have been stored for less than 1 year.

1.2. Soak the seeds in distilled water for 24 h (**Figure 1B**). Transfer the swollen seeds to a seed tray and place them in the greenhouse.

1.3. Seeds start to germinate after 1–2 days. When the epicotyl reaches 1.5 cm, transplant the seedlings to 10 cm (diameter) x 9 cm (height) pots containing soil and sand in a volume ratio of 3:1.

NOTE: The soil consists of a mixture of nutritious soil, vermiculite, and perlite at a 2:1:1 ratio.

1.4. Grow the seedling in a greenhouse.

2. Activation of *A. rhizogenes*

NOTE: The strain used for *A. rhizogenes* transformation was the K599 preserved at -80 °C. The binary vector pROK2 (pBIN438; http://www.biovector.net/product/428388.html) contains green fluorescent protein (GFP) as an indicator gene and a kanamycin resistance gene as a selectable marker to transform *A. rhizogenes.*

2.1. Thaw *A. rhizogenes* on ice.

2.2. Dip the bacteria and line them evenly onto yeast mannitol medium (YEP) supplemented with 8 g/L of agar powder, 25 mg/L of rifampicin, and 25 mg/L of kanamycin (YRK, pH 7.0).

2.3. Incubate at 28 °C for 16 h.

2.4. Select monoclonal colonies. Culture them in a 50 mL tube containing 10 mL of YEB medium supplemented with 25 mg/L of rifampicin and 25 mg/L of kanamycin (YRK, pH 7.0). Place the centrifuge tube on a shaking incubator with a rotational radius of 10 cm, at 28 °C and 200 rpm for 16 h.

3. Plant transformation using *A. rhizogenes*

NOTE: Select healthy plants to infect *A. rhizogenes* using the following injection procedure. This procedure results in transformed hairy roots. To analyze the gene function of *CcCIPK14*, a control is needed. *A. rhizogenes* solutions with empty vector or Cc*CIPK14*-pROK2 plasmids were injected into seedlings to induce hairy roots.

3.1. Inoculate *A. rhizogenes*

3.1.1. Choose pigeon pea seedlings with the same growth status.

3.1.2. Empty the air from the 1 mL syringe and aspirate 0.3 mL of bacterial liquid. Slowly push the pushrod to fill the syringe needle with bacterial liquid[21].

3.1.3. First fix the stem of the pigeon pea seedling with tweezers, and then prick the syringe needle into the stem at 1 cm.

3.1.4. When withdrawing the syringe, completely submerge the needle tip into the stem. Slowly push the pushrod to pump the bacterial liquid out of the penetrating wound.

NOTE: The attachment of residual bacterial liquid to the wound in bacteria droplets can improve infection and transformation efficiency.

3.2. Seedling management

NOTE: High temperature and humidity can improve the infection efficiency of *A. rhizogenes*.

3.2.1. Place seedlings inoculated with *A. rhizogenes* in a tray (30 cm x 60 cm x 6 cm) with 1 L of water. Use a transparent plastic lid (30 cm x 60 cm x 30 cm) as a cover to maintain the temperature and humidity of the internal environment.

3.2.2. Periodically remove fallen leaves and flocculent hyphae attached to leaf tips and fallen leaves.

3.2.3. After a week, the callus begins to appear in the wound. Subsequently, keep the the plastic lid half-open.

3.2.4. After 4 weeks, most of the callus tissue differentiated into adventitious roots. Remove the plastic lid.

3.2.5. Add 1 L of water to the tray every 3 days to keep the soil moist.

NOTE: Pigeon pea is a drought-tolerant plant; too much water can inhibit plant growth.

4. Identification of transformed hairy roots

NOTE: Transformed hairy roots can be identified based on the morphology and gene level. This procedure primarily focuses on reporter gene (GFP) identification assay.

4.1. Collect the root tips of the hairy root and mark the remaining part.

4.2. Evaluate whether there is green fluorescence under a confocal laser scanning microscope.

4.3. Triturate 0.1 g of hairy roots with strong fluorescence signal into fine powder in liquid nitrogen.

4.4. According to the plant genomic DNA kit manufacturer's instructions, prepare the genomic DNA of pigeon pea independent transgenic lines by the modified cetyltrimethylammonium bromide (CTAB) method[23].

4.5. Use 500 ng of genomic DNA template and primers for PCR. The primers are shown in **Table 1.**

4.6. Perform the following amplification cycle: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, annealing of primers at 55 °C for 30 s, and primer extension at 72 °C for 30 s. After 36 cycles and a final extension of 10 min at 72 °C, analyze the amplified products on a 1% agarose gel.

4.7. Stain the gels with nucleic acid staining and visualize them under UV light.

5. Exogenous hormone treatment

NOTE: The positive composite plants were treated with exogenous hormones to study the effect of CcCIPK14 on metabolic. The composited plants induced by *A. rhizogenes* were divided into three groups: JA treatment group, ABA treatment group, and control group (**Figure 3A**).

5.1. Reduce watering 3 days before exogenous hormone treatment.

5.2. Prepare both JA and ABA solutions at a concentration of 5 mg/L.

NOTE: The JA and ABA powder are first dissolved in ethyl alcohol, and then filled to the target volume with distilled water.

5.3. Using a spray bottle, spray JA and ABA solutions uniformly on the leaves of the plants. Treat the control group with water.

NOTE: An average of 10 mL of solution was sprayed on each seedling.

5.4. Cover with the plastic lid immediately after spraying treatment. Put it back in the artificial climate greenhouse.

6. Sample collection and preservation

NOTE: After 3 h of exogenous hormone treatment, plant materials from different treatment groups were collected.

6.1. Remove tawny and contaminated hairy roots and select those with a white appearance. Collect these hairy roots and dry them out with absorbent paper.

6.2. Divide the hairy roots collected from each seedling into two parts. Put one part into a numbered test tube and wrap it using marked tinfoil.

6.3. Lyophilize the tinfoil in liquid nitrogen, and then store all harvested tin foil at -80 °C for further investigation.

REPRESENTATIVE RESULTS:

***A. rhizogenes* -mediated hairy root transformation on pigeon pea**

This study described the step-by-step protocols for the genetic transformation of hairy roots mediated by *A. rhizogenes*, which has significance in the field of plant molecules. It took about 5 weeks to get hairy roots from the roots of pigeon pea infected by *A. rhizogenes*. **Figure 1A** showed an overview of the entire transformation process, from the injection of *A. rhizogenes* to obtain

composite plants with hairy roots. Proliferating tissues were observed around 1 week after infection, and its differentiation to adventitious roots was observed around 2 weeks, and a large number of hairy roots were produced at 35 days (**Figure 1B**). This GFP made it possible to distinguish genetically modified hairy roots from non-transgenic hairy roots through fluorescence. In **Figure 2**, the transformed hairy roots with GFP-pROK2 were visualized under the confocal green fluorescent protein channel. Induction of hairy roots by *A. rhizogenes* with the empty vector did not show green fluorescence (**Figure 2A**). The generated adventitious roots were identified by morphology and GFP reporter gene; the results confirmed that K599 could successfully infect and transform pigeon pea (**Figure 2**). The plant genomic DNA was prepared (CTAB method) for PCR analysis. The GFP was present only in hairy roots induced by *A. rhizogenes* carrying the GFP-pROK2 plasmid; neither the untransformed plant tissues nor the roots induced by *A. rhizogenes* with empty vectors were present (**Figure 2B,C**). These results indicated that the binary vector had been successfully transformed into the adventitious root genome[16,21].

To improve the transformation efficiency of *A. rhizogenes*, the protocol was optimized. Infection with *A. rhizogenes* caused abnormal growth of normal roots and leaves of the host plant and even led to death. There are several possible explanations for this result. In the early vegetative growth stage, the spontaneous propagation and transformation of host cells consumed large amounts of nutrients, which maybe the main reason for inhibiting the development of seedling and even causing plant death[24,25]. Besides, the mechanical damage caused by inoculation may have also affected the seedlings[24,26,27]. Meanwhile, the growth status of seedlings determines the differentiation efficiency of callus. Inoculation is best done when the seedlings are 7 days old and the stems of seedlings are incompletely lignified (**Figure 1A**). Under this condition, the efficiency of *A. rhizogenes*-mediated callus production was 76%. The findings from these studies suggest that relative humidity can affect the proliferation of callus. The proliferation and differentiation of proliferating tissues and the growth of adventitious roots both need to maintain a high relative air humidity. Many roots were created within 4 to 5 weeks of inoculation and have begun to penetrate the soil. The overall transformation efficiency of this experiment can be as high as 72%. Transformation efficiency (%) = (number of positive compound plants / number of infected plants) x 100.

Evaluation of the *CcCIPK14* gene function in pigeon pea using the transgenic hairy root system

Hairy roots of *CcCIPK14*-OE lines were obtained using the above-mentioned hairy root induction protocol. The hairy roots could attach to the infection points on the stems to form a composite plant. As only the hairy roots are genetically modified in composite plants, they can also be used to study signal transduction between the roots and the above-ground part. The phytohormone solutions (JA and ABA) were sprayed uniformly on the leaves of composite plants, and the hairy roots were collected after 3 h (**Figure 3A**). The function of *CcCIPK14* in transgenic roots was verified by gene expression analysis and metabolite determination. The results showed that the expression level of *CcCIPK14* was up-regulated after JA and ABA treatment. Besides, the expression levels of two key enzymes for Genistein synthesis in the *CcCIPK14*-OE line, *CcHIDH1*, and *CcHIDH2* were up-regulated. Compared with the control group, the Genistein content in transgenic roots increased to 0.060 mg/g FW. We compared the phenotypes before and after the transgenic plants were treated with JA/ABA, and there was little difference in morphology

through comparison (**Figure 3B**). *CcCIPK14*, a key functional gene in the flavonoid metabolic pathway, can respond to hormone signals and participate in genistein biosynthesis in pigeon pea.

FIGURE AND TABLE LEGENDS:

Figure 1: Induction of the *A. rhizogenes* -mediated compound plant. (**A**) Flow chart of the hairy roots transformation system, including activating *A. rhizogenes*, seedling cultivation, *Agrobacterium* inoculation, seedling management, and obtaining compound plants. A representative image showing the key stages. (**B**) The three main stages of adventitious root formation at the inoculation site: callus appears, callus proliferates, and differentiates. Scale bars are 1 cm.

Figure 2: Transgenic hairy root analysis. (**A**) GFP signal in transgenic hairy roots, with empty vector-containing *Agrobacterium* solution control. Scale bars are 50 μm. (**B,C**) PCR amplification of genes (GFP) from genomic DNA isolated from wild-type roots and stems, leaves, and transgenic roots of compound plants. CK: Empty Vector #1–4: plasmid (binary vector pROK2 carrying GFP) as the positive control.

Figure 3: CIPK14 function analysis by hairy root method and hormone treatment. (**A**) Process flow chart for verification and analysis of gene function through compound plants. (**B**) Phenotypic changes of compound plants before and after treatment with JA, ABA, and H_2O. Scale bars are 1 cm.

Figure 4: Gene expression level and secondary metabolite content of hairy roots with empty vector and plasmid *CcCIPK14*-pROK2 (binary vector pROK2 with *CcCIPK14*). *CcCIPK14* expression level (**A**), secondary metabolite content in transgenic hairy roots (**B**), and expression levels of *CcHIDH1* and *CcHIDH2* in hairy roots after hormone treatment (**D**). The relative expression level was normalized to that of the actin control. Data are presented as mean ± standard deviation (n = 3), * indicates a significant difference ($P < 0.05$) between *CcCIPK14*-OE and the controls (empty vector lines) using Student's *t*-test. (**C**) The biosynthesis pathway of Genistein. The enzyme catalyzing reaction HIDH, 2-hydroxyiso flavanone dehydratase, CIPK14 (Calcineurin B-like protein-interacting protein kinases). Treatments with the same letters were not significantly different based on the mean comparison by Duncan's test at $p < 0.05$.

Table 1: Primers used in this study.

DISCUSSION:

The rapid characterization of gene function is the common goal in the study of most species, and it is particularly important for the development of resource plants. The *A. rhizogenes*-mediated transformation has been widely used in the hairy root culture. The hairy root culture (HRC), as a unique source of metabolite production, plays a pivotal role in metabolic engineering[18,28]. The application of this technology is mainly limited to the function of genes *in vivo*[21]. Here, we provide a basic method for studying gene function based on the previous *A. rhizogenes* -mediated transformation system. This method can be used to verify a variety of gene functions, such as response to environmental stress and exogenous hormones. The *CcCIPK14-OE* line showed that

CcCIPK14 could promote the accumulation of flavonoid metabolites and respond to exogenous hormones JA and ABA. *CcHIDH1* and *CcHIDH2* are two essential flavonoid synthase genes, which were significantly up-regulated in the *CcCIPK14*-OE line. These results indicate that the method can be used as an effective tool for evaluating gene function and secondary metabolites.

An effective genetic transformation system is a prerequisite for the verification and analysis of gene function[29,30]. *A. rhizogenes* is a bacterium with a natural evolutionary mechanism[31]. It contains a root-inducing (Ri) plasmid that contains *root locus* (*rol*) genes in the T-DNA region, including *rolA*, *rolB*, *rolC*, and *rolD*, which can induce hairy roots from the wound surface of the explants[27,32]. The *A. rhizogenes* -mediated transformation system has many advantages[33]. First, in most species, Ri-transformed cells of the host plant can spontaneously differentiate into roots with normal phenotypes[33]. When additional binary vectors are used to integrate exogenous DNA, Ri plasmids can provide the possibility of obtaining transformed hairy roots without using exogenously applied plant hormones for organogenesis[34]. Second, Ri-transformed roots are genetically stable, because it is speculated that these transformed roots are developed from a single transformed cell[17,33]. Moreover, these hairy roots can maintain attachment to wild-type branches, resulting in composite plants[16,20]. Ri-transformed roots can simulate normal roots, providing experimental materials for verification and analysis of gene functions[35]. This transformation system is suitable for plants that are difficult to regenerate, such as pigeon pea, which has low regeneration frequency[36,37]. However, since only the hairy roots are transgenic, the function of genes on the entire plant level cannot be assessed, and transgenic traits also cannot be passed on to the offspring through sexual reproduction.

In this scheme, *A. rhizogenes* carrying binary vectors can induce transgenic hairy roots by an efficient and stable protocol. The basic steps of the transformation method have been outlined in the form of a flowchart (**Figure 1A**). After 1 week of inoculation with *A. rhizogenes*, calluses were observed on the wounds of the stem. After 3 weeks, the calluses differentiated to hairy roots (**Figure 1B**). This method is of significance because it does not require *in vitro* culture, hence hairy roots were obtained within 5 weeks (**Figure 1**). Meng et al. conducted a series of similar experiments with this method on different plants, and the results showed that most plants could obtain transgenic roots with the target gene within 2 months[21]. Pigeon pea, a protein-rich orphan, is growing in semi-arid tropics regions[37]. The hot and humid culture environment affects the growth of pigeon pea, and can occur deciduous phenotype[21]; the phenotype can be restored by removing the plastic cover.

During these experiments, many factors must be carefully considered to rapidly obtain transformed roots. The health status of the seedlings after inoculation is the key to the success of this experiment[21]. Previous studies have shown that *A. rhizogenes* can induce hairy roots at the inoculation point during multiple stages of seedling growth[38]. However, the growth status of seedings directly affects the transformation efficiency[26]. Premature inoculation of *A. rhizogenes* can inhibit the growth of seedlings and even causes seedling death[39,40]. To obtain maximum transformation efficiency, *A. rhizogene* is usually inoculated on 7-day-old seedlings. Besides, air humidity is also a major factor affecting callus proliferation. Premature removal of plastic after inoculation with *A .rhizogenes* can lead to browning of the callus and dieback of adventitious

roots. This study used two different confirmation methods to identify positive transgenic roots: PCR amplification of the inserted gene and GFP fluorescence detection. The test results confirmed the successful conversion. The conversion efficiency is as high as 75%.

ACKNOWLEDGMENTS:

The authors gratefully acknowledge the financial support by National Natural Science Foundation of China (31800509, 31922058), Outstanding Young Talent Fund in Beijing Forestry University" (2019JQ03009), and National Key R&D Program of China (2018YFD1000602,2019YFD1000605-1) and Beijing Advanced Innovation Center for Tree Breeding by Molecular Design.

DISCLOSURES:

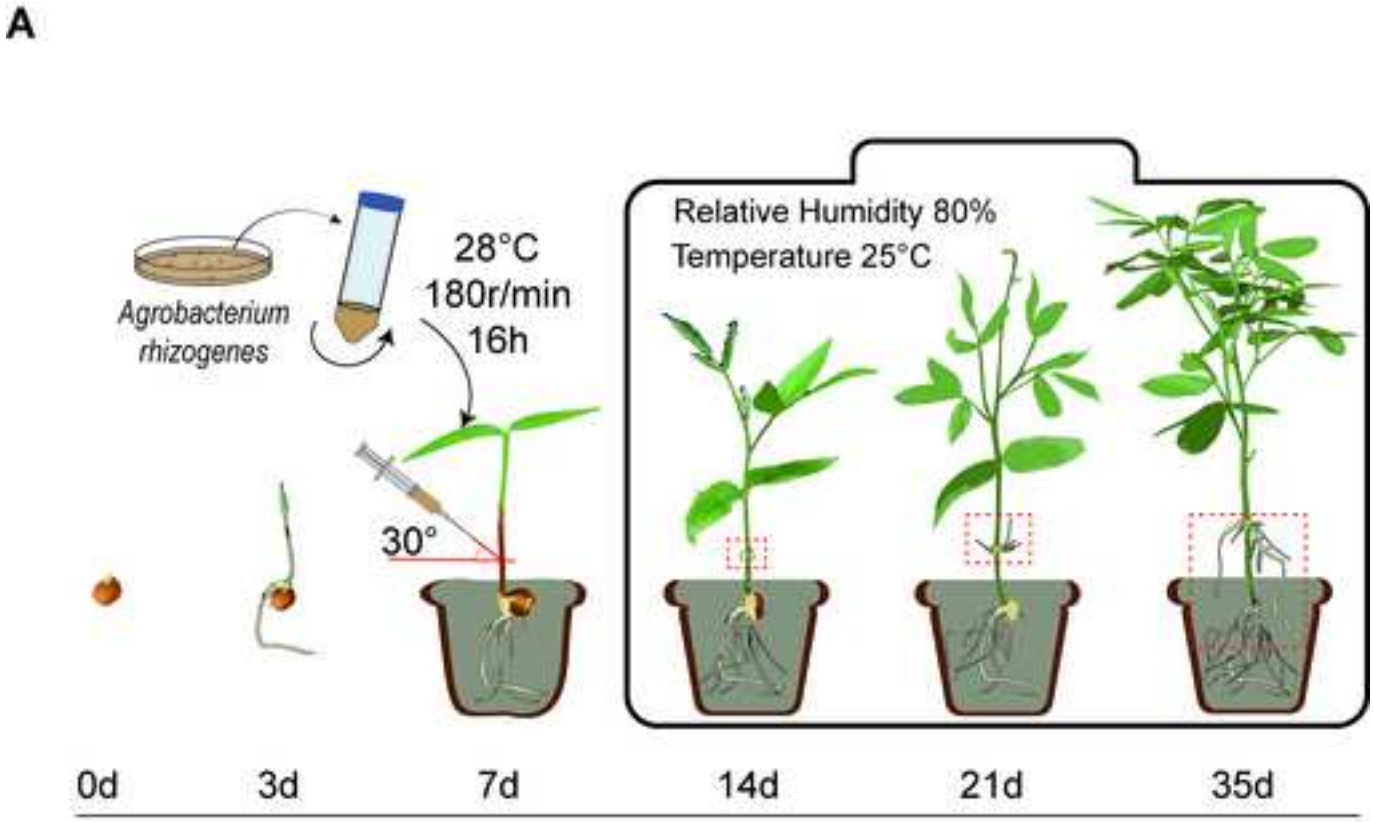
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

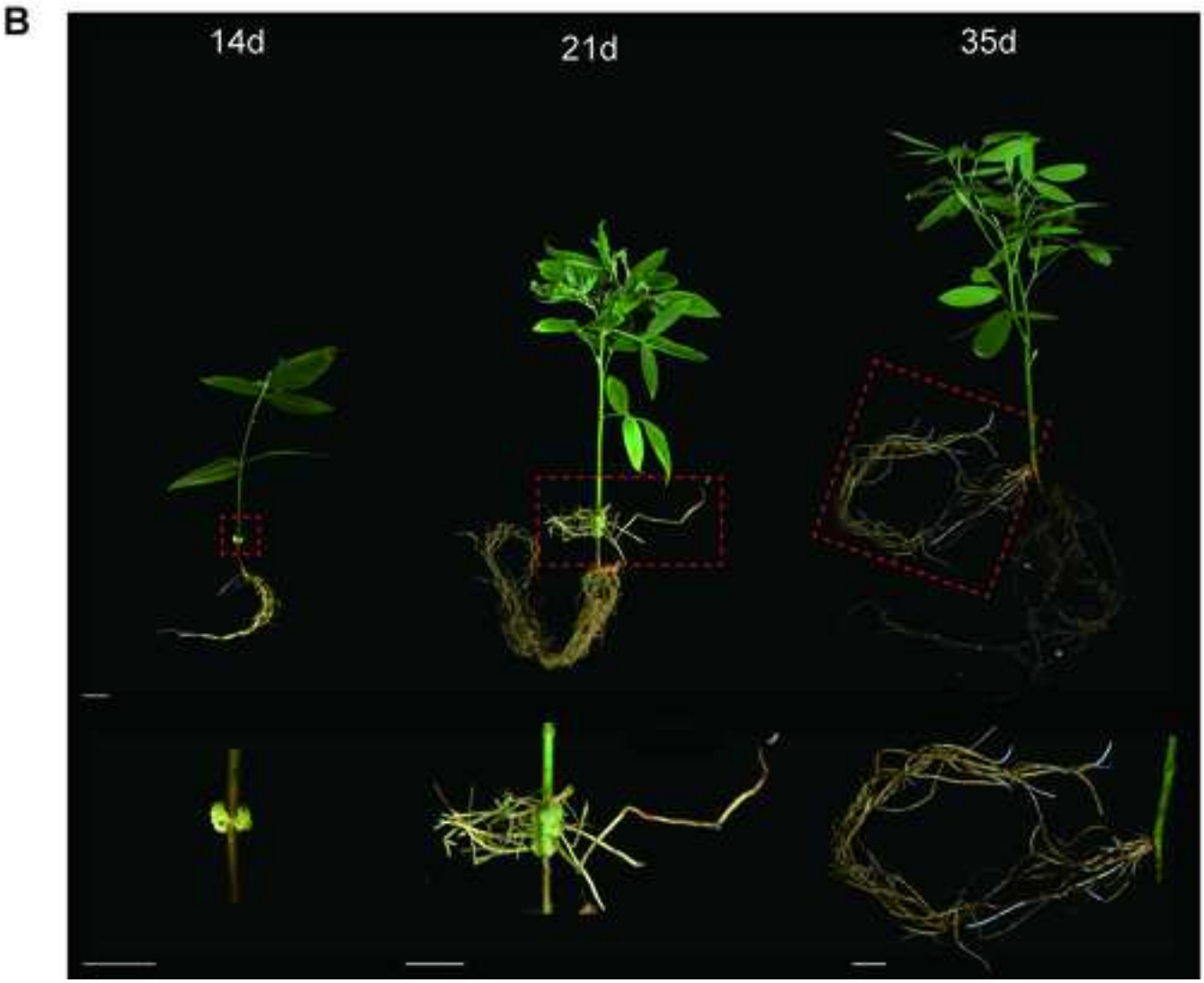
REFERENCES:

1. Poland, J. A., Rife, T. W. Genotyping‐by‐sequencing for plant breeding and genetics. *The Plant Genome*. **5** (3) (2012).
2. Barabaschi, D. et al. Next generation breeding. *Plant Science*. **242**, 3–13 (2016).
3. Petri, C., Webb, K., Hily, J.-M., Dardick, C., Scorza, R. High transformation efficiency in plum (Prunus domestica L.): a new tool for functional genomics studies in Prunus spp. *Molecular Breeding*. **22** (4), 581–591 (2008).
4. Tang, H., Ren, Z., Reustle, G., Krczal, G. Plant regeneration from leaves of sweet and sour cherry cultivars. *Scientia Horticulturae*. **93** (3–4), 235–244 (2002).
5. Yang, L. et al. Response of plant secondary metabolites to environmental factors. *Molecules*. **23** (4), 762 (2018).
6. Ross, J. J., Weston, D. E., Davidson, S. E., Reid, J. B. Plant hormone interactions: how complex are they? *Physiologia Plantarum*. **141** (4), 299–309 (2011).
7. Meng, D. et al. The pigeon pea CcCIPK14‐CcCBL1 pair positively modulates drought tolerance by enhancing flavonoid biosynthesis. *The Plant Journal* (2021).
8. Wen, W., Alseekh, S., Fernie, A. R. Conservation and diversification of flavonoid metabolism in the plant kingdom. *Current Opinion in Plant Biology*. **55**, 100–108 (2020).
9. Shah, A., Smith, D. L. Flavonoids in agriculture: Chemistry and roles in, biotic and abiotic stress responses, and microbial associations. *Agronomy*. **10** (8), 1209 (2020).
10. Zhang, W., Jiang, W. UV treatment improved the quality of postharvest fruits and vegetables by inducing resistance. *Trends in Food Science & Technology*. **92**, 71–80 (2019).
11. Koyama, R. et al. Exogenous abscisic acid promotes anthocyanin biosynthesis and increased expression of flavonoid synthesis genes in Vitis vinifera× Vitis labrusca table grapes in a subtropical region. *Frontiers in Plant Science*. **9**, 323 (2018).
12. Guo, M., Ye, J., Gao, D., Xu, N., Yang, J. Agrobacterium-mediated horizontal gene transfer: Mechanism, biotechnological application, potential risk and forestalling strategy. *Biotechnology Advances*. **37** (1), 259–270 (2019).
13. Veena, V., Taylor, C. G. Agrobacterium rhizogenes: recent developments and promising applications. *In Vitro Cellular & Developmental Biology-Plant*. **43** (5), 383–403 (2007).
14. Lacroix, B., Citovsky, V. Pathways of DNA transfer to plants from Agrobacterium

tumefaciens and related bacterial species. *Annual Review of Phytopathology*. **57**, 231–251 (2019).
15. Guan, Y., Li, S.-G., Fan, X.-F., Su, Z.-H. Application of somatic embryogenesis in woody plants. *Frontiers in Plant Science*. **7**, 938 (2016).
16. Alpizar, E. et al. Efficient production of Agrobacterium rhizogenes-transformed roots and composite plants for studying gene expression in coffee roots. *Plant Cell Reports*. **25** (9), 959–967 (2006).
17. Hwang, H.-H., Yu, M., Lai, E.-M. Agrobacterium-mediated plant transformation: biology and applications. *The Arabidopsis Book*. **15** (2017).
18. Srivastava, S., Srivastava, A. K. Hairy root culture for mass-production of high-value secondary metabolites. *Critical Reviews in Biotechnology*. **27** (1), 29–43 (2007).
19. Porter, J. R., Flores, H. Host range and implications of plant infection by Agrobacterium rhizogenes. *Critical Reviews in Plant Sciences*. **10** (4), 387–421 (1991).
20. Fan, Y.-l. et al. One-step generation of composite soybean plants with transgenic roots by Agrobacterium rhizogenes-mediated transformation. *BMC Plant Biology*. **20**, 1–11 (2020).
21. Meng, D. et al. Development of an efficient root transgenic system for pigeon pea and its application to other important economically plants. *Plant Biotechnology Journal*. **17** (9), 1804–1813 (2019).
22. Ma, R. et al. Agrobacterium-Mediated genetic transformation of the medicinal plant Veratrum dahuricum. *Plants*. **9** (2), 191 (2020).
23. Mi, Y. et al. Inducing hairy roots by Agrobacterium rhizogenes-mediated transformation in tartary buckwheat (Fagopyrum tataricum). *Journal of Visualized Experoments: JoVE*. (157) (2020).
24. Cordovez, V. et al. Plant phenotypic and transcriptional changes induced by volatiles from the fungal root pathogen Rhizoctonia solani. *Frontiers in Plant Science*. **8**, 1262 (2017).
25. Trovato, M., Mattioli, R., Costantino, P. From A. rhizogenes RolD to plant P5CS: exploiting proline to control plant development. *Plants*. **7** (4), 108 (2018).
26. Van Altvorst, A. C. et al. Effects of the introduction of Agrobacterium rhizogenes rol genes on tomato plant and flower development. *Plant Science*. **83** (1), 77–85 (1992).
27. Bahramnejad, B., Naji, M., Bose, R., Jha, S. A critical review on use of Agrobacterium rhizogenes and their associated binary vectors for plant transformation. *Biotechnology Advances*. **37** (7), 107405 (2019).
28. Gutierrez-Valdes, N. et al. Hairy root cultures—a versatile tool with multiple applications. *Frontiers in Plant Science*. **11** (2020).
29. Che, P. et al. Developing a flexible, high‐efficiency Agrobacterium‐mediated sorghum transformation system with broad application. *Plant Biotechnology Journal*. **16** (7), 1388–1395 (2018).
30. Keshavareddy, G., Kumar, A., Ramu, V. S. Methods of plant transformation-a review. *International Journal of Current Microbiology and Applied Sciences*. **7** (7), 2656–2668 (2018).
31. Lacroix, B., Citovsky, V. Beyond Agrobacterium-mediated transformation: horizontal gene transfer from bacteria to eukaryotes. *Agrobacterium Biology*. 443–462 (2018).
32. Sarkar, S., Ghosh, I., Roychowdhury, D., Jha, S. in *Biotechnological approaches for medicinal and aromatic plants*. Springer. 27–51 (2018).

33. Singh, R. S. et al. in *Biotechnological Approaches for Medicinal and Aromatic Plants*. Springer. 235–250 (2018).
34. Bahramnejad, B., Naji, M., Bose, R., Jha, S. A critical review on use of Agrobacterium rhizogenes and their associated binary vectors for plant transformation. *Biotechnology Advances*. **37** (7), 107405 (2019).
35. Meng, D. et al. Development of an efficient root transgenic system for pigeon pea and its application to other important economically plants. *Plant Biotechnology Journal*. **17** (9), 1804–1813 (2019).
36. Surekha, C. et al. Agrobacterium-mediated genetic transformation of pigeon pea (Cajanus cajan (L.) Millsp.) using embryonal segments and development of transgenic plants for resistance against Spodoptera. *Plant Science*. **169** (6), 1074–1080 (2005).
37. Saxena, K. Genetic improvement of pigeon pea—a review. *Tropical Plant Biology*. **1** (2), 159–178 (2008).
38. Hada, A. et al. Improved Agrobacterium tumefaciens-mediated transformation of soybean [Glycine max (L.) Merr.] following optimization of culture conditions and mechanical techniques. *In Vitro Cellular & Developmental Biology-Plant*. **54** (6), 672–688 (2018).
39. Foti, C., Pavli, O. High-efficiency Agrobacterium rhizogenes-mediated transgenic hairy root induction of lens culinaris. *Agronomy*. **10** (8), 1170 (2020).
40. Bhattacharya, A., Sood, P., Citovsky, V. The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. *Molecular Plant Pathology*. **11** (5), 705–719 (2010).





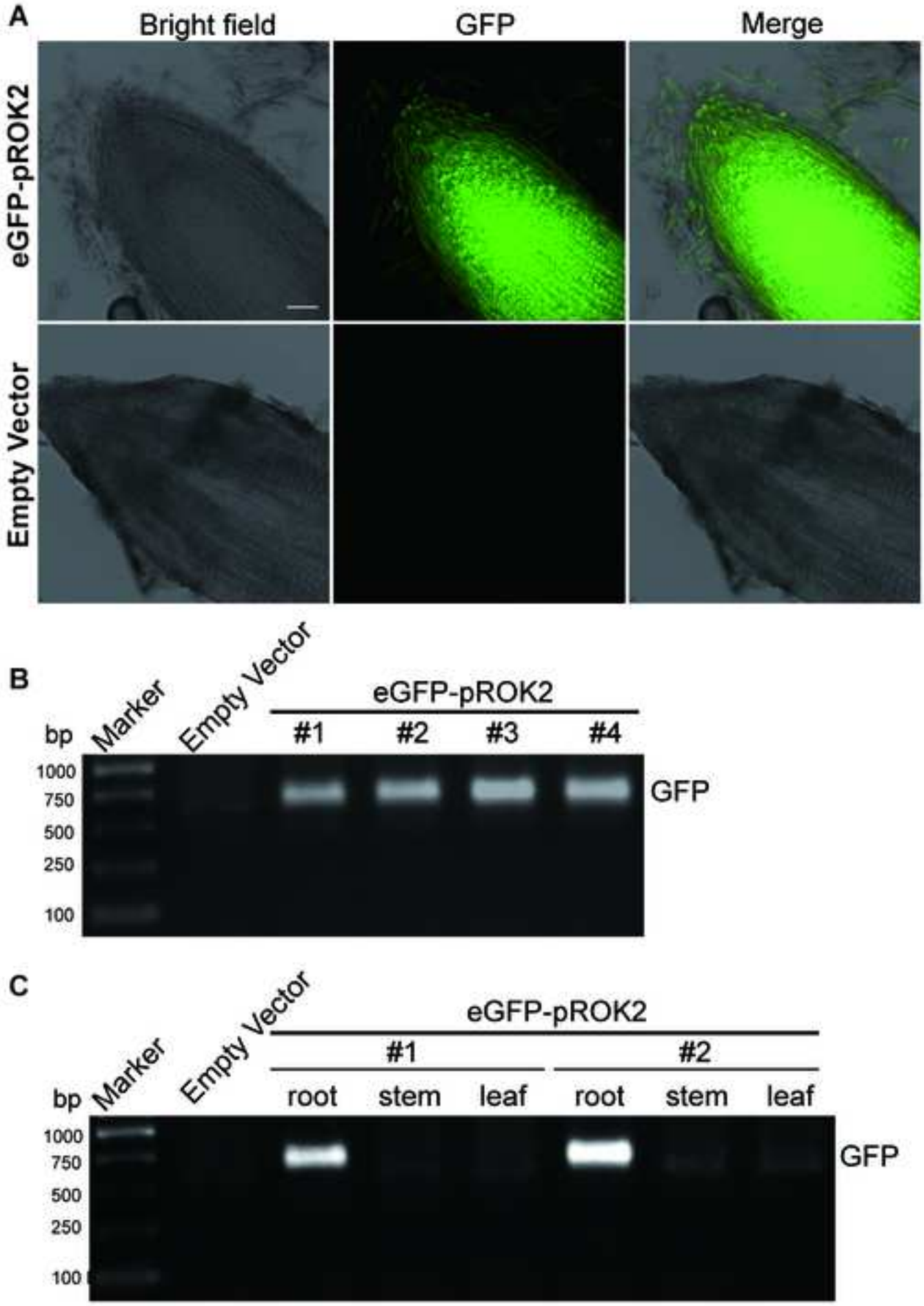


Fig3

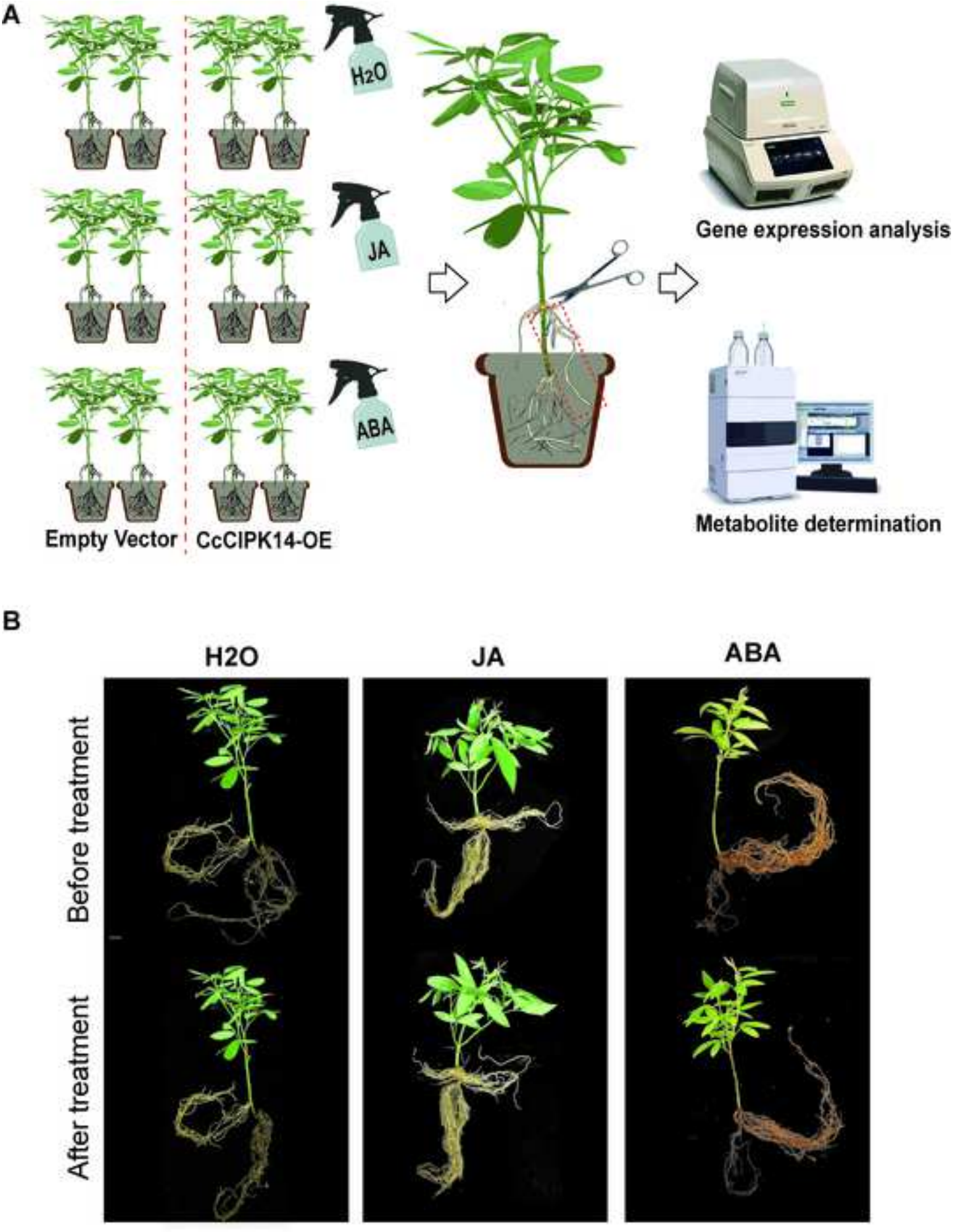
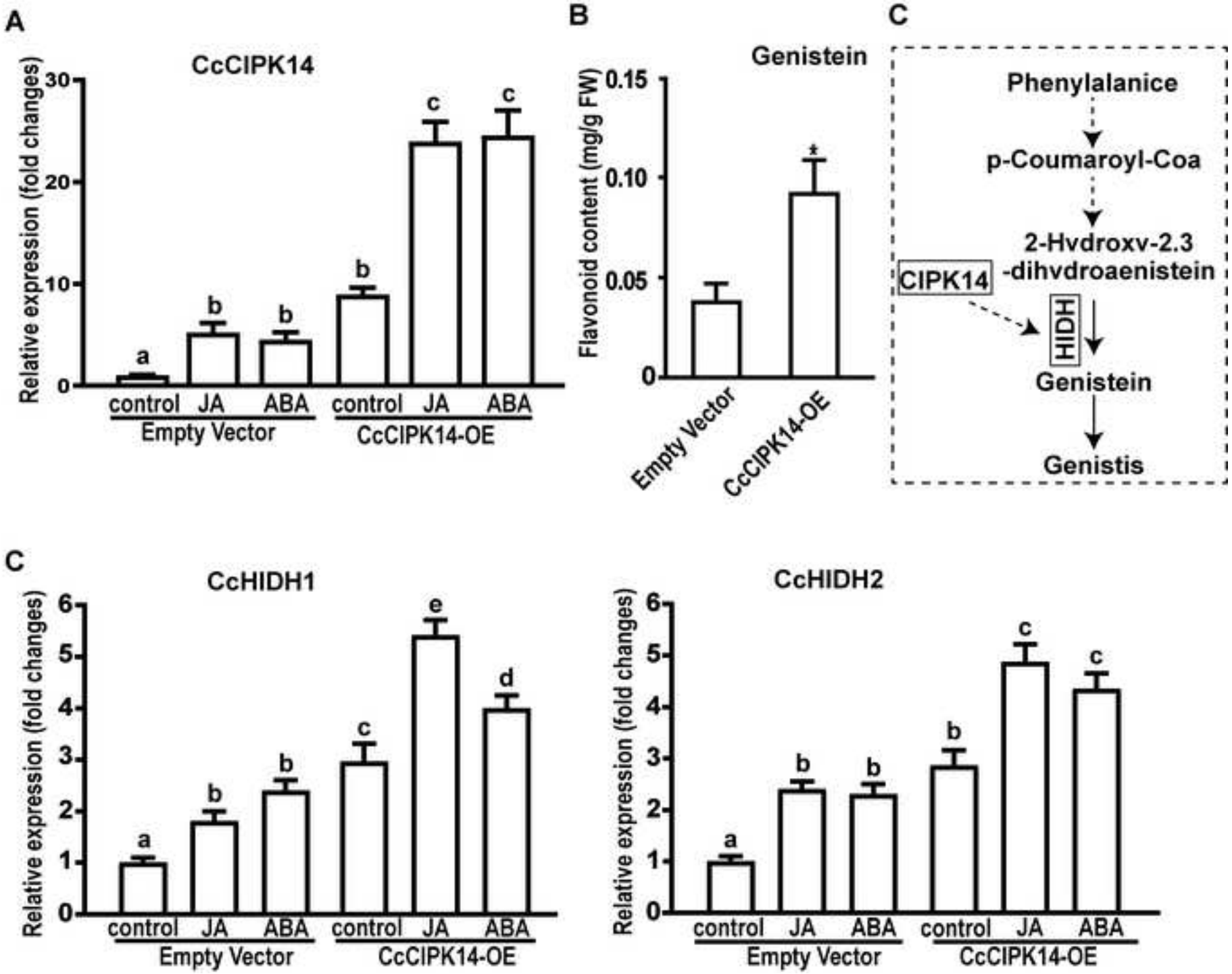


Fig4



Primer	Sequence(5'-3')
CcCIPK14-q-F	GAGACCAACATTGATGATGTGGAAGC
CcCIPK14-q-R	CATTCATTGGTGTGTTGGCTGCTCTTC
CcHIDH1-q-F	GAGGCTGTGCTGGAGTCAAT
CcHIDH1-q-R	AGCTCAGCTAATCTGGTGGC
CcHIDH2-q-F	AGCCCCTATCTCTGTTGGGT
CcHIDH2-q-R	ACTGCTGCAAGTGGCTTACT
GFP-F	CCACAAGTTCAGCGTGTCCG
GFP-R	AAGTTCACCTTGATGCCGTTC

List of Responses

Dear Editor

Thank you for your letter concerning our manuscript entitled "JoVE62304".This is valuable and very helpful for revising and improving our paper. Revised portion are marked in the paper. The main corrections in the paper are as flowing:

Responds to the Editor's comments:

Please employ professional copy-editing services. The language in the manuscript is not publication grade. There are significant issues with the writing that severely reduces the understanding of the science.

Response:*In order to improve the language of the manuscript and bring it up to publication level, the manuscript language was retouched, especially to correct errors in English grammar, spelling, and sentence structure.*

Please revise Table 1 to be an xls/xlsx file.

Response: *We have made revise according to the comments.*