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SHORT COMMUNICATION

Transient expression of the *β-glucuronidase* gene in *Cannabis sativa* varieties

Aleksei Sorokin*, Narendra Singh Yadav*, Daniel Gaudet, and Igor Kovalchuk

Department of Biological Sciences, University of Lethbridge, Lethbridge, Canada

ABSTRACT

In plant biology, transient expression analysis plays a vital role to provide a fast method to study the gene of interest. In this study, we report a rapid and efficient method for transient expression in *Cannabis sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation. *A. tumefaciens* strain EHA105 carrying the pCAMBIA1301 construct with *uid*A gene was used to transform cannabis seedlings and the GUS assay (a measurement of β-glucuronidase activity) was used to detect the *uid*A expression. In the current study, we have also established a rapid germination protocol for cannabis seeds. The all three steps seed sterilization, germination and seedlings development were carried out in a 1% H_2O_2 solution. Transient transformation revealed that both cotyledons and young true leaves are amenable to transformation. Compared with tobacco (*Nicotiana benthamiana*), cannabis seedlings were less susceptible to transformation with *A. tumefaciens*. Susceptibility to *Agrobacterium* transformation also varied with the different cannabis varieties. The method established in this study has the potential to be an important tool for gene function studies and genetic improvement in cannabis.

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Cannabis sativa; rapid germination; *Nicotiana benthamiana*; *Agrobacterium*-mediated transformation; *Agrobacterium* susceptibility; transient expression; GUS assay

Introduction

Cannabis sativa is an annual dioecious herb that belongs to family *Cannabaceae*. The male plant is characterized by heterogametic chromosomes (XY) with homogametic chromosomes (XX) conferring the female plant phenotype.[1] Historically, cannabis has been widely cultivated as a source of seed oil, fiber and intoxicating resin. First written evidence of using cannabis in medicinal practices is described in the compendium of Chinese medicinal herbs by Emperor Shen Nung, dated 2737 B.C.E.[2] In the last decades, the therapeutic potential of cannabinoids has been reported for the treatment of a range of human diseases from complex neurological diseases to cancer.[3] Although cannabis is best known for the psychoactive compound D9-tetrahydrocannabinol (THC), it also contains varying levels of non-psychoactive cannabinoids such as cannabidiol (CBD), cannabigerol (CBG), D9-tetrahydrocannabivarin (THCV), and cannabichromene (CBC), that show promising therapeutic properties and in some cases mitigate the psychoactive effects of THC.[4]

Considering the enormous economic importance, it is worthy to study the functional genomics of cannabis. The transient expression analysis is an important tool for functional genomics study. *Agrobacterium*-mediated transformation is commonly used to achieve both transient and stable gene expression in plants. Wahby *et al.*[5] reported that *C. sativa* hypocotyl tissues exhibited high susceptibility to *Agrobacterium* infection/transformation than other tissues. Recently, Chaohua *et al.* 2016[6] established regeneration protocol that uses cotyledons of *C. sativa* as an explant. In the present study, we employed intact cannabis seedlings for establishment of transient expression protocol. Such protocol can be used for functional genomics study and for the development of stable transformation protocol. In this study, we developed an efficient method for transient expression analysis in *C. sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation and demonstrated that cannabis is less susceptible to *Agrobacterium* transformation than tobacco. Further, we also displayed that susceptibility to *Agrobacterium* infection also varied with the different cannabis varieties.

Results and discussion

Transient expression analysis provides a rapid method to study the function of genes. Transient transformation protocols may also be used to develop stable transformation protocols. In this study, we have reported a rapid and efficient method for transient expression in *Cannabis sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation. The *Agrobacterium tumefaciens* strain EHA105 carrying the pCAMBIA1301 construct was used to transform cannabis seedlings and the GUS assay was used to detect the transgenes.

Hydrogen peroxide (H_2O_2) has been used as a disinfectant for seeds for decades.[7] Nandi *et al.*[8] demonstrated that 1% H_2O_2 was effective in increasing Chili seed germination percentage, vigor index and inhibition of mycelial growth. In current study, we have established all three steps seed sterilization, germination and seedlings development in a 1% H_2O_2 solution. The 1% H_2O_2 solution as a sterilant presents significant advantage over mercuric chloride or bleach that require additional washing of seeds, and separate germination and seedling development steps in Murashige and Skoog (MS) agar medium. The 1% H_2O_2 treatment resulted in significantly higher and rapid germination than water control at 24 h (Figure 1a) which

CONTACT Igor Kovalchuk igor.kovalchuk@uleth.ca Department of Biological Sciences, University of Lethbridge, Lethbridge T1K 3M4, Alberta, Canada
*These authors have contributed equally to this work.

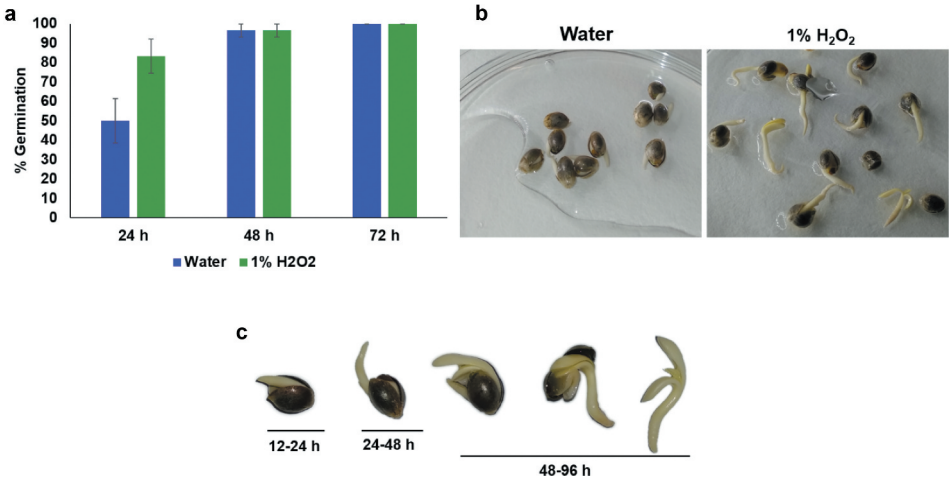

Figure 1. Germination of cannabis seeds in 1% hydrogen peroxide solution and water. (a) Comparison of germination percentage between 1% H_2O_2 and water. Data were shown as mean ± SE ($n = 3$). (b) Representative photographs of germinated seeds/seedlings in the 1% H_2O_2 or water on 4th day. (c) Various stages of germination for cannabis seedlings in 1% H_2O_2 solution. 12–24 h, cannabis embryo absorbs water until radicle breaks through the seed coat; 24–48 h, further development of radicle; 48–96 h, cotyledons emergence and development of two fully opened cotyledons with two early true leaves.

suggested that H_2O_2 enhanced the germination frequency and seedling development (Figure 1a-B). This is a very rapid germination method in 1% H_2O_2 solution as more than 80% germination occurs within 24 h and seedling development to the two cotyledons stage occurred in 72–96 h (Figure 1b,c). After 3–4 days of incubation in 1% H_2O_2 solution, seedlings emerge from seed coats with two fully opened cotyledons and two immature true leaves (Figure 1b,c); seedlings at this developmental stage were used for transformation. Previous literature reports showed that that different varieties of cannabis showed different germination response and revealed optimal germination within 4–7 days by using various germination methods[9] and seedling development in 5–15 days or more.[9] In comparison to aforementioned method, the present germination method provides cannabis seedlings in very short period (3–4 days) with least efforts (Figure 1). Similarly, Çavusoglu and Kabar[10] demonstrated that exogenous application of H_2O_2 to seeds of different plant species increases seed germination rates, coleoptile emergence percentages, radicle and coleoptile elongation, and fresh weights of the seedlings.

The overall workflow for the transient transformation of cannabis seedlings is presented in Figure 2. We have used the intact seedlings (two cotyledons stage or two cotyledons with young true leaves stage) for transformation. To enhance the transformation efficiency, we have used the vacuum infiltration followed by 3-days co-cultivation on MS agar media. Vacuum infiltration has been shown to enhance the transformation efficiency of *Artemisia annua* seedlings.[11] To detect the gene transformation in cotyledons and true leaves, the GUS activity assay was employed (Figure 3). GUS analysis revealed that both cotyledons and young true leaves are amenable to transformation (Figure 3). The transformation experiment was repeated

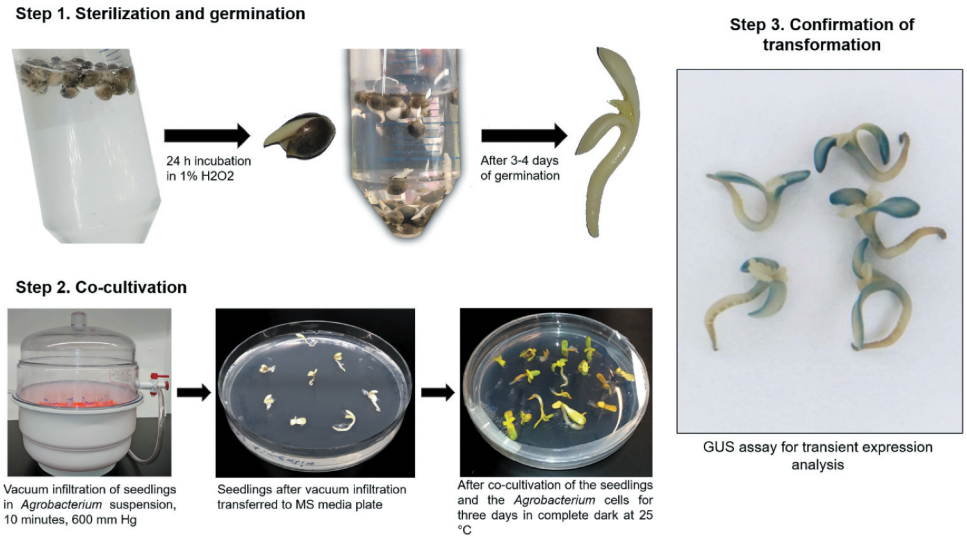

Figure 2. Workflow for *Agrobacterium*-mediated transient transformation of cannabis seedlings. Step 1. Sterilization and germination, seeds are soaked in 1% H_2O_2 solution for 24 hours until germination and then transferred into fresh solution. Seeds are then incubated in 1% H_2O_2 until both cotyledons and epicotyl are visible. Step 2. Co-cultivation, vacuum applied to seedlings submerged in *Agrobacterium* cell suspension, seedlings are then transferred to MS media plates and incubated for three days in complete dark at 25°C. Step 3. Confirmation of transformation, histochemical GUS assay using transformed seedlings.

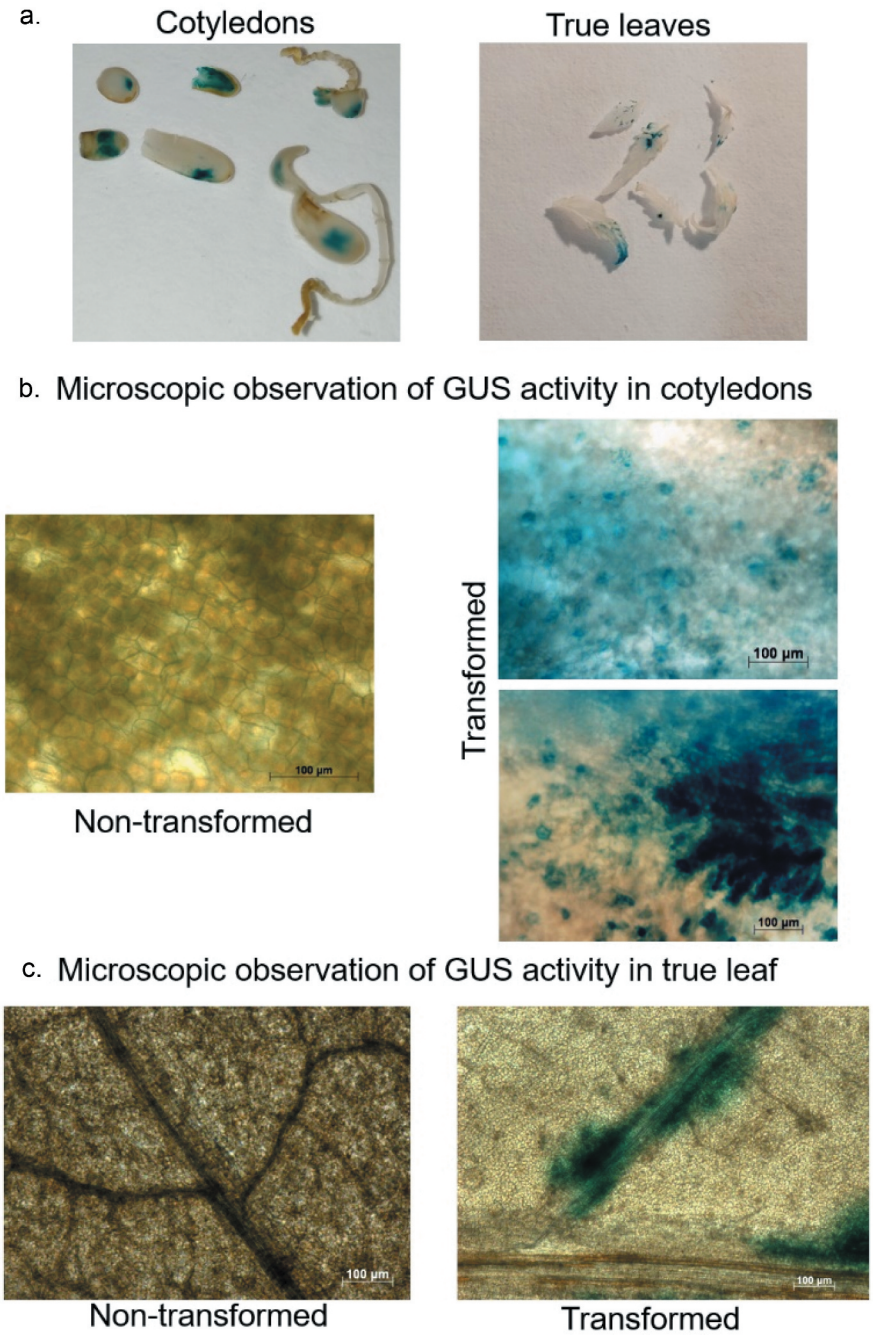

Figure 3. Representative images of GUS activity analysis in cotyledons and leaves tissues of cannabis seedlings to confirm the transient transformation. (a) GUS activity analysis in cotyledons (left panel) and true leaves (right panel). (b) Microscopic observation of GUS activity in cotyledons, non-transformed tissue (left panel) and transformed tissue (right panel). Scale bar 100 µM. (c) Microscopic observation of GUS activity in true leaf, non-transformed tissue (left panel) and transformed tissue (right panel). Scale bar 100 µM.

four times and in one independent experiment, approximately30 seedlings were evaluated. Previously, Feeney and Punja[12,13] successfully demonstrated stable transformation of a hemp cell suspension cultures with *A. tumefaciens* strain EHA101 carrying the binary vector pNOV3635 with a gene encoding phosphomannose isomerase, although they failed to regenerate fully transgenic cannabis plants. Vacuum infiltration-based *Agrobacterium* mediated gene delivery system were used in both protocols. However, there are many differences between protocols. The main difference is that Feeney and Punja 2015[13] used hemp cell suspension culture for transformation, whereas we have used intact cannabis seedlings for transformation. Feeney and Punja 2015[13] used only one hemp cultivar Anka, while we used three different medical cannabis varieties Nightingale, Holy Grail x CD-1, and Green Crack CBD. Feeney and Punja 2015[13] used *Agrobacterium tumefaciens* strain EHA101 carrying binary vector pNOV3635 and we have used EHA105 strain carrying binary vector pCAMBIA 1301. By using our protocol, we have achieved an average transformation frequency with a range of 45–70.6%, while Feeney and Punja 2015[13] method achieved an average transformation frequency with a range of 15.1–55.3 %.

Wahby *et al.* 2013[5] reported that hypocotyls tissues were most susceptible to *A. rhizogenes* infection, while young leaves and cotyledons did not, even when the bacteria were stimulated with acetosyringone. These contradicting results may be due to different *Agrobacterium* strains or different cannabis varieties used in studies.

Comparative qualitative analysis revealed that cannabis seedlings showed less GUS activity than *Nicotiana benthamiana* (tobacco) suggesting that cannabis is less susceptible to *Agrobacterium* infection than tobacco (Figure 4). Susceptibility to *Agrobacterium* infection also varied among the different cannabis varieties. Percentage analysis of transformed seedlings (seedlings which showed at least one visible GUS staining dot in leaves and/or cotyledons) revealed that Nightingale variety showed significantly higher transformed seedlings (70.6%) as compared to Green Crack CBD (45%) and Holy Grail x CD-1 (50%) ($p < .05$) (Figure 5a). The Nightingale exhibited the GUS staining dots throughout the leaves and cotyledons, however Green Crack CBD and Holy Grail x CD-1 strains showed only fewer GUS staining dots which demonstrates that the Nightingale strain showed higher susceptibility than the Green Crack CBD and Holy Grail x CD-1 (Figure 5b). One possible reason behind this differential susceptibility could be the different secondary metabolite profiles (cannabinoid, terpenoid, alkaloid, and polyphenols) of these cannabis varieties which may impact pathogen defense response. Response to *Agrobacterium*-infection can be considered as a pathogen response. It is well established that plant host defense response triggered by *Agrobacterium* infection play crucial role in influencing the susceptibility of plant cells.[14-17] Tie *et al.*[18] reported that defense-related genes play a vital role in interplay between *Agrobacterium* and plant cell. It has been also reported that cultivars of the same species showed differential efficiency to *Agrobacterium* transformation.[18-21] Tie *et al.* 2012[18] reported that the transformation efficiency of the indica rice cultivars was lower as compared to japonica cultivars. Further, they demonstrated that the lower T-DNA integrity resulted in lower transformation efficiency in indica rice. The down-regulation of genes involved in DNA repair early after transformation in indica rice may directly lead to the low integration efficiency. Microarray analysis revealed that some genes necessary for the transformation process were down-regulated in the indica cultivar, highlighting the impact of plant defense response on *Agrobacterium*-mediated transformation.[18] Previously, Feeney and Punja[22] reported that cannabis is amenable to genetic transformation using *Agrobacterium* however the plant is recalcitrant to regeneration, impeding the recovery of transgenic cannabis plants.

In conclusion, we developed a rapid and efficient method for transient expression in *C. sativa* seedlings using *Agrobacterium tumefaciens*-mediated transformation which has potential to be an important tool for gene-function studies and genetic improvement in *C. sativa*.

Materials and methods

Materials

Biological materials

(1) *Agrobacterium tumefaciens* strain (EHA105) carrying binary vector pCAMBIA1301 with uidA gene was used in our study. *Agrobacterium* strain (EHA105) and the plasmid vector pCAMBIA1301 were a gift from Prof. Barbara Hohn, Friedrich Miescher Institute, Basel, Switzerland.

(2) *Cannabis sativa* (Candida CD-1, Nightingale, Green Crack CBD, and Holy Grail x CD-1 varieties) and *Nicotiana benthamiana* seeds were used in this study. All feminized seeds were produced from in-house cannabis varieties. Cuttings from mother plants were subjected to vegetative growth under 18 h light/6 h dark cycle. After 5–6 weeks of vegetative growth selected plants are then masculinized using three times foliar sprays of 3 mM Silver thiosulfate as described by Lubell and Brand 2018.[23] To produce feminized seeds, one masculinized plant and 3 female plants are then placed in a separate closed grow tent. Plants are then subjected to 12 h photoperiod for flowering until seed harvesting. For all cannabis varieties, seeds were harvested in our laboratory and were not older than 6 months when employed in the experiments.

Chemicals

(1) Hydrogen Peroxide 30% (Merck®, catalog number: 1072091000)

(2) Agrobacterium liquid growth medium (YEP liquid medium) (see Recipes)

(3) Agrobacterium liquid induction medium (see Recipes)

(4) Histochemical GUS staining solution (see Recipes)

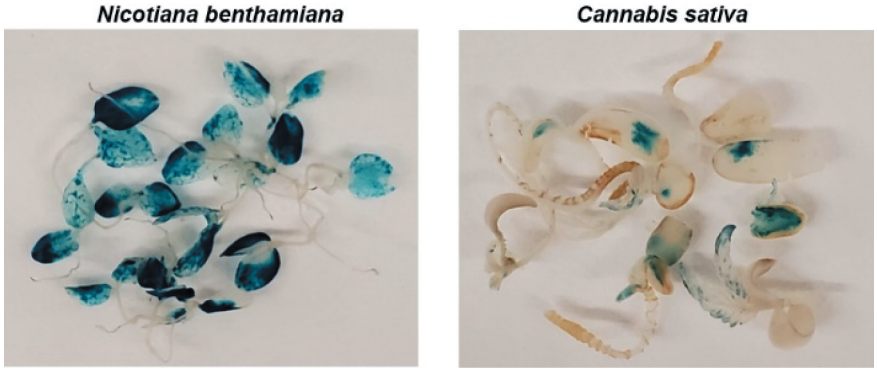

Figure 4. Comparative transient expression analysis between cannabis and tobacco using GUS staining.

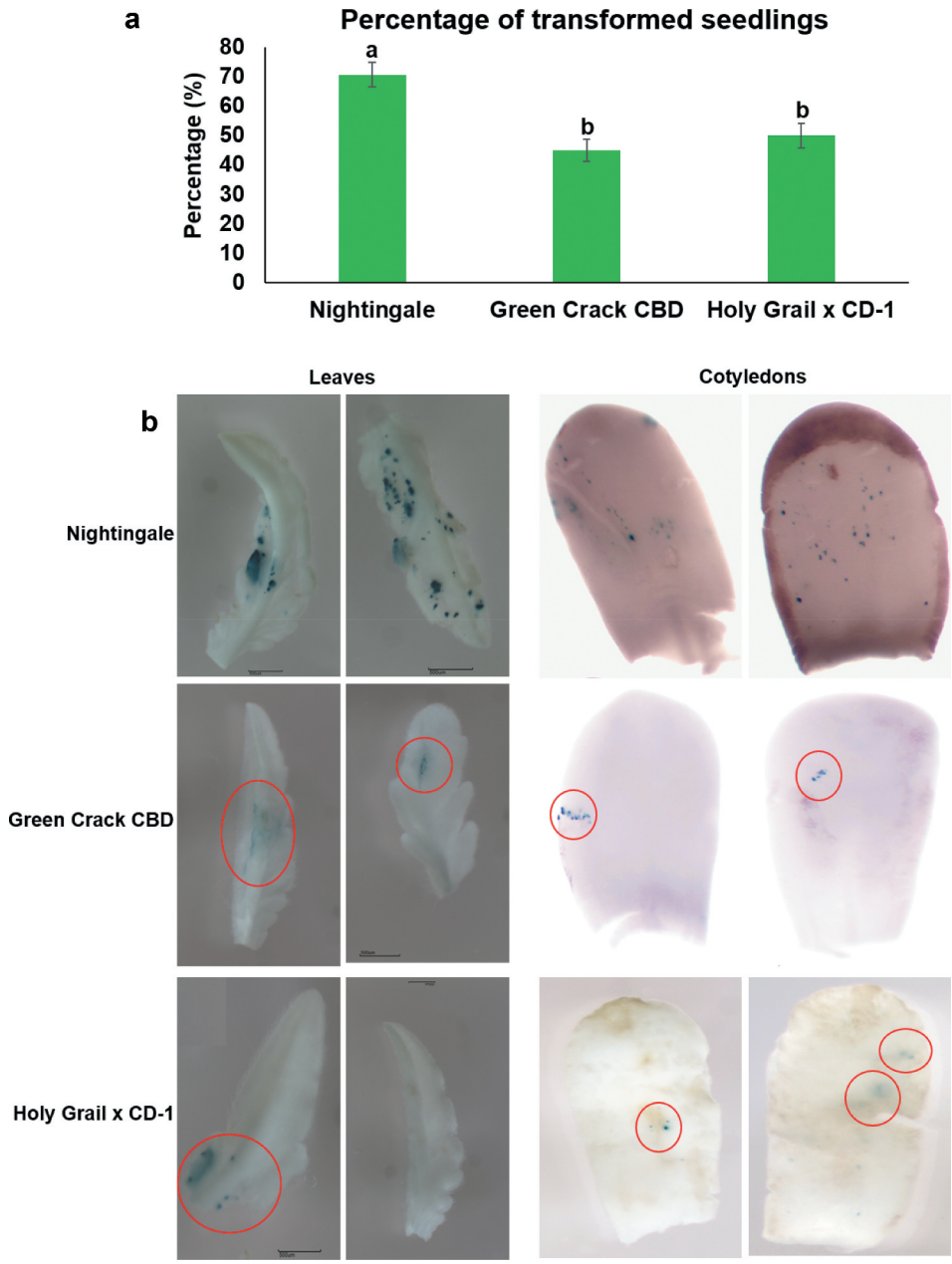

Figure 5. Comparative transient expression analysis among cannabis varieties Nightingale, Green Crack CBD and Holy Grail x CD-1. (a) Percentage of transformed seedlings which showed at least one visible GUS staining dots in leaves and/or cotyledons. Four independent transformation experiments were carried out and in one independent experiment 30 seedlings were used. The data were analyzed by one-way ANOVA with Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. Data are shown as average mean ±SE ($n = 4$). A p-value less than 0.05 ($p \leq 0.05$) were considered statistically significant. Mean values that were significantly different from each other are indicated by different letters. (b) Representative images of comparative GUS staining. The Nightingale exhibited the GUS staining dots throughout the leaves and cotyledons. However Green Crack CBD and Holy Grail x CD-1 varieties showed only fewer GUS staining dots which are red circled.

(5) MS solid media (see Recipes)
(6) MgSO4 (Sigma-Aldrich, catalog number: MX0075-1)
(7) Acetosyringone (Sigma-Aldrich, catalog number: D134406)
(8) Murashige & Skoog Basal Medium with Vitamins (PhytoTechnology Laboratories®, catalog number: M519)
(9) Kanamycin sulfate (PhytoTechnology Laboratories®, catalog number: K378)
(10) Rifampicin (Sigma-Aldrich, catalog number: R3501)
(11) Selective antibiotics: Kanamycin, Rifampicin
(12) 70% Ethanol
(13) Sucrose (Sigma-Aldrich, catalog number: S0389)
(14) MES (Sigma-Aldrich, catalog number: M3671)
(15) Agar
(16) Yeast extract
(17) NaCl
(18) Peptone
(19) EDTA (pH 8.0) (Sigma-Aldrich, catalog number: E9884)

(20) Sodium phosphate buffer (pH 7.0)
(21) Triton X-100 (Sigma-Aldrich, catalog number: 234729)
(22) Potassium ferricyanide (Sigma-Aldrich, catalog number: 702587)
(23) Potassium ferrocyanide (Sigma-Aldrich, catalog number: P3289)
(24) X-Gluc (Sigma-Aldrich, catalog number: R0852)

Plasticware

(1) Sterile empty 100 × 15 mm Petri plates (VWR International, catalog number: 25384–342)
(2) Sterile disposable 50 ml screw-cap centrifuge tubes (BD, Falcon™, catalog number: 352070)
(3) Plastic pipette tips (20, 200, and 1,000 µl)
(4) Disposable Cuvettes
(5) Sterile filter papers

Equipment

(1) Spectrophotometer
(2) Allegra Benchtop Centrifuge X-12 (Beckman Coulter)
(3) Micro-centrifuge
(4) Laminar flow hood
(5) Eppendorf Research® plus 10, 20, 200, and 1,000 µl
(6) Analytical balance
(7) Top loading electronic balance
(8) pH meter
(9) Vortex mixer
(10) Freezer (- 80°C) (e.g. New Brunswick, model:)
(11) Sterile forceps and scalpel (sterilized by heat treatment using a Bunsen burner)
(12) Sterile inoculating loop
(13) A desiccator attached to a vacuum pump (Brinkman DistiVac)
(14) Growth chamber
(15) Shaker incubator (28°C, 220 rpm)
(16) Incubator 37°C
(17) Fluorescent microscope (Zeiss Observer Z1)

Methods

Rapid germination and seedlings development (performed under sterile conditions)

(1) For germination, seeds were soaked in a 1% hydrogen peroxide solution incubated overnight for 24 hrs at room temperature in the dark. The following day, radicles with hypocotyl are visible (Figure 1).
(2) Transfer germinated seeds into fresh 1% H_2O_2 solution and further incubate for 3–4 days until cotyledons have fully opened and two early true leaves are visible.
(3) Remove remaining seed coats using sterile scalpel and forceps.
(4) Sterilize seedlings without seed coats by soaking them in 1% hydrogen peroxide for 5 min.
(5) Prior to transformation rinse seedlings in sterile water 3 times to remove remaining hydrogen peroxide.

Preparation of Agrobacterium cells culture (all steps performed under sterile conditions)

(1) Two days before transformation, inoculate 100 ml of YEP (containing 50 µg/mL Kanamycin and 25 µg/mL Rifampicin) with *Agrobacterium* from glycerol stock and culture at 28°C in an incubator shaker 220 rpm overnight.
(2) Next day centrifuge the *Agrobacterium* cells culture at 4,000 x g for 15 min at RT.
(3) Remove supernatant and add 3 ml of 10 mM MgSO4, resuspend the *Agrobacterium* pellet.
(4) Repeat steps 2 and 3.
(5) Centrifuge a third time, remove supernatant.
(6) Resuspend the *Agrobacterium* pellet in an appropriate volume of induction medium (MS liquid media) so that the final OD600 = 0.6.
(7) Add 100 mM acetosyringone to final concentration 100 µM.

Co-cultivation (all steps performed under sterile conditions)

(1) Place sterilized seedlings in 50 ml Falcon tubes with 30 ml of the *Agrobacterium* cells suspension (*Agrobacterium* cells in induction medium supplemented with acetosyringone).
(2) Place the tubes into a sterile vacuum chamber and apply vacuum for 10–20 min.
(3) Transfer seedlings to a sterile filter paper to remove the excess *Agrobacterium* cell culture.
(4) Transfer the seedlings to 90 mm petri dishes containing MS media (10 seedlings per plate). Spread them evenly on the plate using forceps. Seal the Petri dishes with parafilm.
(5) Co-cultivate the seedlings and the *Agrobacterium* cells for three days in the dark at 25°C.
(6) After co-cultivation, seedlings can be used directly for GUS staining or can be frozen at −80°C for further analysis e.g. MUG assay, PCR analysis.

Transient expression analysis by GUS assay

(1) After 3-days co-cultivation, rinse seedlings in sterile water.
(2) Place seedlings in 50 ml Falcon tubes with Histochemical GUS staining solution.
(3) Apply vacuum for 10 min.
(4) Incubate overnight at 37°C.
(5) After staining, rinse seedlings in 70% ethanol to remove excessive stain.
(6) Keep seedlings in 70% alcohol for distaining of chlorophyll.

Recipes

(1) YEP liquid medium (1 L)

10 g Yeast extract
10 g Peptone

5 g NaCl
pH 7.0 Autoclave

(1) GM medium (1 L)
4.43 g Murashige & Skoog Basal Medium with Vitamins
10 g Sucrose
500 mg MES
pH 5.7, autoclave

(1) MS sold media (1 L)
4.43 g Murashige & Skoog Basal Medium with Vitamins
8 g Agar
pH 5.7, autoclave

(1) Histochemical GUS stain solution
2 mM Potassium ferrocyanide
2 mM Potassium ferricyanide
100 mM Sodium Phosphate Buffer
500 mg X-Gluc (pre dissolve in dimethyl formamide)
0.1% Triton X-100
1 mM EDTA

Statistical analysis

The data were analyzed by one-way with Tukey's multiple comparisons test using GraphPad Prism version 8.4.2 for Windows. Data were shown as mean ±SE. A *p-value* less than 0.05 were considered statistically significant.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORCID

Aleksei Sorokin http://orcid.org/0000-0003-0444-5552
Narendra Singh Yadav http://orcid.org/0000-0003-0122-173X

Author contributions

Conceptualization, N.S.Y. and I.K.; Methodology, A.S. and N.S.Y.; Validation, A.S., N.S.Y. and D.G.; Investigation, A.S., N.S.Y. and D.G.; Resources, I.K.; Data Curation, A.S., N.S.Y., D.G. and I.K.; Writing – Original Draft Preparation, N.S.Y; Writing – Review & Editing, A.S., N. S.Y., D.G. and I.K.; Visualization, A.S., N.S.Y. and I.K.; Supervision, N.S. Y. and I.K.; Project Administration, I.K.; Funding Acquisition, I.K.

Competing interests

The authors declare that they have no competing interests.

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