Protoplast Isolation, Transient Transformation, and Flow-cytometric Analysis of Reporter-gene Activation in *Cannabis sativa* L.

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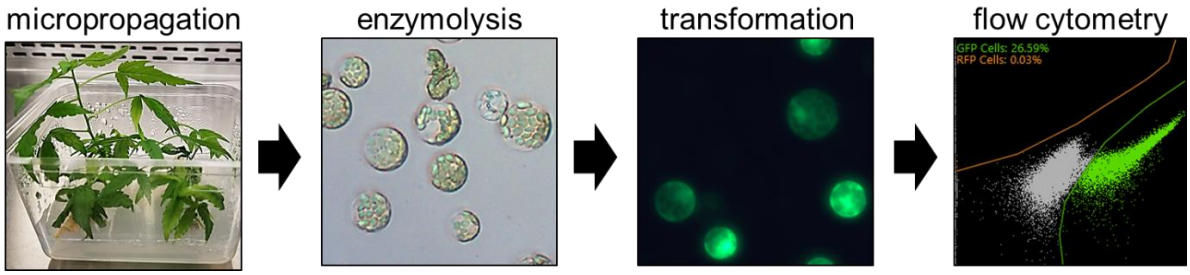
Graphical abstract



Highlights

- A simple, hormone-free micropropagation procedure for *Cannabis sativa*
- Isolation of cannabis leaf protoplasts with 2 million/g yield and 82% viability
- Transient transformation of protoplasts with up to 31% efficiency
- Auxin-responsive reporter-gene activation measured using flow cytometry

Abstract

Cannabis sativa L. is a valuable, up-and-coming industrial crop with a substantially growing market. However, due to an extended period of legal restriction, research with cannabis has been limited, particularly in laboratory settings. Expanding the application of biotechnological techniques to cannabis can facilitate addressing species-specific impediments to improving crop traits and further fundamental understanding of its intricacies. Here, we describe application of protoplast transformation for the study of transient gene expression in a low-THC cannabis cultivar. To produce explant tissue as a source of protoplasts, a method for hormone-free *in vitro* micropropagation is established. Protoplasts are isolated from young leaves of the micropropagated stocks and transiently transformed with plasmid DNA carrying a fluorescent marker gene. This is the first report of protoplast transformation in this species. A protoplast isolation yield is achieved of up to 2 x 10^6 cells per gram of leaf material, vitality staining shows that up to 82% of isolated protoplasts are viable, and quantification of the cells expressing a fluorescent protein indicates that up to 31% of the cells can be successfully transformed. Additionally, protoplasts are transformed with an auxin-responsive reporter gene and the reaction to treatment with indole-3-acetic acid is quantified using flow cytometry. This work demonstrates that relatively minor modification of standard techniques can be used to study this important emerging crop.

Keywords

biotechnology; *Cannabis sativa*; flow cytometry; micropropagation; protoplast transformation

1. Introduction

Cannabis sativa has numerous varieties with vastly differing properties (de Meijer and Keizer, 1996). The crop has various applications, including use of the seed for its nutritional value, fibers for textile, and metabolites for medicinal use (Farinon et al., 2020; Freeman et al., 2019; Vandepitte et al., 2020). Cannabis has been grouped into "drug-type" varieties with high Δ^9-tetrahydrocannabinol (THC) content and "industrial hemp" varieties with a low ($\leq$0.3%) THC content. Historically, industrial hemp has been used predominantly for fiber and oilseed production. However, novel pharmaceutical uses for the plant's non-psychoactive cannabinoids as well as the US federal legalization of industrial hemp have led to a recent resurgence in interest for this crop and the application of biotechnology to improve its genetics (Fike, 2016; Gray et al., 2016; Thomas and ElSohly, 2016; Hesami et al., 2020).

Due to the highly heterozygous, outcrossing nature of many of the elite hemp varieties, clonal propagation is essential to maintaining genotypic fidelity. *In vitro* micropropagation is used to minimize the space needed for mass multiplication and maintenance of elite germplasm. Several studies have shown that the inclusion of plant growth regulators (phytohormones), especially cytokinin analogs, enhance the propagation rate (Wang et al., 2009; Lata et al., 2010, 2016; Piunno et al., 2019; Mestinšek-Mubi et al., 2020). However, there have been reports of somaclonal variation or genetic instability in micropropagation, potentially due to the use of plant growth regulators in the procedure (Kodym and Leeb, 2019). Studies on the optimization of cannabis micropropagation have shown that genotype, medium nutrient composition, and growth regulator supplementation can all be of significant influence on propagation efficiency, both qualitatively and quantitatively, *e.g.* (Page et al., 2020; Wróbel et al., 2020). With the purpose of the production of plant material for use in a cell- and molecular biology lab (as well as for the exponential multiplication of germplasm), it is desirable to establish procedures for iterative micropropagation, where material can be maintained and multiplied through successive rounds

of propagation. Optimal conditions for singular and iterative micropropagations may differ substantially, as discussed in Wróbel et al. (2020).

Hemp is a prime candidate for the application of new plant breeding technologies (NPBT), *e.g.* CRISPR/Cas9-mediated gene editing. An obvious target would be THC synthase; germplasm guaranteed to lack THC production, and perhaps with increased production of alternate cannabinoids, could be engineered if this gene function can be knocked out. However, the use of NPBT in hemp is hindered by its recalcitrance to tissue culture, precluding the (re)generation of genetically modified lines (Monthony et al., 2020). Advances in enhanced plant regeneration may alleviate such obstacles in the near future (Gordon-Kamm et al., 2019; Debernardi et al., 2020).

An important step in deploying CRISPR/Cas9 strategies in crop trait improvement is to have a system to test the effectiveness of the designed guide RNAs. Transient transformation of protoplasts is a quick and effective method to assess the nuclease efficiency and specificity (Nadakuduti et al., 2019; Sant'Ana et al., 2020). Although there is one report of protoplast isolation (Morimoto et al., 2007), protoplast transformation has not been demonstrated in cannabis. Protoplast isolation is not always a straightforward procedure; the species/genotype, growth circumstances, source tissue, pretreatment conditions, employed cell-wall digestion enzymes, and buffer composition of the enzymolysis solution can all be of crucial importance and influence the quantity and viability of the isolated cells (Choury et al., 2018; Gajdová et al., 2007; Jones et al., 2012; Wang et al., 2020). Aside from testing editing efficiency, protoplasts can be used for other types of experiments, such as visualization of subcellular protein localization, measurement of enzyme activity, transcriptional network analysis, and even regeneration of non-transgenic edited plants through protoplast culture (Davey et al., 2005; Lin et al., 2018).

Here, we demonstrate hormone-free *in vitro* micropropagation of cannabis using shoot-tip and nodal explants, the isolation and transient transformation of protoplasts from leaves, and the flow-cytometric quantification of fluorescent reporter-gene activation in protoplasts. These methods are expected to be widely applicable in fundamental and translational research.

2. Materials and methods

2.1 Plant material

Cannabis sativa var. Cherry x Otto II: Sweetened was obtained as *in vitro* explants from the Institute for Advanced Learning and Research (IALR; Danville, VA, USA). *In vitro* culture was initiated at the IALR in March 2019, the protoplast experiments described herein were conducted July-October 2020. Cherry x Otto II: Sweetened (Horizon Hemp & Agriculture, USA) is a low THC/high CBD strain (average CBD-A: 14% and average THC: 0.03% per dry weight). Plants were micropropagated in Phytatray II trays (11.4 cm × 8.6 cm × 10.2 cm; Sigma-Aldrich, USA) with 100 ml MS2 media (4.4 g/l Murashige and Skoog [MS] salts with vitamins [Caisson Labs, USA], 2% w/v sucrose, 0.8% w/v agar [Sigma-Aldrich, USA]) (Murashige and Skoog, 1962). Plants were incubated in a growth chamber (CMP6010, Conviron, USA) with a 16 h light and 8 h dark regime, 75 µmol m^{-2} s^{-1} PAR (Philips F39T5/841 HO fluorescent bulbs), at 22° C. After approximately two months, apical shoot-tips and stem segments with 2-3 nodes (approximately 2 cm in length) were transplanted to new trays (4-6 plantlets per tray). *In vitro* work was conducted under aseptic conditions in a biosafety cabinet (NU-425-600, NUAIRE, USA).

2.2 Protoplast isolation and transformation

Young, not fully expanded leaves were harvested from micropropagated plantlets 1-2 months after transplantation and cut into pieces approximately 5 mm x 5 mm while submerged in deionized water. Harvesting was conducted under aseptic conditions; the rest of the procedure was conducted under non-sterile conditions. Macerated leaves were strained from the water and dried using a sieve. 0.3 g of leaf material was added to 15 ml enzymolysis solution in a 50 ml flask and incubated on a rotary shaker at 75 rpm for 16 h at room temperature under ambient light. Enzymolysis solution was modified from Yoo et al. (2007) and contained 1.25% w/v Cellulase R-10 (Yakult, Japan; Kanematsu USA), 0.3% w/v Macerozyme R-10 (Yakult, Japan; Kanematsu USA), 0.4 M mannitol, 20 mM MES (2-(N-morpholino)ethanesulfonic acid), 20 mM potassium chloride, 0.1% w/v bovine serum albumin, and 10 mM calcium chloride, adjusted to pH 5.7 with 1 M TRIS hydrochloride pH 7.5 (Sigma-Aldrich, USA) and heated to 55° C for 10 min. Modified enzymolysis solution contained the same ingredients with the addition of 0.075% w/v Pectolyase Y23 (Desert Biologicals, USA).

The solutions with digested leaves were passed over a 40 µm cell strainer (BD Falcon, USA) and centrifuged at 500 G for 5 min in a 15 ml conical tube. The supernatant was removed and pellets were resuspended in 2 ml fresh enzymolysis buffer (without enzymes). At this point, protoplasts were counted for yield calculations using a hemacytomer. Protoplast suspensions were purified using an Optiprep (Sigma-Aldrich, USA) density gradient. The 2 ml protoplast suspension was gently mixed with 1.33 ml Optiprep to give a 40% v/v Optiprep solution, 3 ml of 20% Optiprep (mixed with enzymolysis buffer, v/v) was layered on top of the 40% solution, 3 ml enzymolysis buffer was layered on top of that. Protoplasts were recovered from the 20%-0% interface after 5 min centrifugation at 500 G and washed with enzymolysis buffer (resuspended in 15 ml, centrifuged for 5 min at 500 G, and resuspended in 2 ml of fresh buffer). Purified protoplasts were stained with 1 µg/ml fluorescein diacetate (Sigma-Aldrich,

USA) and imaged with an inverted epifluorescence microscope (IX81, Olympus, USA). Viability was calculated as the percentage of apparently intact protoplasts that stained positive with fluorescein diacetate.

Purified protoplasts were washed and resuspended in MMg solution (0.4 M mannitol, 15 mM magnesium chloride hexahydrate, 4 mM MES, adjusted to pH 5.7 with 1 M potassium hydroxide [Sigma-Aldrich, USA]) at a density of 2 x 10^6 protoplasts per ml. 250 µl (5 x 10^5 protoplasts) was added to 25 µg plasmid DNA (pBeaconGFP_GR-GUS (Brooks et al., 2019), pEVTV_DR5 (Lieberman-Lazarovich et al., 2019), pBeaconRFP_GUS (Bargmann and Birnbaum, 2009)) and mixed with 250 µl PEG solution (40% w/v polyethylene glycol 1500, 0.4 mM mannitol, 0.1 M calcium chloride [Sigma-Aldrich, USA]) for 5 s. We have found that a 5 s incubation with PEG solution works as well or better than the 15 min incubation used previously (Yoo et al., 2007; Bargmann and Birnbaum, 2009). Protoplasts were washed and resuspended in 1 ml enzymolysis buffer. Protoplast suspensions were transferred to a 24-well plate and incubated at room temperature in the dark. For reporter-gene activation studies, protoplasts were treated with 5 µM indole-3-acetic acid (Sigma-Aldrich, USA; as in Bargmann and Birnbaum (2009)) during the 16 h incubation period, or mock treated.

2.3 Flow cytometry

Protoplasts were analyzed by flow cytometry using a Sony SH800S cell sorter running PBS (phosphate-buffered saline) as the sheath fluid and a 100 µm nozzle chip. Protoplasts were run through the cytometer in the enzymolysis buffer, as opposed to previously used W5 buffer (Bargmann and Birnbaum, 2009), because it prevents potential clogging issues due to precipitation of the calcium ions in the W5 buffer with the phosphate in the PBS sheath fluid. Green vs. red fluorescence was measured

using 488 nm excitation - 530/30 nm emission and 561 nm excitation - 583/30 nm emission, respectively. Cells were separated from debris by back-gating GFP-positive events to a back-scatter vs. forward-scatter plot. Data were recorded for between 2 x 10^4 and 5 x 10^4 cell events.

3. Results and discussion

3.1 Hormone-free micropropagation

In order to have access to sterile plant material, a micropropagation method was developed to grow cannabis plantlets *in vitro*. Preference was given to a method that did not involve the use of plant growth regulators in order to avoid the potential influence on subsequent experiments and optimize chances of maintaining genetic and morphological stability through iterative rounds of micropropagation. Two cm shoot tips or stem segments with two to three nodes were harvested and transplanted to trays with Murashige and Skoog medium supplemented with vitamins and sucrose. After two months, rooted plantlets ranging from four to eight cm had developed for the majority (>75%) of transplanted explants and could be harvested for further propagation or to be used in various experiments (Figure 1). On average, each explant generated two to three new explants suitable for the next round of propagation. This method worked for the variety Cherry x Otto II: Sweetened, shown here, but also worked well for several other varieties grown in the lab (data not shown).

A number of reports of successful micropropagation of cannabis have used methods that involve the use of phytohormones (Wang et al., 2009; Lata et al., 2010, 2016) but this work shows it can be achieved with an efficiency sufficient for the production of material for experimental study without the use of plant growth regulators. One previous report of hormone-free micropropagation used rockwool in

specialized containers with aeration (Kodym and Leeb, 2019). Here, micropropagation was achieved using simple off-the-shelf trays with a common plant growth medium. This process could likely be optimized further by assessing the effect of different light intensities, growth temperatures, medium supplements, or explant characteristics.

3.2 Protoplast isolation and transformation

Young, not fully expanded leaves were harvested from micropropagated plantlets and macerated before incubation in enzymolysis solution for 16 h. The enzyme cocktail was based on the recipe used for Arabidopsis (*Arabidopsis thaliana*) seedlings and leaves (Yoo et al., 2007) and contained cellulase and macerozyme. The yield was 9.05 x 10^5 protoplasts per gram of leaf material. The yield could be improved more than two-fold, to 2.27 x 10^6 protoplasts per gram of leaf material, by the inclusion of pectolyase (Figure 2A). These results indicate that supplementary pectin lyase activity (which is also present in Macerozyme R-10) provided by the inclusion of pectolyase positively influences the release of protoplasts from cannabis leaf material. The addition of pectolyase has previously been shown to increase protoplast yield in several species, including strawberry, white lupin, and Japanese lawngrass (Inokuma et al., 1996; Nyman and Wallin, 1988; Sinha et al., 2003). Potentially, the addition of extra pectin lyase makes the other cell wall polymers more accessible to digestion enzymes. Yield could perhaps be increased further by altering the enzyme concentrations or osmoticum levels. Ideally, the duration of the enzymolysis is kept as brief as possible to optimize protoplast health.

Protoplasts were further purified by centrifugation through a density gradient and viability was subsequently assessed by staining with fluorescein diacetate. Staining showed that 80% (without pectolyase) and 82% (with pectolyase) of purified protoplasts were viable, indicating that the inclusion

of pectolyase did not significantly influence protoplast viability (Figure 2B and C). This is the second report of protoplast isolation from cannabis. Morimoto et al. (2007) described the isolation of protoplasts from cannabis cell culture for use in the study of the induction of cell death but did not report results on yield or viability. We show here for the first time that high-quality protoplasts can be isolated from the leaves of *in vitro* micropropagated plantlets. Furthermore, we demonstrate that only a minor modification (inclusion of pectolyase) of procedures routinely used for the isolation of protoplasts from Arabidopsis was enough to enable efficient protoplast isolation from cannabis.

A polyethylene glycol-mediated transformation procedure based on that commonly used with Arabidopsis protoplasts (Yoo et al., 2007) was used to introduce plasmid DNA carrying a p35S:GFP expression cassette to the isolated cannabis leaf protoplasts. The next day, protoplast transformation efficiency was assessed by microscopic inspection. Whereas mock-treated protoplasts did not give a visible signal in the GFP channel (Figure 3A), protoplasts transformed with the GFP plasmid showed clear GFP expression (Figure 3B). A manual count of four independent micrographs indicated that 27% of protoplasts were successfully transformed. This is without taking into account that viability staining indicated that 18% of protoplasts were inviable after isolation (which would readjust the transformation efficiency closer to 33% of viable protoplasts).

Protoplast transformation efficiencies vary widely between different studies, tissues, and species; 5%-20% in Arabidopsis root protoplasts, 50%-70% in tomato leaf protoplasts, and up to 90% in Arabidopsis leaf protoplasts (Xing, 2020). The transformation rate achieved here falls well within the range that is compatible with various transient transformation assays. Higher efficiencies may be reached by altering the growth conditions of the starting material or varying the plasmid DNA concentration.

There is a previous report of transient transformation of cannabis using *Agrobacterium tumefaciens*-mediated transformation of seedlings to express β-glucuronidase (Sorokin et al., 2020); in comparison,

transient transformation of protoplasts is quicker, less labor-intensive, and could be used in conjunction with cytometry or cell-sorting to isolate the successfully transformed cells. This result paves the way for the use of cannabis protoplasts in transient transformation assays, for instance to test CRISPR/Cas9 editing efficiency (Sant'Ana et al., 2020) or reporter-gene activation (Bargmann and Birnbaum, 2009).

3.3 Flow-cytometric analysis

Flow cytometry can be used to assess and quantify fluorescent properties of plant protoplasts, and fluorescence-activated cell sorting can also be used to isolate cells with particular fluorescent properties (Bargmann and Birnbaum, 2010). When a suspension of mock-treated protoplasts was run through the cytometer, it could be seen that most events fell on a diagonal axis of a red vs. green fluorescence dotplot (Figure 4A). These events included both protoplasts and cell debris. When protoplasts transformed with the GFP expression cassette were analyzed, a clear GFP-positive population could be observed off the diagonal axis (Figure 4B). Since the GFP signal was only observed in live cells, this population could be used to define and isolate apparently intact cells in a back-scatter vs. forward-scatter plot (Figure 4C). A gate defined by the back-scatter vs. forward-scatter signal of the GFP positive events was back-gated onto a back-scatter vs. forward-scatter plot of all events to isolate cells from debris (Figure 4D) and indicated that 42% of all measured events were cells. Looking at the red vs. green fluorescence properties of just the cells, 27% of cells were identified as GFP-positive, matching the manual count (Figure 4E and F). These results show that cannabis leaf protoplasts can be transiently transformed with standard plant vectors and accurately analyzed by flow cytometry to identify and quantify GFP-positive cells.

Protoplasts were also transformed with a plasmid containing the DR5::GFP auxin-sensitive reporter gene (Ottenschlager et al., 2003), allowing for the cytometric quantification of the response to auxin treatment (Bargmann and Birnbaum, 2009). The synthetic DR5 promoter is activated directly by components of the auxin signaling pathway, auxin response factors (Ulmasov et al., 1997). Auxin is a phytohormone that plays pivotal roles in numerous plant developmental processes and environmental responses and whose signaling pathway is relatively well-studied (Bargmann and Estelle, 2014), including studies in cannabis (Huang et al., 2016). Auxin, its analogs, and antagonists are commonly used in cannabis tissue culture (Thacker et al., 2018; Smýkalová et al., 2019). Recently, auxin signaling in cannabis has also been associated with female flower development (Petit et al., 2020).

Analysis of protoplasts transformed with the reporter gene alone does not allow for accurate quantification of DR5::GFP activation. Quantification of the GFP signal in all cells would underestimate the response due to the inclusion of the non-transformed cells and quantification of just the GFP-positive cells would overestimate the response because any non-responding cells would be omitted (Supplemental Figure S1). Therefore, protoplasts were co-transformed with the DR5::GFP reporter and a plasmid containing a p35S:RFP expression cassette (Figure 5). Cytometric quantification of the RFP-positive cells indicated that 31% of protoplasts were successfully transformed in this experiment (Figure 5A). Using this approach, the GFP signal in just the strong RFP-positive cells (thereby avoiding any highly autofluorescent non-transformed cells) could be quantified to give an accurate readout of the auxin response solely in transformed cells. The analysis showed that cannabis leaf protoplasts respond strongly to a 16 h treatment with 5 µM indole-3-acetic acid (IAA), with an almost four-fold increase in DR5::GFP signal. These results demonstrate that leaf protoplasts can be used to investigate transcriptional responses in cannabis and that the auxin-response element isolated from Arabidopsis and present in the DR5 synthetic promoter is responsive to auxin treatment in cannabis.

4. Conclusion

This work is not intended as an optimization study of the different procedures described herein, rather to show the feasibility of bringing cannabis into a lab setting and using standard techniques to study this important industrial crop species. Sterile propagation of germplasm is an essential first step in providing plant tissue for further investigation. We demonstrate that cannabis can be micropropagated using readily available tissue culture medium and vessels (Figure 1). Obtaining protoplasts in sufficient quantity and of adequate quality is often a challenge, with low yield and deficient viability being a common problem. We show here that the young leaves of *in vitro* micropropagated plantlets are a good source tissue for the isolation of protoplasts from cannabis. The inclusion of pectolyase in the enzymolysis solution significantly improved protoplast yield during enzymolysis (Figure 2). A protoplast transformation efficiency of 27% to 31% was attained using polyethylene glycol treatment (Figure 3, 4, and 5). This was achieved using established procedures, with the exception of the shortened polyethylene-glycol treatment (5 s, as opposed to 15 min) and incubation buffer (see Materials and methods). Flow cytometry was performed using the relatively affordable and user-friendly Sony SH800S fluorescence-activated cell sorter (Figure 4 and 5, Supplemental Figure S1). We were able to use a back-gating strategy to distinguish cells from debris, thereby increasing the accuracy of the cytometric quantification of transformation efficiency.

Overall, the results presented here illustrate the use of tissue culture, cell-, and molecular biology techniques in the study of *Cannabis sativa* and show that this valuable industrial crop is amenable to such methods. We expect the research community will be able to build upon these findings and apply biotechnology innovations to improve crop traits and broaden its utilization. We anticipate that especially the procedures for protoplast isolation and transformation can be used by others, for instance

to test the efficiency of gene-editing tools and to assess the activity of enzymes involved in cannabinoid synthesis and metabolism.

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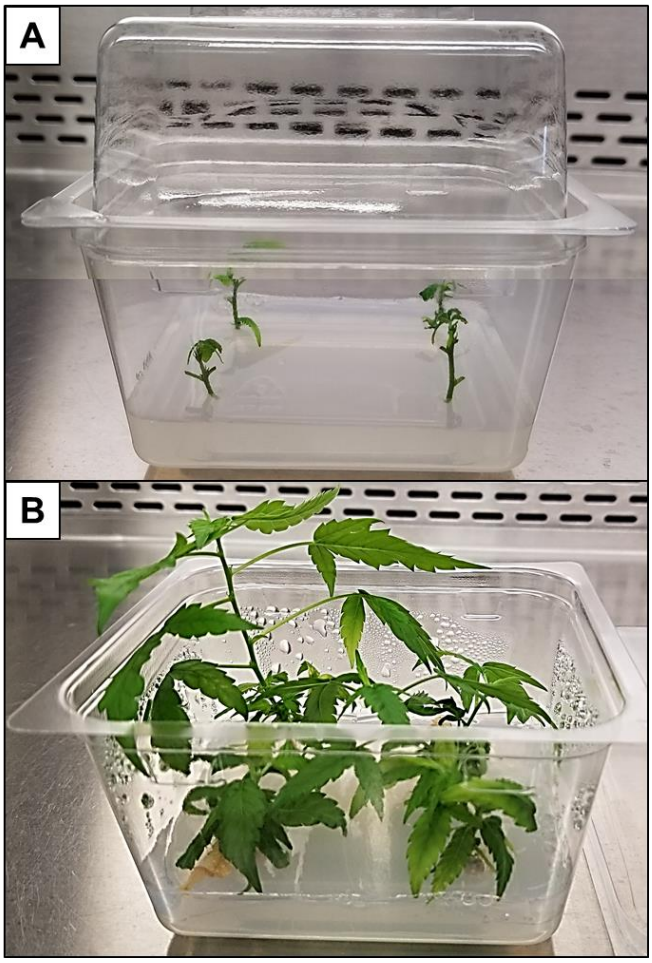
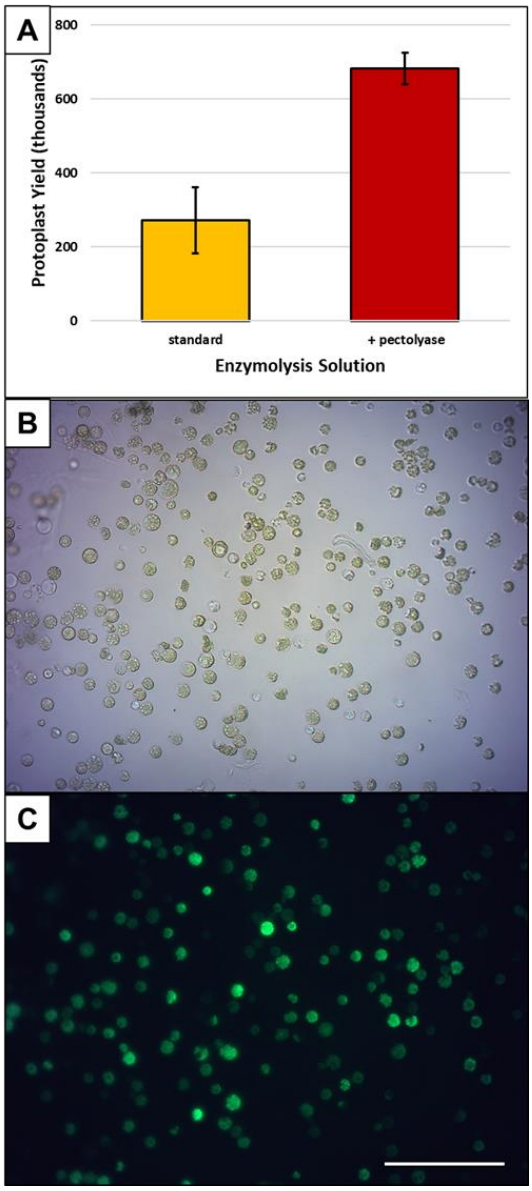


Figure 1. Hormone-free micropropagation of *Cannabis sativa*. Shoot tips or stem segments with two to three nodes (A) are transplanted to a tray with MS2 medium. After approximately two months, rooted plantlets (B) are ready for harvesting tissue. Shoot tips and stems can be excised and transplanted to new trays.



438

439 **Figure 2. Mesophyll protoplasts isolated from *Cannabis sativa* leaves.** Protoplast isolation efficiency
440 from 0.3 g of leaf material was significantly increased (A) by the inclusion of Pectolyase Y23 in the
441 enzymolysis solution, error bars indicate standard deviation, n=3. After purification using a density
442 gradient, the suspension was enriched for live protoplasts. Viability of protoplasts purified though a
443 density gradient was assessed by staining with fluorescein diacetate (FDA) indicating an 82% viability.
444 Micrographs of the same protoplasts (digested with inclusion of pectolyase) are shown under brightfield
445 (B) and under a green fluorescence filter to show FDA-positive cells (C). The scale bar indicates 100 µm.

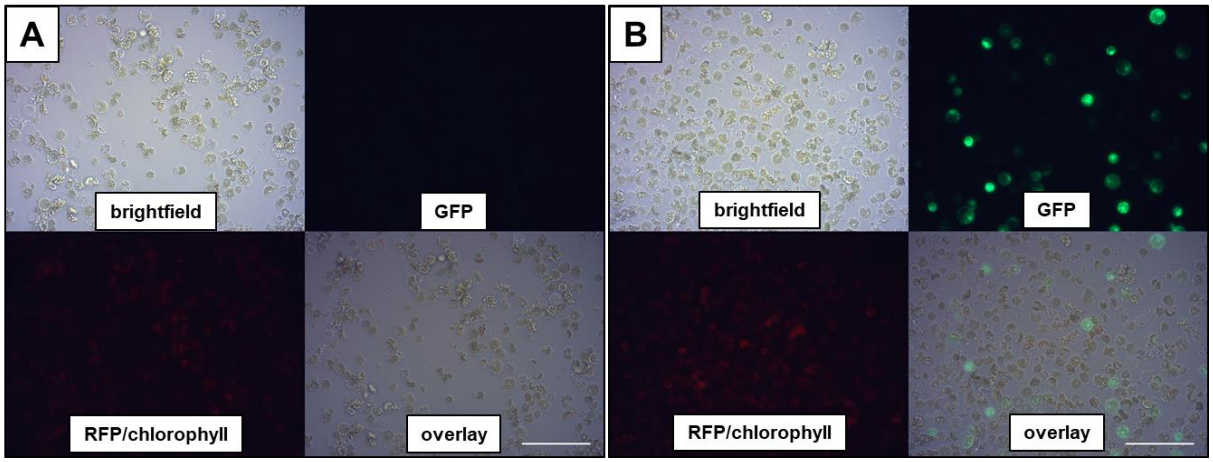
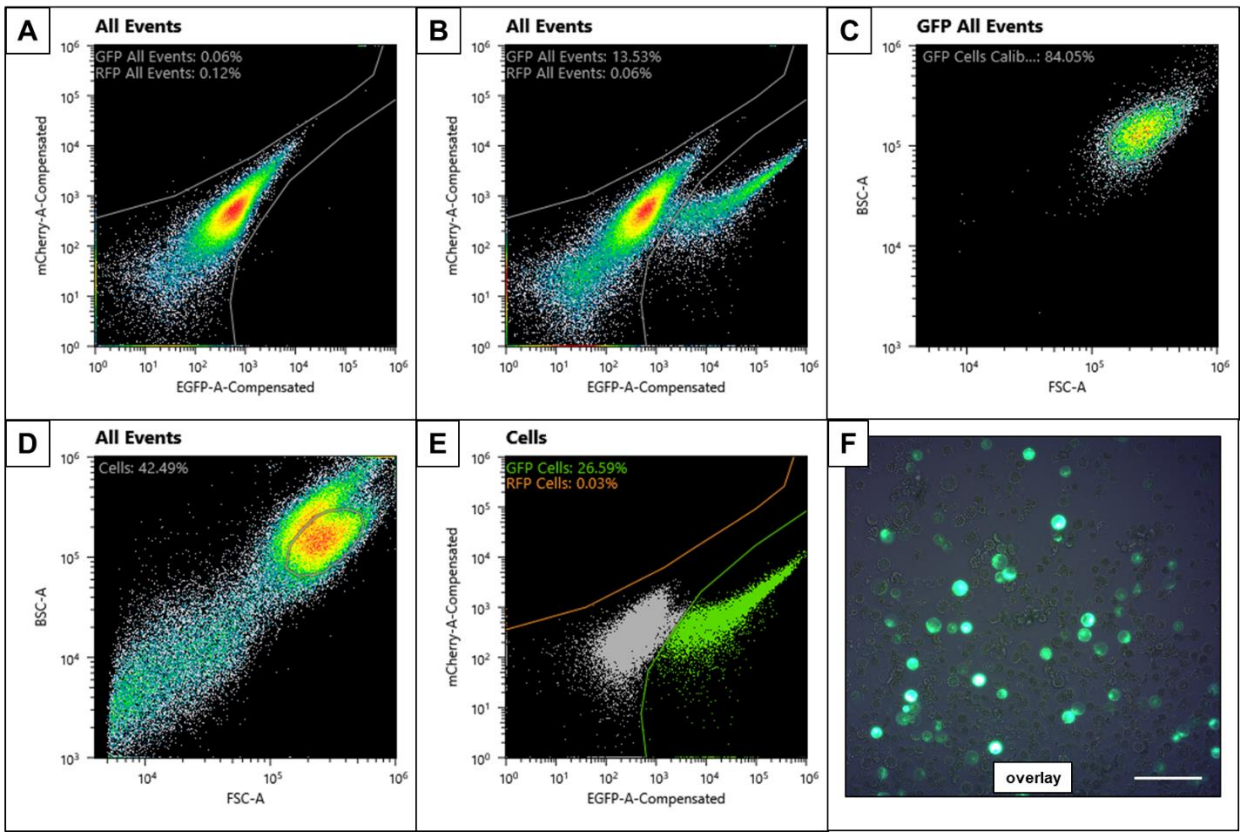


Figure 3. Transient transformation of *Cannabis sativa* mesophyll protoplasts. Protoplasts were transformed using polyethylene glycol-mediated delivery of plasmid DNA. Micrographs are represented for brightfield, GFP, RFP/chlorophyll, and an overlay of all three channels of either mock-treated protoplasts (A) or protoplasts transformed with a plasmid carrying a GFP expression cassette (B). The scale bars indicate 100 µm.



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453 **Figure 4. Flow cytometric analysis of *Cannabis sativa* protoplasts transformed with a GFP expression**
454 **cassette.** Mock-treated protoplasts are represented in a dotplot of red fluorescence (mCherry) vs. green
455 fluorescence (EGFP) (A) and used to set up gates for GFP- and RFP-positive events. Protoplasts
456 transformed with a GFP expression cassette (B) displayed a clear GFP-positive population in a dotplot of
457 red fluorescence vs. green fluorescence. GFP-positive events were back-gated on a dotplot of back-
458 scatter vs. forward-scatter (C) to create a gate for live cells. The gate for live cells was applied to a
459 dotplot of back-scatter vs. forward-scatter for all events (D) to separate live cells from debris. A dotplot
460 of red fluorescence vs. green fluorescence of events gated by the live cells gate (E) was used to estimate
461 the transformation efficiency. The cytometric quantification of transformation efficiency was compared
462 with a manual count of GFP-positive and -negative cells in micrographs (F). The scale bar indicates 100
463 µm.

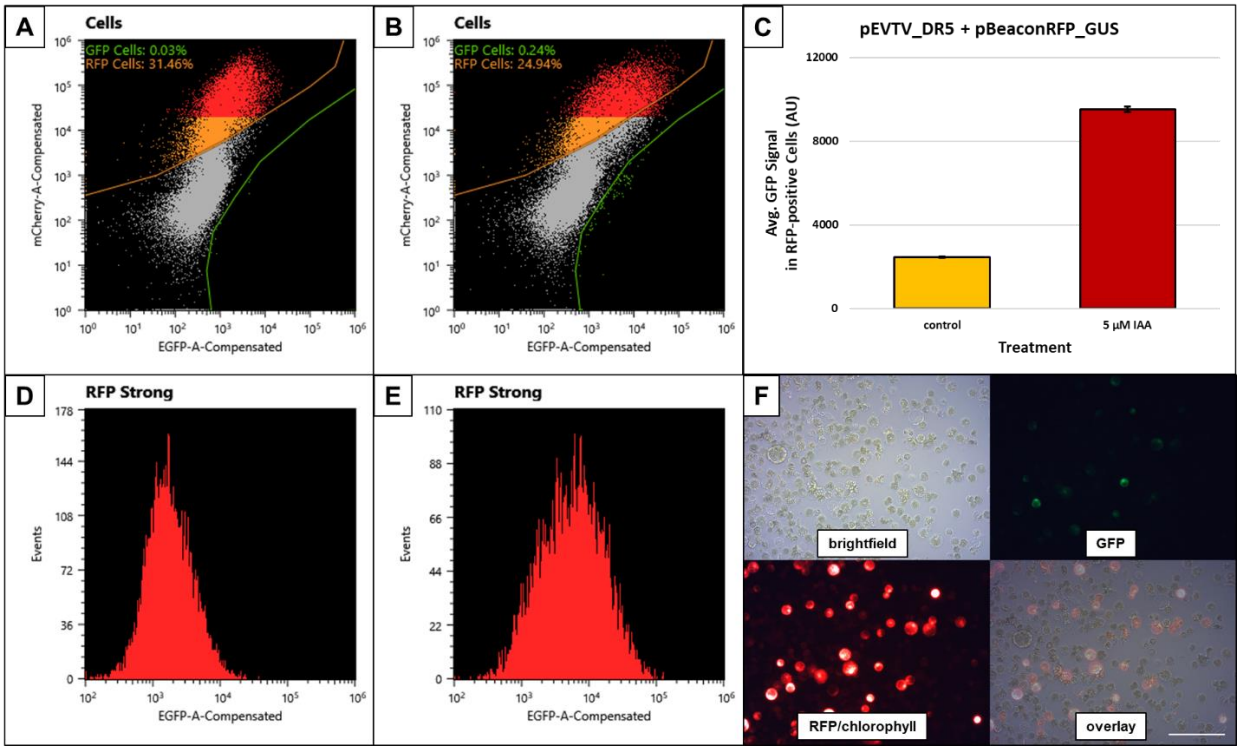

Figure 5. Flow cytometric analysis of *Cannabis sativa* protoplasts transformed with a DR5::GFP auxin-sensitive reporter gene and an RFP expression cassette. Protoplasts transformed with a plasmid carrying the DR5::GFP reporter gene (pEVTV_DR5) and a plasmid carrying an RFP expression cassette (pBeaconRFP_GUS) were mock-treated (A) or treated with 5 µM IAA (B) for 16 h and are represented in a dotplot of red fluorescence (mCherry) vs. green fluorescence (EGFP). The average GFP signal (arbitrary units) of protoplasts that fall within the strong RFP-positive gate (red) is represented in a bar graph for the mock-treated control and the IAA treated protoplasts (C), error bars represent standard error, n=7033 for control and 6374 for IAA treatment. Mock-treated (D) or treated protoplasts treated with IAA (E) are represented in histograms showing the number of strong RFP-positive events with a given GFP signal intensity (arbitrary units). Micrographs are shown for brightfield, GFP, RFP/chlorophyll, and an overlay of all three channels of protoplasts treated with 5 µM IAA for 16 h (F). The scale bar indicates 100 µm.

Supplemental Figure S1. Flow cytometric analysis of *Cannabis sativa* protoplasts transformed with a DR5::GFP auxin-sensitive reporter gene. Protoplasts transformed with a plasmid carrying the DR5::GFP reporter gene (pEVTV_DR5) were mock-treated (A) or treated with 5 µM IAA (B) for 16 h and are represented in a dotplot of red fluorescence (mCherry) vs. green fluorescence (EGFP). Mock-treated (C) or protoplasts treated with IAA (D) are represented in histograms showing the number of cells with a given GFP signal intensity. Mock-treated (E) or protoplasts treated with IAA (F) are represented in histograms showing the number of cells with a given GFP signal intensity. The average GFP signal (arbitrary units) of protoplasts that fall within the Cells gate (G) or GFP-positive gate (H) are represented in a bar graph for the mock-treated control and the IAA treated protoplasts, error bars represent standard error, Cells: n=21697 for control and 37048 for IAA treatment, GFP-positive: n=880 for control and 5543 for IAA treatment.