Using *Agrobacterium tumefaciens* to assemble multi-step metabolic pathways in *Nicotiana benthamiana*

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Running head: Assembling metabolic pathways by agroinfiltration

Abstract

Within the realm of the natural world, plants emerge as prolific producers of diverse bioactive compounds with pharmaceutical, nutritional, and industrial applications. However, many of these compounds are scarce with low concentrations and specific distributions among species, prompting the exploration of methods for producing them in plant biofactories. Typically, pathways comprising several enzymatic steps need to be engineered in plant hosts to produce the desired product of interest from available metabolic precursors. Transient expression systems, specifically agroinfiltration of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens*, is a potent and cost-effective method for testing synthetic gene combinations. Here we present a protocol to produce metabolites through a multi-step pathway, exemplifying the assembly of a carotenoid synthesis pathway within the plant cell cytosol. The approach showcases the efficiency and simplicity of agroinfiltration-mediated transient expression systems in reconstructing metabolic pathways, offering a valuable and sustainable alternative to stably transformed lines.

Keywords:

Agroinfiltration, Biofactory, Carotenoids, Metabolic Engineering, Transient Expression Systems

1. Introduction

In the natural realm, plants serve as highly productive generators of a diverse range of bioactive compounds, encompassing a broad spectrum of molecules applicable in pharmaceuticals, nutrition, and industry [1,2]. Unfortunately, many plant-synthesized bioactive compounds are present only in some tissues or species and in relatively low concentrations, restricting their availability and commercial feasibility. These limitations have been prompting the investigation of methods to increase their yield from plant sources, harnessing the industrial-scale production potential of these bioactive compounds [3]. Central to achieve this goal has been the exploration of the plant biofactory concept (i.e., genetically engineered plant systems designed to act as specialized factories for the increased production of targeted compounds). This approach involves manipulating the genetic make-up of plants to boost their capacity for synthesizing and/or storing specific bioactive compounds [4]. As an alternative to the generation of genetically transformed stable lines, transient expression systems offer several advantages including the ability to efficiently test the functionality of synthetic gene combinations in a fast and cost-effective manner. In particular, the development of non-edible crops such as *Nicotiana* species as sun-powered biofactories represents a strong asset in the development of a sustainable bioeconomy [5]. Among the techniques most employed in transient expression systems, agroinfiltration of *Nicotiana benthamiana* leaves with *Agrobacterium tumefaciens* stands out as a potent strategy [5–7]. Many metabolites have been produced in *N. benthamiana* leaves following agroinfiltration, including isoprenoids such as carotenoids and tocopherols [7–11]. Virtually all these approaches involve the orchestrated participation of more than one gene. Rather than trying to combine all the required genes together in a single, gargantuan plasmid, co-infiltration of several cultures with different gene combinations is a common practice. Combining multiple *A. tumefaciens* cultures, each carrying a single gene construct, enables the reconstruction

of entire metabolic pathways with high efficiency. Such combinatorial approach offers several advantages, as large transformation vectors or knowledge on the stoichiometry of individual enzyme contributions to the pathway flux are not required. Compared to the generation of stably transformed lines, agroinfiltration-mediated transient expression systems requires much simpler and easier cloning steps and it does not need the generation, screening and analysis of large numbers of transgenic plants for the desired metabolic phenotype. Furthermore, agroinfiltration was initially optimized for *N. benthamiana* but it can also be adapted to economically important crop plants [12].

Here we report a protocol to produce metabolites using a multi-step pathway that involves the utilization of several constructs in a single agroinfiltration procedure. As an example, the assembly of a carotenoid synthesis pathway within the plant cell cytosol (where pigment biosynthesis typically does not occur) is showcased using genes encoding carotenoid biosynthetic bacterial enzymes.

2. Materials

2.1. Biological materials.

1. *Nicotiana benthamiana* seeds
2. *Agrobacterium tumefaciens* cells transformed with the constructs of interest. In our example, we transformed *A. tumefaciens* strain GV3101 cells with plasmid constructs encoding bacterial (*Pantoea ananatis*) enzymes that divert cytosolic isoprenoid precursors to sequentially produce geranylgeranyl diphosphate (crtE), phytoene (crtB), lycopene (crtI) and β-carotene (crtY). Additionally, an inhibitor of gene silencing (HcPro from *Watermelon Mosaic Virus*) was added to the construct combination [13]. As a result, five distinct bacterial strains were generated, each harboring to a different construct:

- pGWB702-HcPro
- pGWB405-crtE
- pGWB405-crtB
- pGWB405-crtI
- pGWB405-crtY

2.2. Equipment.

1. Growth chamber or greenhouse for the cultivation of *N. benthamiana* plants at 24–22°C under long-day photoperiod (16 h of light 150 µmol m^{-2} s^{-1} and 8 h of darkness).
2. Shakers with constant temperature (28°C) and agitation (200 rpm).
3. Centrifuge compatible with 50 ml Falcon tubes (e.g., Thermo Scientific Multifuge X1R).
4. Cell optical density meter to quantify the density of bacterial suspensions at 600 nm, OD_{600} (e.g., Biowave Cell Density Meter CO8000)

2.3. Reagents.

1. 50 mg/ml rifampicin in DMSO.
2. 50 mg/ml spectinomycin in distilled water (to select for pGWB702 and pGWB405 constructs).
3. LB (Luria–Bertani) liquid and solid media. Alternatively, YEB medium can be used for the growth of *A. tumefaciens*. In this nutrient-rich medium, the culture

will take longer to reach the stationary phase but can be sustained for extended periods of time [14].

4. Agroinfiltration solution: 10 mM MES pH 5.7, 10 mM MgCl2, and 150 µM acetosyringone.

2.4. Plant and cell growth and agroinfiltration.

1. Plastic pots, tray and greenhouse soil
2. Transparent film
3. Falcon tubes of 15 ml and 50 ml.
4. 1.0 ml syringes for tissue infiltration.
5. 0.3 x 13 mm needles (e.g., BD MicrolanceTM)

3. Methods

The steps described here are schematically represented in Figure 1.

3.1. Preparation of biological materials (steps 1 to 3 in Figure 1).

1. Sow *N. benthamiana* seeds in pots filled with wet soil and place them in a tray in the greenhouse or in a growth chamber under long-day conditions.
2. Cover the trays with transparent film until the first true leaves appear.
3. Grow them until week 4-5 (*see* **Note 1**).
4. One week before agroinfiltration, grow each transformed *A. tumefaciens* strain on solid LB plates with 50 µg/ml rifampicin and 50 µg/ml spectinomycin at 28ºC until colonies appear (*see* **Note 2**). Place the plate with the colonies in the refrigerator if not used immediately.

5. Two days before agroinfiltration, pick 2-3 colonies per strain to be included in the experiment and inoculate 5 ml of liquid LB supplemented with 50 µg/ml rifampicin and 50 µg/ml spectinomycin in a 15 ml Falcon tube. Tape a loose lid on the tube (to allow proper airflow) and grow overnight at 28ºC with agitation at 200 rpm (*see* **Note 3**).

6. Also two days before agroinfiltration, ensure that the *N. benthamiana* plants intended for the experiment are adequately watered. If not, provide them with the necessary water. (*see* **Note 4**).

7. The day before agroinfiltration, use approximately 100 µl of one of the pre-cultures of each strain to inoculate 10 ml of fresh medium in 50 ml Falcon tube. Grow at 28ºC with agitation at 200 rpm. Again, make sure that the tube cap is loosely taped, allowing proper aeration.

3.2. Preparation of the agroinfiltration mix *(steps 4 to 6 in Figure 1)**.***

1. Measure and record the OD at 600 nm (OD_{600}) of each overnight culture (*see* **Note 5**).
2. Based on the measured OD_{600}, calculate the volume of culture that should be centrifuged to obtain the chosen OD_{600} in the infiltration solution (*see* **Note 6**) according to the following equation:

$$V_{culture} = (OD_{600\ infiltration} / OD_{600\ culture}) \times V_{infiltration}$$

For example, if aiming to prepare a 50 ml infiltration solution combining 5 individual strains to a final OD_{600} of 0.8, it would be necessary to mix 10 ml of each individual solution with an OD_{600} of 0.16 of each. In this case, the volume of a particular strain culture showing an OD_{600} of 2 to be centrifuged will be the following:

$$V_{culture} = (0.16 / 2) \times 10 = 0.8\ ml$$

3. Centrifuge the volume of culture calculated for each individual strain in a fixed angle centrifuge at 4400 rpm for 10 min (*see* **Note 7**).

4. Remove the supernatant and resuspend each cell pellet in agroinfiltration solution either by gently shaking the tubes or by gently pipetting the cells using a 1 ml plastic tip with a cut end.

5. Incubate each of the tubes for at least 2 h at 28ºC with 200 rpm agitation.

6. Mix the different solutions of strains carrying the constructs of interest in a single 50 mL Falcon tube avoiding the formation of bubbles (*see* **Note 8**). In our example, the combination to test will contain 5. As indicated above, to achieve a global OD_{600} of 0.8, individual solutions with an OD_{600} of 0.16 will need to be mixed (5 x 0.16 = 0.8). Combinations with a lower number of constructs can be prepared by adjusting the individual OD_{600} values or/and by including non-transformed *A. tumefaciens* cells or, even better, an empty plasmid control strain (Table 1).

Table 1. Some examples of OD600 adjustments in different combinations.

Construct	s**EBIY**	s**EBI**	s**EBI**0	s**EBI**-	s**EB**	s**EB**0
pGWB702-HcPro (s)	0.16	0.2	0.16	0.16	0.27	0.16
pGWB405-crtE (E)	0.16	0.2	0.16	0.16	0.27	0.16
pGWB405-crtB (B)	0.16	0.2	0.16	0.16	0.27	0.16
pGWB405-crtI (I)	0.16	0.2	0.16	0.16		
pGWB405-crtY (Y)	0.16					
pGWB405 (0)			0.16			0.32
Untransformed (-)				0.16		
Global OD600	**0.8**	**0.8**	**0.8**	**0.8**		**0.8**
Expected product	β-carotene	lycopene	lycopene	lycopene	phytoene	phytoene

3.3. Agroinfiltration and analysis *(steps 7 to 8 in Figure 1)**.***

1. About 2h before starting the experiment, move the plants from the greenhouse or growth chamber to the place where infiltration will be performed, if possible, to allow their acclimation. Avoid exposing plants to strong wind, temperature or light shifts (in particular, illumination with direct sunlight should be prevented).

2. Pierce a small hole in the leaf by using a syringe needle (the lower the diameter the better; we recommend a 0.3 mm needle). Then, use a 1 ml syringe (without the needle) to slowly inject the infiltration mix in the leaf. Gently place the syringe mouth on the pierced hole on the abaxial side of the leaf, holding a finger in the same position of the adaxial side while gently pressing the piston (*see* **Note 9**). The infiltration will be visible as a dark-green halo on the leaf.

3. Leave the plants resting for at least 1 h before moving them back to the greenhouse or growth chamber. This resting period helps in minimizing the stress due to the infiltration procedure.

4. Metabolites are typically produced in 3 to 5 days after agroinfiltration. In many cases, the agroinfiltration halo will still be visible. In the case of our experimental example, the production of different carotenoids can also be visually detected on agroinfiltrated areas of the leaves as a halo with color ranging from yellow/pale green to orange [10,13]

5. Collect samples and analyze their metabolic phenotype using the proper methodology.

4. Notes

1. The selection of *N. benthamiana* plants of different ages for agroinfiltration presents both benefits and constraints. Leaves from younger plants (i.e., 3-4 weeks old) are better to support rapid processes due to a more active cellular metabolism. Their smaller size also facilitates handling and provides increasing agroinfiltrated area as the leaf grows. However, their limited biomass could be a handicap for some assays (e.g., tissue sampling for metabolite quantification or protoplast isolation). Conversely, leaves from mature plants (i.e., 5-6 weeks old) provide increased biomass, potentially resulting in higher yield and scalability. Moreover, using mature plants allows for the selection of multiple leaves per plant, thereby increasing the potential for obtaining a higher number of technical replicates if needed. By contrast, the lower cellular activity and slower growth rate of older leaves may affect the efficiency of transient protein production [6,15,16]. Understanding these differences is pivotal for optimizing agroinfiltration protocols, balancing factors such as expression efficiency, yield, and scalability based on the specific needs of each experiment.

2. Initiating the preculture from a single colony cultivated in solid media, instead of directly transferring a bulk amount from the glycerol stock to liquid media, ensures genetic purity, minimizing the potential for contamination or genetic variability within the experiment.

3. Agitation speed may affect *A. tumefaciens* growth. About 200-250 rpm is optimal for growth, as it facilitates oxygen transfer and nutrient distribution and promotes bacterial growth by preventing clumping and allowing aeration throughout the culture with no effect on mechanical stress. On the other hand, a too high agitation speed (> 700 rpm) may cause mechanical stress to the bacteria and affect their viability and growth without enhancing any metabolic production compared with lower speeds [17].

4. It is key that plants to the agroinfiltrated have an optimal water status. The water content of leaves is important to facilitate the passage of the infiltration solution through the leaf tissues, thus avoiding the necessity of doing more than one agroinfiltration wound and preventing cell death. To mitigate the risk of overwatering, it is recommended to water the plants, if needed, at least two days prior to the agroinfiltration experiments, avoiding watering them on the day before the agroinfiltration.

5. To measure the OD600 is recommended to employ a 1:5 dilution of the culture in antibiotic-supplemented LB medium (e.g., 200 ml of culture in 800 ml of medium). This practice helps alleviate potential limitations associated with measuring culture optical densities exceeding the sensitivity range of your spectrophotometer.

6. The optimal OD600 of *A. tumefaciens* transformants in the infiltration solution can vary from 0.1 to 1, considering several factors such as construct expression efficiency, protein accumulation or agglomeration, and potential toxicity resulting from metabolic stress, which could induce tissue necrosis, especially when the infiltration is carried out in a thin leaf. Within this spectrum, our experiments conducted within the OD600 range of 0.5 to 0.8 revealed no statistically significant differences in necrosis occurrence, indicating that this can be a viable and non-detrimental OD600 range.

7. It is important not to use excessive centrifugation forces to avoid stress on the bacterial cells that could result in a reduction of agroinfiltration efficiency.

8. The required final volume of solution will vary depending on the desired number of replicates. For triplicates, three leaves can be infiltrated with 5 ml of infiltration solution.

9. Agroinfiltration must be carried out with patience and it should be slow to avoid cell breakage caused by excessive pressure. Depending on the leaf age and growth conditions, infiltration can vary in difficulty. It is crucial to avoid excessive force during infiltration, as it may lead to excessive cell damage and necrosis formation.

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Tables

Table 1. Some examples of OD600 adjustments in different combinations.

Construct	s**EBIY**	s**EBI**	s**EBI**0	s**EBI**-	s**EB**	s**EB**0
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pGWB405-crtE (E)	0.16	0.2	0.16	0.16	0.27	0.16
pGWB405-crtB (B)	0.16	0.2	0.16	0.16	0.27	0.16
pGWB405-crtI (I)	0.16	0.2	0.16	0.16		
pGWB405-crtY (Y)	0.16					
pGWB405 (0)			0.16			0.32
Untransformed (-)				0.16		
Global OD600	**0.8**	**0.8**	**0.8**	**0.8**		**0.8**
Expected product	β-carotene	lycopene	lycopene	lycopene	phytoene	phytoene

Figures

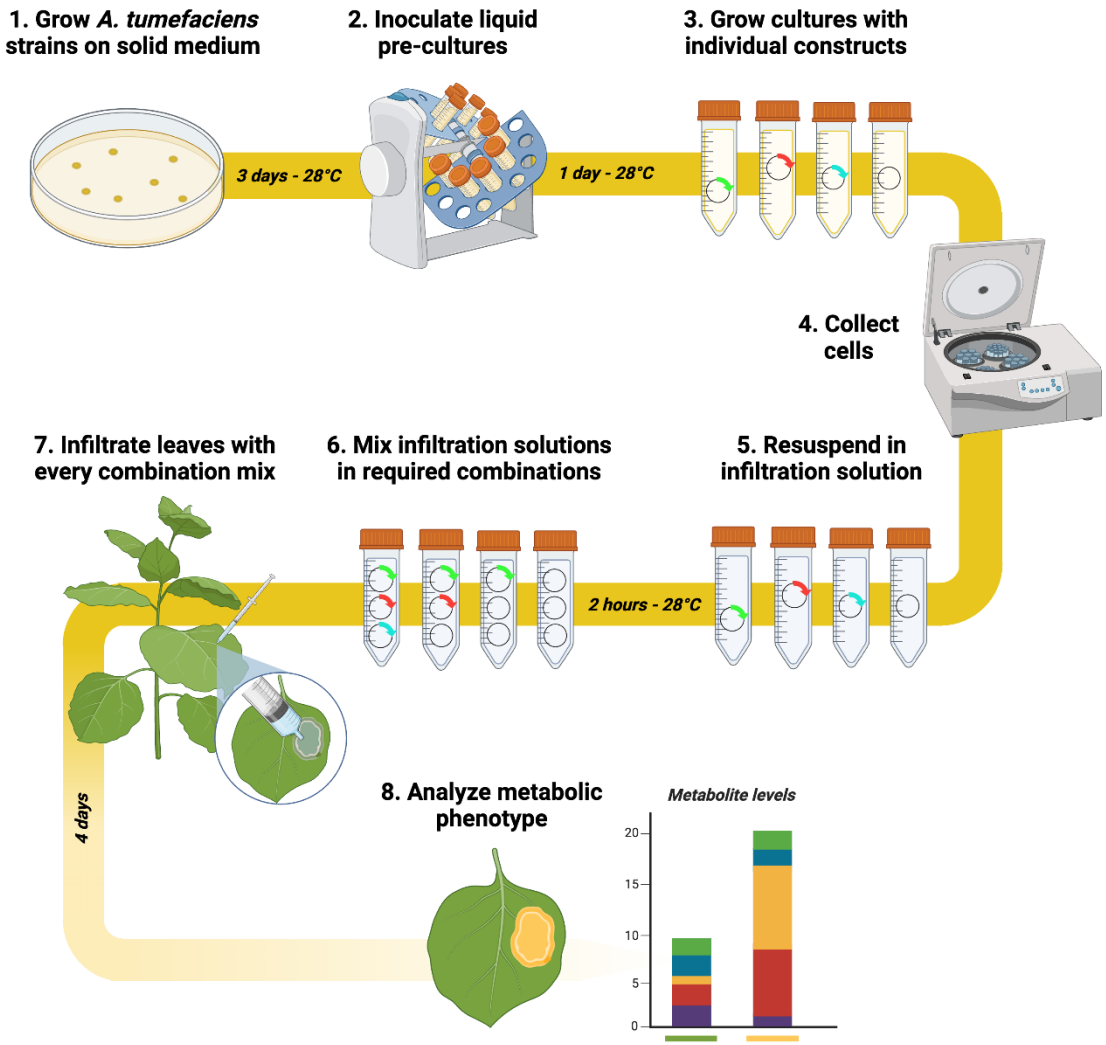

Figure 1. Schematic presentation of the workflow for assembling multi-step metabolic pathways in *Nicotiana benthamiana* by agroinfiltration. See text for details.