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Engineering Metabolism in *Nicotiana* Species: a Promising Future

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Abstract

Molecular Farming intends to use crop plants as biofactories for high-value-added compounds following application of a wide range of biotechnological tools. Particularly the conversion of non-food crops into efficient biofactories is expected to be a strong asset in the development of a sustainable bioeconomy. The "non-food" status combined with the high metabolic versatility and the capacity of high-yield cultivation forward the plant genus *Nicotiana* into one of the most appropriate "chassis" for Molecular Farming. *Nicotiana* species are a rich source of valuable, industrial, active pharmaceutical ingredients and nutritional compounds, synthesised from highly complex biosynthetic networks. Here, we overview and discuss the potential approaches currently used to design enriched *Nicotiana* species for Molecular Farming using New Plant Breeding Techniques (NPBTs).

Highlights

- Molecular Farming uses crops as biofactories for high-value-added compounds by applying a wide range of biotechnological and engineering tools.

- The "non-food" status combined with the high metabolic versatility and the high biomass production make the genus *Nicotiana*, specifically the species *N. tabacum* and *N. benthamiana*, one of the most promising "chassis" for molecular farming.

- Tobacco is a rich source of pyridine alkaloids: analysing the intermediates of the pathway in diverse species and in a temporally and spatially resolved manner will reveal which metabolite pools could serve as precursors for new high-value-added compounds.

- The understanding of the chemical diversity combined with the recent technological advances in genome sequencing, the development of New Breeding Techniques and synthetic biology tools will open new avenues for metabolic engineering in *N. tabacum* and *N. benthamiana*.

Plant Biofactories

In the past decade, **Plant Molecular Farming** (PMF) (see Glossary) has become an emerging research area that designs crops as **biofactories** for high-value-added compounds by applying a wide range of biotechnological tools. This research field shows very significant potential, which resides in the genetic transformability of plants, first demonstrated in the 1980s [1]. Plants gather more favourable conditions than other systems, such as mammalian cells, to produce valuable small molecules and proteins. The main benefits include *safety*, due to the absence of replicating human pathogens, *simplicity*, because sterility is not required during production, *scalability*, due to the potential for open-field cultivation, and/or the *speed* of **transient expression,** potentially providing gram quantities of product [2, 3].

A key point in PMF is the selection of the appropriate host organism, the *chassis*, which has to go beyond scientific criteria. *Arabidopsis thaliana*, the **model organism** for plant research, provides a great advantage in the development of tools and knowledge. However, it is a non-crop plant with low productivity and thus is unsuitable as an elite biofactory platform. The use of edible, industrial, or minority crops is an option, but discouraged by the fact that their farming for non-food or feed purposes would endanger the production chains, considerably affecting their market value. By contrast, the conversion of non-food crops into efficient biofactories is expected

to be an efficient solution in a modern and sustainable **bioeconomy**. The "non-food" status, combined with its high metabolic versatility and capacity of high-yield cultivation, turn the genus *Nicotiana*, specifically the species *N. tabacum* (cultivated tobacco) and *N. benthamiana* (Australian dwarf tobacco), into one of the most appropriate chassis to be used for PMF. *N. benthamiana* is well suited to produce both recombinant proteins and small molecules in controlled conditions, due to its permissiveness to propagation of transient expression vectors, while *N. tabacum* is best suited for large-scale production in open field conditions [2, 4-6]. Owing to these advantages, *N. benthamiana* has reached maturity as manufacturing platform for protein-based biologics such as biocides, antibodies and especially recombinant vaccines, with at least two vaccine candidates, influenza and COVID-19, reaching advanced stages in clinical trials [7].

In this review, we give an up-to-date overview of cutting-edge research that is providing new designer crops enriched in high-value-added metabolites for PMF using **New Plant Breeding Techniques** (NPBTs) (Figure 1, Key Figure). We summarise the recent advances in the genomic knowledge and NPBT applications, including sequence-specific nuclease (SSN)-mediated genome editing, cis/intra-genesis, grafting and agro-infiltration, which, added to the advances in synthetic biology are enabling the breeding of *Nicotiana*-based biofactory crops for high-value-added compounds.

The Genus *Nicotiana*: a Workhorse for Genetic Innovations

The genus *Nicotiana* comprises around 75 species in 13 sections, originating mostly in the Americas and Australia [8]. The basic chromosome number is 12, as in most Solanaceae, and the haploid DNA content ranges from 1.37 Gb in *N. obtusifolia* to 6.27 Gb in *N. suaveolens* [9]. A large portion (36 species) derived from allotetraploidization events, a common phenomenon in the genus. The diploid progenitors of most allotetraploids, or their close relatives, are known, with the exception of the ancient allotetraploidy event that gave rise to the section *Suaveolentes*, which comprises *N. benthamiana* [10]. Ancient polyploidization events gave rise to the radiation of whole sections, such as *Suaveolentes* (6.4 mya), *Repandae* (5.1-3.5 mya) and *Polydicliae* (1.5-1.2 mya), while recent ones (≤ 1 mya) are only represented by single species, like *N. tabacum, N. arentsii* and *N. rustica* [10].

Since the 1930s, the genus *Nicotiana* has been key to establish genetic innovations and to discover novel biological phenomena, including synthetic hybrids, androgenesis, tissue culture and parasexual hybridization, nuclear and chloroplast transformation, homology-dependent gene silencing, and interorganellar and graft-mediated gene and genome transfer (Table S1). A large amount of publicly available genomic and transcriptomic resources is available for this genus: 462 (mostly transcriptome) bioprojects and genome assemblies for 12 species (Table S2). An

unpublished, chromosome level assembly of *N. benthamiana* is also available upon request at https://nbenth.com/. Five ecotypes, named according the geographic zones, have been defined: Northern Territory (NT), North Western Australia (NWA), Western Australia (WA), Queensland (QLD), and South Australia (SA). These are referred to as wild strains in contrast to the laboratory isolate (LAB) strain [11]. The wild strains show a considerable diversity. Within each of the wild accessions, there is no obvious morphological variability, but there are clear differences between the accessions in plant form, leaf shape, hercogamy, flower size, and seed size [12]. The website http://benthgenome.com provides access to genome and transcriptome assemblies of both laboratory and wild strains of *N. benthamiana*, as well as the seed source.

Metabolic Engineering for the Production of Biomass and High-Value-Added Metabolites

Metabolomic analysis of tobacco has generated a comprehensively annotated metabolome with over 4,000 and 8,000 metabolites identified in tobacco tissues and tobacco smoke, respectively [13]. The classes of compounds identified to date include terpenoids, phenolics, alkanes, alkynes and polyketides. Because of the effects on the human organism, alkaloids deserve a separate mention and have been thoroughly studied. Nicotine and all minor tobacco alkaloids have been shown to be pharmacologically active upon binding to several nicotinic acetylcholine receptors (nAChRs). Nicotine, anatabine, anabasine, anabaseine, and cotinine display protective effects in animal models of several inflammatory conditions, including sepsis, Parkinson's disease, Alzheimer's disease, and IBD [14]. Due to their amenability to both nuclear and plastid transformation, some *Nicotiana* species have been extensively used for metabolic engineering efforts based on classical transgenesis. Prominent examples of such efforts include the engineering of xanthophyll cycle pigments to improve photosynthesis by accelerating recovery from photoprotection [15], the overexpression of the Photosystem II PsbS protein to improve water use efficiency [16], and the engineering of synthetic glycolate metabolism to boost biomass production in the field [17].

In addition to chemical fingerprinting, a series of NPBTs, including agro-infiltration, virus-mediated overexpression/gene silencing, and gene editing have been used to alter endogenous metabolite levels, or to express non-native metabolites in *Nicotiana* leaves (Table S3). Some examples are discussed below in more detail.

Terpenoid compounds are also present but have been underutilised in tobacco to date. Nonetheless, opportunities exist to generate valuable terpenoids from the precursors present in tobacco cells. Artemisinin, phytosterols, vitamin D, taxanes and keto- and apocarotenoids are examples of high-value-added compounds that have been produced from endogenous precursors through engineering, including compartmentation of the final products [18-22]. Considering the

metabolic catabolism of the precursor pools combined with organelle-specific engineering may help to boost yields (see below for more examples).

Cutting-Edge Methods for Multigene Metabolic Engineering of Tobacco

Combinatorial Transformation

The production of new metabolites in plant cells often requires the synthesis of multiple enzymes and thus the orchestrated expression of several (trans)genes. Multigene expression in plants can be achieved by a number of techniques [23, 24], of which combinatorial nuclear transformation and the construction of synthetic operons for plastid (chloroplast) transformation being particularly versatile and efficient.

Combinatorial transformation represents a large-scale biolistic co-transformation method that involves the simultaneous introduction of several independent transgenes, each present on a separate plasmid vector. The carotenoid and artemisinin pathways have been successfully engineered by this means in maize and tobacco, respectively [25, 26]. A combinatorial approach has also allowed the simultaneous manipulation of several vitamin metabolic pathways in maize [27]. This technology provides several key advantages. In particular, (i) there is no upper limit for the number of transgenes, (ii) no large transformation vectors need to be built, and (iii) no prior knowledge about the quantitative contributions of the individual enzymes (and auxiliary proteins) to the pathway output is required (Figure 2; Box 1). However, it should be borne in mind that the engineering of complex pathways involving large cascades of enzymatic reactions necessitates the generation and analysis of a large population of stable transgenic lines.

Multigene engineering of chloroplast genomes exploits the bacterial-type gene organization in plastids, with multiple genes being co-expressed as polycistronic transcripts. A number of studies have identified suitable genetic elements to ensure proper expression, processing and translation of operon-encoded transgenes [28, 29]. Several metabolites have been synthetized by expressing synthetic or natural bacterial operons in plastids [23, 30-32], including the ketocarotenoid astaxanthin [33-35], and the antimalarial drug precursor artemisinic acid [26]. High-level accumulation of some (endogenous or foreign) metabolites can disrupt essential chloroplast functions, thus compromising photosynthetic efficiency and plant growth [23]. The development of new tools for inducible transgene expression [36] or the use of synthetic organelles [37] will likely facilitate the rational design of tightly regulated synthetic metabolic pathways in the future [38].

In an approach dubbed combinatorial supertransformation of transplastomic recipient lines (COSTREL), plastid operon design has been combined with combinatorial nuclear transformation

to facilitate the high-level production of artemisinic acid in plastid [26]. While a synthetic operon residing in the plastid genome ensures strong and stable expression of the core pathway enzymes as well as confinement of the metabolite, additional combinatorial supertransformed nuclear transgenes fine-tune the flux through the pathway and maximize the metabolic output [26].

Grafting as a New Plant Breeding Technique

For many centuries, grafting has been used as an agricultural and horticultural technique for asexual plant propagation and the generation of composite plants with rootstocks providing useful traits (e.g., resistance to root-borne diseases). Only recently, grafting has become a promising NPBT [39-42].

The discovery of horizontal nuclear, mitochondrial and plastid DNA transfer across graft junctions between plants of different species [39-41, 43, 44] (Box 2) opened up new possibilities in the field of metabolic engineering. Unlike sexual hybridisation, grafting is not restricted to sexually compatible species (although anatomical, genetic or metabolic incompatibilities can also pose limitations to the range of graft-compatible species) [35].

The observation that plastid genomes move across graft junctions permitted the extension of the plastid engineering toolbox to new species, especially species that are currently recalcitrant to chloroplast transformation. By interspecies grafting and selection for plastid genome transfer, *N. tabacum* plastids harbouring a synthetic operon for astaxanthin production were successfully moved into the nicotine-free woody species *N. glauca* [21]. As the organellar genotype also substantially contributes to plant fitness [45], this methodology additionally provides an opportunity to evaluate different combinations of nuclear and organellar genomes in plant breeding.

Besides plastid genomes, also entire nuclear genomes can move across the graft junction. This type of horizontal genome transfer results in novel plant species that are allopolyploid, thus highlighting an asexual pathway of speciation [41]. By grafting of *N. tabacum* and *N. glauca*, followed by selection for horizontal nuclear genome transfer, the new synthetic plant species *N. tabauca* was created [41]. Polyploidy often confers superior growth properties and, in addition, provides new genetic raw material for the evolution of novel adaptive traits [46]. Hence, grafting-based approaches offer the possibility to generate new synthetic crops with improved traits, including novel pathways in specialized metabolism.

Transient Expression

Transient expression [47] can be achieved in *Nicotiana* leaves through: i) infiltration with *Agrobacterium tumefaciens* carrying a recombinant Ti plasmid (agro-infiltration) [48]; ii) inoculation with recombinant viral vectors or iii) agro-infiltration with Ti plasmids carrying parts

of viral vectors (agro-infection). Agro-infection combines the advantages of the easy delivery by *A. tumefaciens* with the generation of a systemic viral infection, leading to higher expression levels. The system can be further engineered by deconstructing viral vectors into different pro-vector modules, which can be assembled into a functional vector in the plant cell after infiltrating with a mixture of two or more different *Agrobacterium* strains [49, 50]. In some cases, genes essential for replication are deleted from the viral genome and transferred to the plant host's genome, serving a dual purpose: to provide more space for cloning heterologous genes of interest and to create a biocontainment system in which only transgenic plant hosts can be infected by the viral vector [51].

A wide diversity of compounds has been produced in *Nicotiana* species following agro-infiltration or agro-infection (Table S3). For example, through pathway engineering, large quantities of a suite of triterpene analogues, carotenoids with nutritional value, and intermediates of the anticancer drug paclitaxel were produced in *N. benthamiana* [5, 51, 52]. These studies highlight the potential of *Nicotiana* species as an alternative platform for the production of multiple high-value-added compounds.

Nicotiana and Synthetic Biology: Chassis, Circuits and Effectors

Nicotiana species are at the forefront of **synthetic biology**. Mostly, plant synthetic biologists follow top-down approaches consisting of engineering new genetic capacities in a performant plant species, often referred to as "the chassis". Engineered features range from the production of high-value-added compounds such as fine chemicals or recombinant proteins, to the ability to control such biosynthetic capacities in response to environmental cues or even management decisions. Challenging as it is, working with *Nicotiana* species can facilitate this endeavour, as *Nicotiana* chassis are genetically well-described and amenable to engineering, thus facilitating the implementation of rapid design–build–test cycles as required for the discipline [53].

Chassis Improvement

N. benthamiana has an adequate size for indoor production, a shorter life cycle than other related species, its leaf anatomy favours massive infiltration, and most importantly, the endogenous gene silencing mechanisms of the LAB strain are partially defective, facilitating the use of DNA delivery vectors [54]. Nevertheless, improvement of additional traits will influence the capacity as biofactory of *N. benthamiana* and also of cultivated tobacco. Indeed, whereas most current food crops have gone through thousands of years of breeding, the focus was never on improving biofactory-specific traits. From a bioproduction perspective, targeting traits such as yield, growth habits or favourable chemical composition can contribute to the amelioration of the biofactory's

chassis. In a remarkable example of yield improvement in tobacco, engineered alternative photorespiratory pathways reached record 24% gains in biomass production [17]. Non-flowering for biosafety and biomass [55], extended leaf juvenility for enhanced transformability, or low nicotine and polyphenol-oxidase activity for easy downstream processing [56] are examples of traits that could significantly enhance the biofactory value of *Nicotiana* species and varieties. Most of these traits are controlled by multigene families in polyploid *Nicotiana* species and would have been hard to breed in the pre-genome editing era (Box 3). However, fast-breeding by gRNA multiplexing offers unprecedented possibilities for biofactory chassis improvement [57]. Since a one-fit-all chassis is unlikely, a foreseeable outcome is the development of specialist varieties, each displaying a certain combination of biofactory traits.

An additional target for specialized metabolite production are the glandular **trichomes,** epidermal outgrowths that are the site for biosynthesis and storage of large quantities of specialized metabolites. The specialized metabolites secreted by glandular trichomes might represent up to 17% of the leaf dry weight in tobacco [58]. This high productivity in terms of specialized metabolites makes them into a suitable platform for metabolic engineering of valuable natural products. This requires the availability of trichome-specific promoters that could be used to efficiently drive the expression of the transgenes coding for the enzymes needed to control the pathway in a cell-type specific manner. In this respect, the identification of the *NtMALD1* and *NtRbcS-T1* promoters and their comparison with previously identified trichome-specific promoters are promising tools for expressing entire biosynthesis pathways in glandular trichomes of *N. tabacum* [59]. Additionally, *Nicotiana* organs with higher trichome numbers would also enable increased production of valuable compounds. The ectopic expression of a C2H2-zinc-finger transcription factor, *NbGIS*, or the tomato *Woolly* gene both lead to more glandular trichomes in tobacco [60, 61].

Genetic Circuits

Next to chassis improvement, one of the initial steps in top-down synthetic biology engineering comprises the design of synthetic genetic circuits that enable a tuneable control of physiological and/or biosynthetic outputs. Typically, a gene circuit comprises three types of elements, namely sensors, processors and actuators [62]. Sensors use environmental signals as inputs, responding with a transcriptional output. In most cases, synthetic sensors involve a chimeric transcriptional regulator that conditionally responds to an environmental cue, and which binds specifically a DNA operator (*cis*-acting elements) upstream of a plant core promoter that drives the transcriptional output signal (e.g., the expression of the gene of interest [63]). The number of orthogonal synthetic sensors available for plants is relatively limited. The best known are chemical sensors, extensively used for conditional activation of transgenes, as those based on

glucocorticoids [64], ethanol [65], copper [66] or agrochemicals [67]. Recently, the range of synthetic sensors has been expanded to light-driven sensors [68], which overcome some of the limitations of chemical inducers such as the elevated cost and toxicity of chemicals, as well as the limited spatiotemporal regulation. These elements, aka optogenetic sensors, involve light-dependent protein–protein interactions. The application of light of a specific wavelength results in the reconstitution of a transcription factor able to produce the transcriptional output. Optogenetic sensors have been successfully tested in both tobacco cell cultures and whole plants [68-71], and could be of paramount importance in the advent of LED-controlled plant growth.

Processors transform transcriptional inputs into different types of transcriptional outputs. Transcriptional regulators, either activators or repressors, are the most basic processor elements. Recently, programmable transcriptional regulators (PTRs) based on CRISPR/Cas have provided additional possibilities to the design of more complex processors, allowing the switching/selection of transcriptional outputs by using different gRNAs. The first generation of PTRs involved the direct translational fusion of activator or repressor domains (AD or RDs) to dCas9 or TALENs (Box 3). The second generation of PTRs entailed additional anchorage points to the ADs and RDs, for instance through the recruitment of single chain variable antibody fragments (scFv) fused to ADs, resulting in up to 10,000-fold transcriptional activation in *N. benthamiana* [72-74]. While excellent activation levels were achieved using these new generation of PTR, repression strategies need novel approaches and further optimization, including a better understanding of the chromatin state [75].

More complex processor elements have been described operating at the *Nicotiana* chassis, such as logic gates performing Boolean operations, developed using viral vector elements [76], or a memory switch based on the bacteriophage ϕC31 site-specific integrase [77]. The switch is designed to control the transcriptional state (on or off) of two target genes by an alternative inversion of a central DNA regulatory element. The state of the switch can be externally operated by the action of the ϕC31 integrase, and its recombination directionality factor.

Cell and Hairy Root Cultures. Adaptable Production Tools for Multiple High-Value-Added Compounds

The large-scale production of valuable compounds is limited by growth rates, climate dependency, restricted cultivation areas, plant diseases, pests and overharvesting. All these issues reinforce the need for developing alternative methods and protocols for the industrial-level fabrication of plant-derived metabolites. Suspension culturing of plant cells has been considered as another promising source for the biosynthesis of valuable specialized metabolites. Production of specialized metabolites using plant cell suspension cultures is usually a challenging task,

because these compounds are typically produced at specific developmental stages or during environmental conditions [78]. The Bright Yellow-2 tobacco suspension cell line (BY-2) is one of the most popular plant expression systems [79]. BY-2 cells are characterized by a fast growth rate relative to other plant cell suspension lines, and the ease of cell cycle synchronization and transformation with *Agrobacterium*. Several compounds, such as the betalain amaranthin [80], stilbenes [81], and more than 20 pharmaceutical proteins and peptides have already been successfully produced in BY-2 cell suspension cultures, including interleukins and antibodies [82].

Like plant cell cultures, hairy roots have been exploited for the production of added-value compounds less abundant in nature and whose complex structures make their chemical synthesis inviable [83]. Hairy root cultures (HRCs) obtained from the infection of a tobacco explant by the bacteria ***Rhizobium rhizogenes*** (classically referred to as *A. rhizogenes*) allow the production of highly diverse molecules. HRCs are appropriated for the production of specialized metabolites due to characteristics such as genetic stability, high biomass production and efficient biosynthetic capacity, and are able to produce specialized metabolites for a long period of time [84]. Some examples of specialized metabolites produced using *Nicotiana* transgenic HRCs are tropane alkaloids such as scopolamine and hyoscyamine [85], stilbenes [81] and geraniol [86]. Additionally, HRCs have also been proposed as an alternative for the use in phytoremediation programmes [87]. This adaptability and the capacity to be developed as large-scale **bioreactors** [88] position hairy root platforms as powerful tools to produce complex molecules, like pharmaceutical, cosmetics, and food supplements. However, several other factors also have to be considered, such as the stability of the compounds produced, as well as their toxicity, which can negatively affect root growth when accumulating to a certain level; this toxicity poses another challenge that limits the practical application of hairy roots [89].

Concluding Remarks and Future Perspectives

Nicotiana ssp. offer multiple advantages for the production of sustainable, high-value-added, plant-derived products. Here, we have shown a large number of examples of how *Nicotiana* species, especially *N. tabacum* and *N. benthamiana*, could be biotechnologically redesigned using NPBTs to increase their economic competitiveness when producing different metabolites. To date, the high-value-added molecules that have been engineered in tobacco only correspond to a minor fraction of its potential to produce natural compounds, as well as nutritional supplements, biopolymers or industrial and pharmaceutical compounds. In the future, it will be crucial that the new varieties and/or edited lines can be brought to an actual field production process with scalable and profitable results. To exploit tobacco as a sustainable feedstock from which multi-fractional

cascades can be used to isolate valuable compounds and enriched extracts, further metabolomic studies would be necessary. For example, transcriptome and metabolome analysis of a tobacco diversity panel would be useful to (i) assess the baseline of metabolites present, (ii) determine what species contain unique metabolites, and (iii) elucidate the genetic basis of metabolic capacities in the genus. It would be interesting to know which natural products are present in different fractions, typically generated when assessing multiple fractional separations. In this way, the remaining biomass from varieties developed to produce targeted speciality chemicals or pharmaceuticals could be further used to generate valuable co-products, by implementing a biorefining concept. From an industrial perspective, the identification of novel products that can be derived cost-effectively from the plant biomass to increase productivity and reduce the extraction and purification costs, will be fundamental.

The emerging field of synthetic biology offers the potential to engineer superior tobacco chassis. For instance, introducing more efficient photorespiratory pathways into tobacco, while inhibiting the native pathway, markedly increased both photosynthetic efficiency and vegetative biomass, also addressing the optimization of carbon sources [90]. Compartmentation of particular reactions, like the creation of cyanobacterial-like carboxysomes, could also contribute to their increased efficiency [91]. Likewise, the majority of therapeutic proteins are glycosylated and it is well known that the glycan profiles have a significant impact on protein stability and functionality [92-94]. Also, many plant-specialized metabolites accumulate as glycosides. Glycosylation plays an important role in the molecular structure of plant natural products, influencing their reactivity and solubility, cellular localization and bioactivity. Unlike yeast, the *N. benthamiana* transient expression platform offers a considerable potential as a system for glycodiversification of plant metabolites and small pharmaceuticals [95]. This platform has the potential to be glycoengineered for the production of high added-value chemicals. Compartmentation is another key strategy used by plants to accumulate high levels of potentially toxic metabolites without affecting viability. Improvement of our knowledge on both intra- and extracellular metabolite compartmentation is essential for the conversion of *Nicotiana* species into efficient metabolite biofactories.

In conclusion, developing a plant-based biofactory implies advantages over other conventional microbial culture factories or animal cells when the total costs are considered. However, some challenges remain (see Outstanding Questions), namely determining which are the most interesting products to be obtained, engineering the host to improve the plant chassis, and optimizing the production and purification steps. Further research should focus on the integration of the steps outlined above in an enclosed plant-based production facility such as a vertical farm in combination with established biorefineries. Such a conversion of a non-food crop like tobacco into an efficient biofactory is expected to be a solution in a modern and sustainable bio-based economy. In parallel, the Plant Molecular Farming field is opening new possibilities and

strategies to drive rural development and slow down the exodus to which these population nuclei are being subdued in the recent decades.

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Outstanding Questions

- In order to exploit tobacco as a sustainable feedstock, what novel mid-to-high value side products are present and can be derived in the different fractions generated when assessing multiple fractional separations?
- Could the remaining biomass be further used to generate valuable co-products, by implementing a biorefining concept?
- Compartmentation is used by plants to accumulate potentially toxic metabolites without affecting viability. How can we improve our technologies for engineering metabolite compartmentation towards the conversion of *Nicotiana* species into efficient metabolite biofactories?
- How can we implement new strategies for genetic and genome-wide engineering? Can we control the expression of genes and gene clusters to desired levels in synthetic pathways?
- How can we improve synthetic gene circuits whose complexity requires more processor elements? How can we further tailor these systems towards advanced production of added-value compounds?
- Hairy roots and plant cell cultures are valuable tools for the characterization of gene function and enzyme activity *in vivo*, but can biosynthetic pathways be modified in a way that cell or hairy roots cultures become comparable to crop plants in the field regarding the efficient production of valuable compounds?
- How can genetically modified *Nicotiana* species be safely used for sustainable agricultural development? Can we make genetically modified crops that are economically viable for molecular farming applications? Can public perception of genetically modified crops be improved?

Glossary

Bioeconomy. The bioeconomy comprises those parts of the economy that use renewable biological resources from land and sea – such as plant, animal and microbial biomass – to produce food, materials and energy.

Biofactory. Any system that produces useful amounts of biologically active compounds of economic interest.

Bioreactor. An apparatus for growing cells or organisms (yeast, bacteria, plant or animal cells) under controlled conditions. Used in industrial processes to produce pharmaceuticals, vaccines, or antibodies.

Grafting. Horticultural technique used to join parts from two or more plants so that they appear to grow as a single plant. In grafting, the upper part (scion) of one plant grows on the root system (rootstock) of another plant.

Model organism. An organism suitable for studying specific traits or phenomena, often due to its short generation time and characterized genome, and whose biology is well known and accessible to laboratory studies.

New Plant Breeding Techniques. Group of methods, including genome editing, cis- and intragenesis, transient expression and silencing, grafting, that allows the development of new plant varieties with desired traits.

Operon. A functioning unit of DNA containing a cluster of genes under the control of a single promoter.

Plant Molecular Farming. Refers to the production of recombinant proteins (including pharmaceuticals and industrial proteins) and specialized metabolites, in plants. This involves the growing, harvesting, transport, storage, and downstream processing of extraction and purification of the protein or metabolite.

Rhizobium rhizogenes. Gram-negative soil bacterium occurring near plant roots and ultimately causing the so-called "hairy root syndrome" in the infected plant host. This syndrome consists of a non-geotropic branching root overgrowth at the infection site.

Sequence-specific nuclease (SSN). SSNs are programmable molecular tools for recognition and modification of specific DNA sequences. SSNs include meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR/Cas (clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein).

Synthetic biology. The discipline aiming to apply engineering principles to plant biotechnology.

Transgenic plant. A plant that has been genetically engineered, a breeding approach that uses recombinant DNA techniques to create plants with new characteristics.

Transient expression. Temporary expression method in which plant cells are transiently transfected with a construct harbouring a T-DNA without stable transformation. The genes carried by the episomal T-DNA are expressed for a short time (days) before degradation and will not be passed to future generations of the cell or plant.

Trichomes. Epidermal outgrowths that cover aerial tissues in a large number of higher plant species. They often are rich in secondary metabolites and protect plants from biotic and abiotic stresses.

Virus-induced gene silencing. VIGS is a sequence-specific technique that exploits the natural defence mechanisms employed by plants to protect themselves against viruses. For VIGS, viral vectors bearing a fragment of a target gene (non-viral insert) are used to silence that particular gene.

Box 1. Combinatorial Nuclear Transformation

Combinatorial nuclear transformation involves the simultaneous integration of several independent transgenes into the nuclear genome [24] (Figure 2). The delivery of DNA by particle bombardment-mediated transformation (biolistics) ensures the frequent incorporation of multiple copies of the transforming plasmids (each harbouring a single transgene). The plasmids often co-integrate into a single genomic location [96], resulting in the segregation of all transgenes as a single Mendelian locus. For transformation, all transgene-containing vectors are mixed and another plasmid that harbours a selectable marker gene is added. Large numbers of transgenic plants are selected and screened for the desired phenotype (e.g., a particular metabolic output [26]).

The number of independent transgenic lines that should be analysed depends on several factors, including (i) the number of transgenes that are co-transformed, (ii) the variation in expression level of the individual transgenes that likely needs to be screened, and (iii) the difficulty to obtain the optimum stoichiometry of the enzymes and auxiliary proteins to be expressed. Thus, complex metabolic pathways that are composed of many enzymatic steps and that are highly regulated require the generation of large populations of transgenic lines (typically several hundred) to cover a sufficient combinatorial space. The large number of transgenic lines to be analysed necessitates high-throughput screening methods to identify the desired (metabolic) phenotypes [25, 26].

Box 2. Grafting-Mediated Horizontal Genome Transfer

Recent work has uncovered that both plastid and nuclear genomes can move across graft junctions, thus producing cytoplasmic hybrids (cybrids) [21, 39, 40] or new allopolyploid species [41].

Grafting-mediated horizontal genome transfer can be achieved experimentally in three steps: (1) generation of transgenic and/or transplastomic plants that carry different selectable markers in the genome of interest, (2) interspecies grafting, and (3) identification of horizontal genome transfer events by subjecting graft unions to double selection on plant regeneration medium. Since each graft partner is only resistant to one selection agent, double selection for both agents kills all cells except those that received the other marker gene by horizontal DNA transfer.

Horizontal transfer of plastid genomes generates new combinations of plastid and nuclear genomes (equivalent to cybrids). Such events are selected by grafting of a plastid-transformed (transplastomic) donor plant harbouring an antibiotic resistance marker (typically a chimeric *aadA* gene conferring spectinomycin resistance) and a nuclear-transgenic recipient line transformed with a different resistance marker (e.g., the kanamycin resistance gene *nptII*, or the hygromycin resistance gene *hpt* [21, 40]).

Horizontal transfer of nuclear genomes leads to new allopolyploid species. Events are identified by grafting of transgenic individuals that harbour different nucleus-encoded selectable markers [41], followed by double selection.

Stem grafting is by far the most commonly used grafting technique, but other tissues (including roots and calli) can also be grafted experimentally or engage in natural grafting [42, 97]. Recently, *in vitro* grafting of callus tissues has been shown to also trigger horizontal genome transfer [97].

To employ grafting-mediated genome transfer as a new breeding technology, it is recommended to use hemizygous transgenic marker lines, thus facilitating the removal of the selectable marker gene by a single cross (in plastid genome transfer) or by self-pollination (in nuclear genome transfer). Consequently, while the method would involve a transgenic intermediate, the end-product would be transgene-free. This is somewhat analogous to the use of genome editing techniques and the post-editing removal of the transgenes encoding the editing reagents (e.g., TALENs, CRISPR/Cas systems) by outcrossing.

The cellular mechanisms underlying horizontal genome transfer are not yet understood, and it is currently unclear whether genome travel is restricted to graft unions or occurs also in intact somatic tissues.

Box 3. Sequence-Specific Nuclease-Mediated Genome Editing

Sequence-specific nucleases (SSNs), such as Zinc-finger nucleases (ZFN), TAL effector nucleases (TALENs) and CRISPR/Cas all produce double-strand breaks (DSBs) at designated DNA positions, which are subsequently repaired via non-homologous end joining (NHEJ) or homologous-directed repair (HDR). NHEJ often results in small insertions or deletions that may lead to loss-of-function mutations in the targeted genes (SSN-1). The addition of a homologous donor template can trigger HDR, resulting in the incorporation of a truly edited DNA sequence at the DSB site. When the donor template contains only small changes from the native sequence, the technology is classified as SSN-2. Alternatively, a donor template containing a new DNA sequence flanked by homology arms would result in gene replacements, targeted gene insertions or gene stacking at the DSB (SSN-3). The CRISPR/Cas technology has partially overcome previous SSNs with its efficiency and programming simplicity, requiring only a small guide RNA (gRNA) with a 20 nucleotides-specific protospacer. The concurrence of multiple gRNAs in the same cell, along with Cas, is sufficient to simultaneously target several genes. The CRISPR "multiplexing" capacity has a strong impact on breeding, speeding up trait pyramiding or targeting functionally redundant gene families, which are especially relevant in polyploid crops.

The delivery of Cas9 and gRNA to the plant cell, and the subsequent obtainment of edited offspring, are major technical challenges in plant editing. The CRISPR/Cas machinery can be deployed directly to the cells (protoplasts) as a ribonucleoprotein complex, or indirectly via transgenesis. CRISPR-encoding transgenes can be either stably integrated in the genome or delivered transiently as episomal DNA via biolistics or Agrobacterium, either alone or in combination with inducers of somatic embryogenesis [98]. A final delivery option consists in the use of viral vectors, a strategy known as virus-induced gene editing (VIGE). In all cases, first-time examples were implemented in the *Nicotiana* chassis due the extraordinary regeneration capacity and the availability of viral vectors [99].

Nuclease-inactive (dead) and nickase versions of Cas9 (dCas9 and nCas9) have been engineered to produce alternative editing strategies, named base editing and prime editing. Cytosine and adenosine "base editors" contain base deaminases fused to the nCas9 protein [100]. Base deamination results in precise single nucleotide substitutions. More recently, efficient prime editing was also demonstrated in plants. Here, a reverse transcriptase (RT) fused to nCas9 targets specific genome loci with an engineered gRNA that includes a "donor" RT template, therefore producing highly precise base substitutions [101].

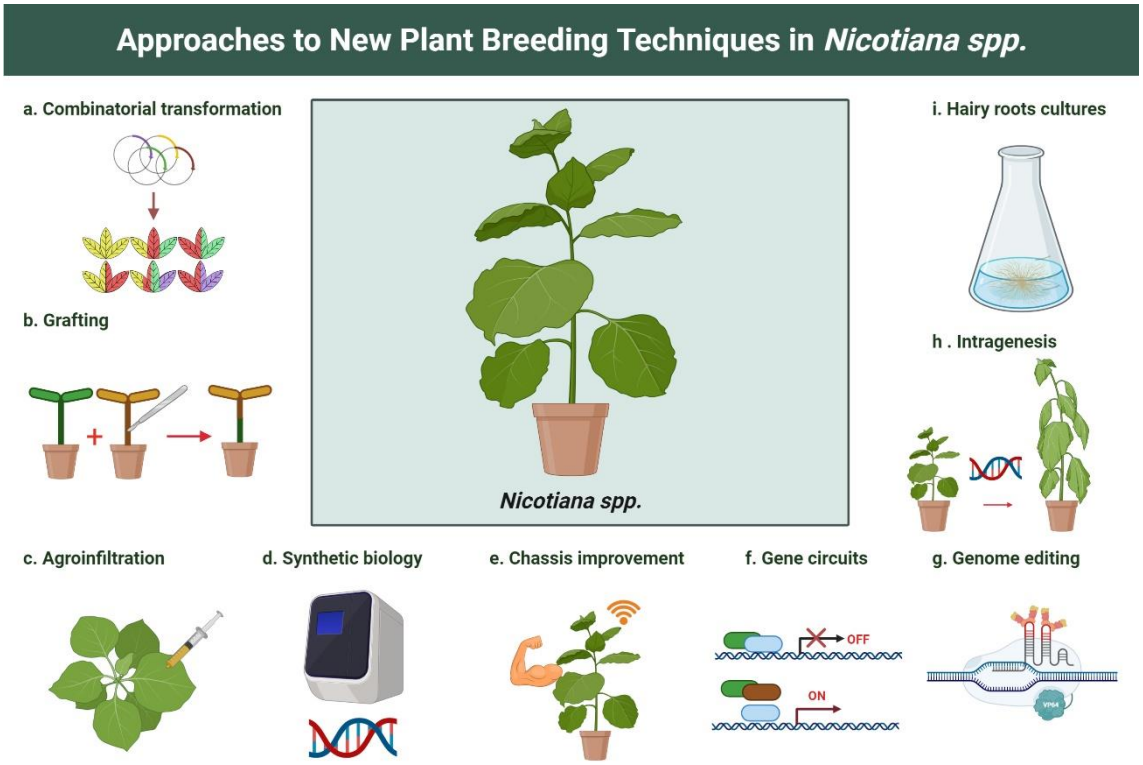

Figure 1 (Key Figure). Schematic Overview of the New Plant Breeding Techniques Used in *Nicotiana* species and Discussed in This Review. The figure includes images from Biorender (https://biorender.com/).

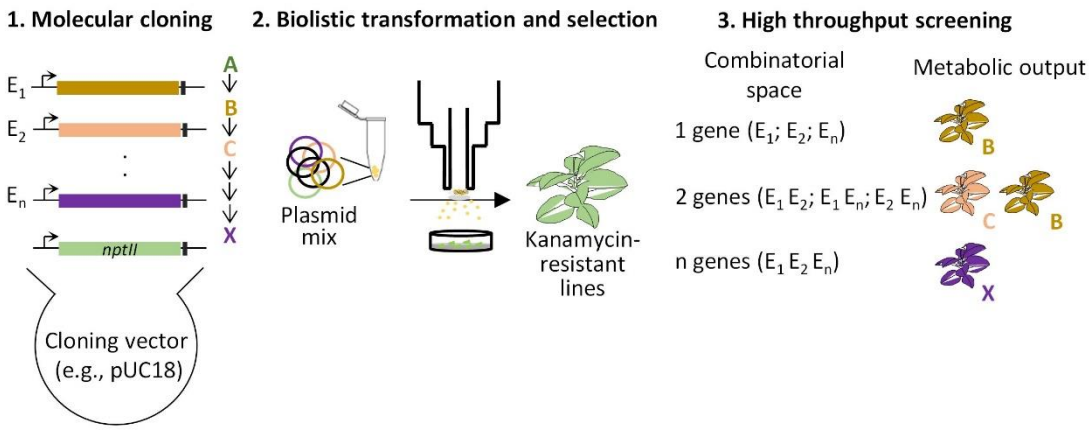

Figure 2. Schematic Representation of the Combinatorial Transformation Approach. Combinatorial transformation involves three major steps: **(1)** Transgenes for metabolic enzymes (E) and, if applicable, additional auxiliary factors involved in a pathway of interest (metabolites A to X) are cloned into simple vectors that harbour an expression cassette with a promoter (arrow) and terminator (black box). Subscript numbers (1,2, … n) in transgene-encoded enzymes (E) indicate their position in the metabolic pathway. Enzyme-product pairs are marked with the same colour. An additional plasmid vector contains a resistance marker for selection of transgenic plants (e.g., an *nptII* gene encoding kanamycin resistance; green). **(2)** All constructs are mixed (usually in equal quantities) and loaded onto gold particles to perform particle bombardment-mediated plant transformation. Kanamycin-resistant plants are obtained by *in-vitro* regeneration in selective medium. **(3)** Transgenic plants are subjected to high-throughput screening for metabolite accumulation from the pathway of interest. Random co-integration of different transgenes results in many different combinations (combinatorial space) that promote accumulation of different metabolites of the pathway (metabolic output). Plants harbouring different sets of transgenes are coloured based on their expected metabolic phenotype. For simplicity, plants harbouring gene combinations that do not metabolize the endogenous precursor molecule are not shown.

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