Combination of plant metabolic modules yields synthetic synergies

Zur Erlangung des akademischen Grades eines

DOKTORS DER NATURWISSENSCHAFTEN

(Dr. rer. nat.)

Fakultät für Chemie und Biowissenschaften

Karlsruher Institut für Technologie (KIT)-Universitätsbereich

genehmigte

DISSERTATION

von

Fatemeh Rajabi

aus

Shiraz, Iran

Referent: Prof. Dr. Peter Nick

Korreferent: Prof. Dr. Holger Puchta

Tag der mündlichen Prüfung: 05.02.2018

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT), Botanisches Institut, Lehrstuhl 1 für Molekulare Zellbiologe, im Zeitraum von Juni 2013 bis Dezember 2017 angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Zudem erkläre ich, dass ich mich beim Anfertigen dieser Arbeit an die Regeln zur Sicherung guter wissenschaftlicher Praxis des KIT gehalten habe, einschließlich der Abgabe und Archivierung der Primärdaten, und dass die elektronische Version mit der schriftlichen übereinstimmt.

Karlsruhe, im Dezember 2017

Fatemeh Rajabi

The central parts of the thesis have been published in;

Rajabi, F., Heene, E., Maisch, J. & Nick, P. (2017). Combination of plant metabolic modules yields synthetic synergies. *PLOS ONE* 12(1).

Acknowledgments

I would like to take this opportunity to express my gratitude to all those who offered me their help in any way during the entire course of my graduate study.

I would like to express my special appreciation and thanks to my advisor Prof. Peter Nick, who has been a tremendous mentor for me. I would like to thank him for encouraging my research and for allowing me to grow as a research scientist. Meanwhile, I deeply appreciate all the time and attention he devoted, all the suggestions and ideas he provided in supervising me.

My sincere thank also goes to Dr. Jan Maisch for his continuous supports, motivation and his priceless guidance and comments that without his precious supports it would not be possible to conduct this research.

I am grateful that Prof. Holger Puchta agreed to be my co-examiner immediately and I really appreciate his time, devotion and expertise.

I also would like to express my sincere gratitude to Dr. Christian Gusbeth from Institute for Pulsed Power and Microwave Technology (IHM), Karlsruhe Institute of Technology (KIT), who performed and maintained a stable condition of nanosecond pulsed electric fields (nsPEFs) for our project.

I would especially like to thank Ernst Heene for his great guides and assistance in analyzing natural compounds by HPLC.

I would like to thank Dr. Michael Riemann for his help and suggestions during my work. I appreciate the excellent work of our lab technician Nadja Wunsch, Sabine Purper and Sybille Wörner as well as our Azubi Lena Draheim and Jonas Müller for their assistance in this project.

My sincere thanks also go to all the doctoral students as well as all the members who work in Botanical Institute 1 for such a pleasant, friendly and helpful working atmosphere. My special thanks also go to Dr. Qiong Liu whom I learned many things as well as get help from so many times.

Last but not least, I would like to thank my family: My parents, my sisters and brother for their continuous supports with endless love. At the end, I would like to express appreciation to my

beloved husband, Mohammad for all of the sacrifices that he has made on my behalf and was always my support in the moments when there was no one to answer my queries.

This work is financially supported by the German Academic Exchange Service (DAAD), and by funds from the German Federal Ministry of Education and Research (BMBF, Bioeconomy Initiative, 031B0065A).

Fatemeh Rajabi

Table of Contents

Abbreviation

2, 4-D: 2, 4-dichlorophenoxyacetic acid

BY-2: Tobacco *Nicotiana tabacum* L. cv. Bright Yellow 2

DMSO: Dimethyl sulfoxide

EF-1 α: Elongation factor 1α

GFP: Green fluorescent protein

HPLC: High-performance liquid chromatography

IAA: Indolyl-3-acetic acid

JA: Jasmonic acid

MI: Mitotic index

MPO: N-methylputrescine oxidase

MS-Medium: Murashige and Skoog medium

NAMN: Nicotinic acid mononucleotide

NND: Nicotine N-demethylase

nsPEF: nanosecond pulsed electric fields

PI: Pulsing indices

PMT: Putrescine N-methyltransferase

PTS1-Cherry: Peroxisome-targeted mCherry marker

QPT: Quinolinate phosphoribosyltransferase

ROS: Reactive oxygen species

WT: Wild type

Zusammenfassung

Das große Potenzial pharmakologisch wirksamer pflanzlicher Sekundärmetabolite ist oft durch eine geringe Ausbeute und Verfügbarkeit der produzierenden Pflanzen begrenzt. Die chemische Synthese dieser komplexen Verbindungen ist oftmals zu teuer. Die Pflanzenzellfermentation bietet eine alternative Strategie zur Überwindung dieser Einschränkungen. Die Produktion in Batch-Zellkulturen bleibt jedoch oft ineffizient. Ein Grund könnte die Tatsache sein, dass verschiedene Zelltypen für die Produktion der Metabolite interagieren müssen, was in Suspensionszellkulturen nur schlecht nachgebildet werden kann. Die Sekretion des Endprodukts in das Kulturmedium stellt ein weiterer limitierender Faktor dar, wenn das Aufschließen der Zellen für die Produktgewinnung vermieden werden soll. Am Beispiel des Alkaloidstoffwechsels von Tabak untersucht diese Arbeit eine alternative Strategie, bei der die Wechselwirkungen im Stoffwechsel verschiedener Zelltypen eines Pflanzengewebes mittels verschiedener pflanzenzelltypischer Stoffwechselmodule technisch nachgebildet werden. Diese Studie bildet die Wechselwirkung zwischen den Nicotin-sekretierenden Zellen der Wurzel und den Nicotin-konvertierenden Zellen des seneszenten Blattes nach, wobei die Zielverbindung Nornicotin in der Modellzelllinie Tabak BY-2 erzeugt wird. Wenn die Nikotin-Demethylase *Ntom*CYP82E4 in BY-2 Tabakzellen überexprimiert wurde, wurde die Nornicotinsynthese ausgelöst. Die Nornicotinsynthese erfolgte allerdings nur in einem geringen Ausmaß. Es wird jedoch in dieser Arbeit gezeigt, dass die Produktion von Nornicotin in dieser Zelllinie durch Zuführen des Vorläufers Nicotin verbessert werden konnte. Das Metabolic Engineering einer anderen Zelllinie, die das Schlüsselenzym *Ntab*MPO1 überexprimiert, erlaubte es, die Bildung und Sekretion dieses Vorläufers anzuregen. Diese Studie zeigt ferner, dass die Nornicotinakkumulation der *Ntom*CYP82E4-Zellen ohne irgendeinen negativen Effekt auf die Physiologie der Zellen signifikant stimuliert werden konnte, indem konditioniertes Medium der *Ntab*MPO1-Überexpressionslinie zugeführt wurde.

Die Cokultivierung der *Ntom*CYP82E4 mit den *Ntab*MPO-Zellen regte die Nornicotinakkumulation sogar noch weiter an und zeigte, dass die physikalische Gegenwart von Zellen besser war, als nur das konditionierte Medium zuzuführen, das aus den gleichen Zellen gewonnen wurde. Diese Ergebnisse liefern einen Beweis für das Konzept, dass in der Pflanzenzellfermentation die Kombination verschiedener metabolischer Module die Produktivität von Zielverbindungen verbessern kann. Für eine effiziente Downstream-Rückgewinnung der Produkte wurde eine Strategie getestet, die sich nanosecond Pulsed Electrical Fields (nsPEFs) zunutze macht. Um die Lebensfähigkeit der Zellen zu erhalten und die weitere Verwendung der Biomasse zu ermöglichen, wurden die Zellen starken (1-20 kV cm^{-1}), aber sehr kurzen (10-100 ns) elektrischen Impulsen ausgesetzt, was zu einer vorübergehenden Permeabilisierung der Zellmembranen führte.

Abstract

The great potential of pharmacologically active secondary plant metabolites is often limited by low yield and availability of the producing plant. Chemical synthesis of these complex compounds is often too expensive. Plant cell fermentation offers an alternative strategy to overcome these limitations. However, production in batch cell cultures remains often inefficient. One reason might be the fact that different cell types have to interact for metabolite maturation, which is poorly mimicked in suspension cell lines. Secretion of the final product to the culture medium to avoid cell rupture for product recovery is another limiting factor. Using alkaloid metabolism of tobacco, this work explores an alternative strategy, where the metabolic interactions of different cell types in a plant tissue are technically mimicked based on different plant-cell based metabolic modules. This study simulates the interaction found between the nicotine secreting cells of the root and the nicotine-converting cells of the senescent leaf, generating the target compound nornicotine in the model cell line tobacco BY-2. When the nicotine demethylase *Ntom*CYP82E4 was overexpressed in tobacco BY-2 cells, nornicotine synthesis was triggered, but only to a minor extent. However, it is shown here that the production of nornicotine in this cell line could be improved by feeding the precursor, nicotine. Engineering of another cell line overexpressing the key enzyme *Ntab*MPO1 allows to stimulate accumulation and secretion of this precursor. This study shows that the nornicotine production of *Ntom*CYP82E4 cells can be significantly stimulated by feeding conditioned medium from *Ntab*MPO1 overexpressors without any negative effect on the physiology of the cells. Co-cultivation of *Ntom*CYP82E4 with *Ntab*MPO1 stimulated nornicotine accumulation even further, demonstrating that the physical presence of cells was superior to just feeding the conditioned medium collected from the same cells. These results provide a proof of concept that combination of different metabolic modules can improve the productivity for target compounds in plant cell fermentation. For efficient downstream recovery of the products, a

nanosecond pulsed electric fields (nsPEFs) strategy was tested. To maintain cell viability and allow for the further use of biomass, cells were exposed to strong (1-20 kV cm^{-1}), but very short (10-100 ns) electric pulses which leads to a temporary permeabilization of cell membranes.

1 Introduction

Plants are able to produce a wide variety of specific secondary metabolites, which makes them unique among multicellular organisms (Goossens et al. 2003; Anarat-Cappillino & Sattely 2014). Storage of secondary metabolites in relatively high concentrations, sometimes in organs which do not involve in their biosynthesis or as inactive prodrugs is a characteristic feature of secondary metabolites. When plants are exposed to the danger, these prodrugs are enzymatically activated and can act as defense compounds. Therefore, contrary to what was previously assumed, secondary metabolites are not useless waste products but important tools of plants needed against herbivores, microbes (bacteria, fungi) and viruses (Wink 1988; Wink 2010). Some secondary metabolites also function as signal molecules to attract pollinating or seed dispersing animals. Plants have evolved secondary metabolites with a wide array of biochemical and pharmacological properties during millions years of evolution. The molecular targets of many secondary metabolites are proteins (receptors, ion channels, enzymes, cytoskeleton, and transcription factors), DNA/RNA and/or biomembranes. Some of these interactions are highly specific for instance most of alkaloids interfere with neuroreceptors (Wink 2000; Wink 2007).

Starting from traditional medical systems, humans have exploited this metabolic proficiency of plants since ancient times. Due to the growth of the world population and the popularity of phytomedicinal compounds, the global demand for natural compounds has been growing steadily, and pharmaceutically interesting plants are increasingly exploited on an industrial scale. However, low yield (Georgiev et al. 2009; Barbulova et al. 2014), limited seasonal availability, low abundance of active compounds, and slow growth of many medical plant species (Roberts 2007; Wilson & Roberts 2012) are progressively hampering this approach. In addition, in some countries, cultivated or collected medicinal herbs have raised concern due

to pollution by herbicides, insecticides, and heavy metals (Gaosheng & Jingming 2012). Moreover, especially in taxonomically difficult groups, or in plants that are rare, adulteration by surrogate material of similar morphology has become a serious issue (Han et al. 2016). Chemical synthesis does not provide alternatives because due to the complex structures of secondary metabolites, production *in vitro* is not cost-efficient in most cases. Thus, innovative strategies to produce medicinal natural products in sufficient quantity, quality, and standardized conditions have a considerable international impact for the development of novel pharmaceutical products (Gaosheng & Jingming 2012).

Plant cell cultures certainly represent a valid alternative for the sustainable production of valuable secondary metabolites, but the success of plant cell fermentation has been limited by low product yields and cell culture variability (Wilson & Roberts 2012). One reason for this limitation is the fact that, in contrast to the situation in a plant, the metabolic activity in a plant cell culture system is not partitioned into different cell types. In other words, plant cell fermentation in batch cell cultures might often not be very efficient, because it is based on just one type of cells and therefore cannot provide the interaction of different cell types required for the maturation of the metabolites. Optimization of downstream processing for the efficient recovery of metabolites from a cell culture system is a matter of great importance. Secretion of secondary metabolites into the liquid medium is often limited by pecto-cellulosic tissue and membranes enclosing organelles within cells (Georgiev et al. 2009). Excretion of these metabolites to the culture medium can significantly decrease downstream processing costs (Wilson & Roberts 2012). Permeabilizing cells using pH shock (Thimmaraju et al. 2003) or chemical treatments are alternatives to achieve both cells viability and further use of biomass. In other words, there is no need to kill cells if the metabolites are released into the medium and therefore biomass can be separated from the medium before downstream processing. However, the aforementioned methods have limited potential for cell permeabilization while

preserving cell viability and applying these methods are not always successful for different cell types (Brodelius 1988; Park & Martinez 1992). Cell membranes permeability could be obtained by exposure of cells to nanosecond pulsed electric fields (nsPEF) provided that physical characteristics of exposure become optimized (Brodelius 1988; Hapala 1997; Knorr & Angersbach 1998).

Although combinations of different methods have been successfully employed to achieve high yields for the final product, like elicitor treatment, precursor feeding and metabolic engineering, the significant importance of compartmentalization of metabolism into different cell types remained relatively an uninvestigated field and therefore, it is clearly worth investing some effort into this aspect.

1.1 Ecological function of secondary metabolites

A general feature of plants is the production of a high range of secondary metabolites, including many nitrogen-free (such as terpenes, polyketides, phenolics, saponins and polyacetylenes) and nitrogen-containing compounds (such as alkaloids, amines, cyanogenic glycosides, non-protein amino acids, glucosinolates, alkamides and peptides) (Osbourn & Lanzotti 2009). Involvement of specific enzymes in the synthesis of these metabolites in a tissue-, organ- and developmental-specific way give rise to different complex mixtures of secondary metabolites, which is quite diverse from organ to organ and commonly between individual plants (Facchini & De Luca 2008; Murata et al. 2008). Gene regulation of corresponding genes is as complex as genes encoding enzymes of primary metabolism. Secondary metabolites are usually accumulated and stored in high concentrations in the plant organs that are important for survival and reproduction (Wink 2010).

Principally, hydrophilic substances are accumulated in the vacuole, whereas lipophilic compounds are stored in resin ducts, laticifers, trichomes, oil cells, or in the cuticle (Shoji et al. 2008; Cai et al. 2012). Despite a huge application of secondary metabolites by humans for more than thousands of years, their putative biological functions have been a matter of argument (Mann, 1992; Roberts and Wink, 1998). These compounds have been used as dyes (e.g. indigo, shikonin), flavours (e.g. vanillin, capsaicin, mustard oils), fragrances (e.g. rose oil, lavender oil and other essential oils), stimulants (e.g. caffeine, nicotine, ephedrine), hallucinogens (e.g. morphine, cocaine, scopolamine, tetrahydrocannabinol), insecticides (e.g. nicotine, piperine, pyrethrin, rotenone), vertebrate and human poisons (e.g. coniine, strychnine, aconitine, colchicine, cardiac glycosides) and even as therapeutic agents (e.g. atropine, quinine, cardenolides, codeine) (Wink 2010). Contrary to animals, plants cannot run or fly away when attacked by a predator or protect themselves against invading microbes or parasites by an activated immune system. Indeed, plants have the ability to replace wounded or infected parts which provide a certain tolerance towards herbivores and microbes. Some plants employ mechanical and morphological protection, such as thorns, spikes, glandular and stinging hairs (often filled with poisonous chemicals), or develop an almost impenetrable bark (especially woody perennials) (Wink 2010). Besides, biosynthesis of some special secondary metabolites by plants can significantly protect them against viruses, bacteria, fungi, competing plants and herbivores (Osbourn & Lanzotti 2009). In addition, secondary metabolites can serve as signal compounds to attract animals for pollination (fragrant monoterpenes, colored anthocyanins or carotenoids) and for seed dispersal (Cipollini & Levey 1997). There are several cases in which one single compound act as both defense compound and signal molecule. For example, anthocyanins or monoterpenes can attract insects to the flowers but have insecticidal properties at the same time. This makes sense, since insects need to be attracted as pollinators, but should not eat the flowers. Indeed the pollinators are rewarded by nectar. In addition, some secondary metabolites concomitantly exhibit physiological functions,

for example, they can serve as mobile and toxic nitrogen transport and storage compounds or ultraviolet-protectants. These multiple functions are typical and do not contradict their main role as chemical defense and signal compounds. Literally, serving multiple functions by a single trait increase probability of that trait to be conserved by natural selection (Wink 2010).

1.2 Plant secondary metabolites production routes

Millions of years of evolution have resulted in the production of a high variety of natural products by plants. Owing to these natural products, humans have developed a broad knowledge of useful plants over time through continuous contact with their natural environment (Gaosheng & Jingming 2012). It has been reported that 80% of the people in developing countries are still relying on traditional medicine based on plant extracts for their primary health care needs (Farnsworth, 1988). The reason behind this interest in traditional medicine is lower costs compared to western medicine and also low or no toxicity of plant-based medicinal compounds. Currently, over 60% of anticancer drugs and 75% of drugs for infectious disease are either natural products or analogs of natural products (Wilson & Roberts 2012). The great potential of pharmacologically active secondary plant metabolites is often limited by low yield and availability of the producing plant and therefore natural sources can't fulfill the need of increasing market.

In addition, for the harvesting of these compounds from the plant source, some parts of their tissues such as root, leaves, seeds or flowers should be cut off for extraction and will directly cause problems for the reproduction of these plants (Gaosheng & Jingming 2012). The dimeric indole alkaloids vinblastine and vincristine are used in the treatment of leukemia and tons of dried leaves of *Catharanthus roseus* have to be extracted to isolate only a few grams of these

compounds (Pais et al. 1988). The anticancer drug paclitaxel is another illustration in which 340,000 kg of *Taxus* bark or 38000 trees are required to produce the 25 kg per year demand for this valuable compound (Wilson & Roberts 2012). In addition, most medicinal plants are highly endangered and therefore providing these valuable natural products from natural sources is economically unsustainable (Gaosheng & Jingming 2012; Barbulova et al. 2014). Therefore, since a long time, laboratories worldwide are trying to produce natural products for the development of pharmaceutical industry using different approaches instead of natural harvesting (Wink 2010; Gaosheng & Jingming 2012). Despite all the efforts exerted by scientists all over the world, several compounds continue to be harvested from their native plant owing to lack of better commercialization options. For instance, camptothecin, vinblastine and vincristine are still commercialized through natural harvesting from *Camptotheca acuminate* and *Catharanthus roseus*, respectively (Lopez-Meyer et al. 1994; Ishikawa et al. 2010). Morphine and codeine which are used as an analgesic are still taken from *Papaver somniferum* plants (Odell et al. 2008). Another example is artemisinin with an application in malaria treatment harvested from *Artemisia annua* (Covello 2008). Alternate routes to valuable secondary metabolites supply are included total or partial chemical synthesis, heterologous expression of the biosynthetic pathway in other organisms and *in situ* production via plant cell culture (Wilson & Roberts 2012). Each method offers distinct advantages and disadvantages depending on the specific system of interest which is illustrated in the following sections.

1.2.1 Chemical synthesis and limitations

Total chemical synthesis is an alternative way for the production of plant natural products. One of the best examples is vanillin from the plant *Vanilla planifolia* which is a very popular flavor used worldwide. However, most of the annual demand of vanillin is supplied by chemical

methods that use guaiacol, eugenol or lignin as a precursor (Schwab et al. 2008; Wilson & Roberts 2012). Although several attempts have been made for chemical synthesis of some precious natural compounds like morphine and codeine, but commercially chemical synthesis of these compounds are still not feasible. The large size and complex chemical synthesis routes lead to the conclusion that for some natural products, this method is not cost efficient (Gerardy & Zenk 1992). Despite the existence of sophisticated methodologies for complex natural products, the multiple steps involved in their synthesis can result in low overall yields. For instance, it has been reported that total synthesis of taxol® has a very low overall yield (Nicolaou et al. 1994; Chemler & Koffas 2008). Usually, a synthetic route for a complex molecule with over 10 separate steps is economically and environmentally unfavorable, since with each step the overall yield decreases and the waste and resources increase (Chemler & Koffas 2008; Wilson & Roberts 2012).

1.2.2 Heterologous production and limitations

Heterologous synthesis of plant secondary metabolites has been investigated in prokaryotic or eukaryotic organisms. Several techniques such as metagenomics, transcriptomics, proteomics and metabolomics are necessary for the identification of involved genes, enzymes, and metabolites of a plant natural product biosynthetic pathway. Origin, type and complexity related to natural products play important roles in the heterologous biosynthesis of these compounds. In addition, attention to the biology of the native host system is of great importance while choosing a heterologous host. In other words, similarities in gene expression and cellular environment between native and heterologous host system would most likely support the metabolic requirements of the natural product pathway to be reconstructed. Another crucial factor is determining the availability of genetic transfer techniques for the selected

heterologous host given that host-specific technical limitations still exist (Chemler & Koffas 2008; Zhang et al. 2011).

The secondary metabolic pathways for taxadiene as one of the dedicated precursor of paclitaxel have also been introduced into heterologous plant hosts, such as *Arabidopsis* (Besumbes et al. 2004) and tomato fruits (Kovacs et al. 2007). In addition, genes involved in the early biosynthetic pathway of paclitaxel have been heterologously expressed in *Escherichia coli and Saccharomyces cerevisiae*. Several further modifications such as codon optimization, combinatorial biosynthesis, and introducing regulatory elements to inhibit competitive pathways resulted in the production of two committed taxol intermediates, taxadiene and taxadiene-5α-ol (Ajikumar et al. 2010; Zhang et al. 2011). Also, several attempts have been made to produce the antimalarial drug artemisinin in *E. coli* and *S. cerevisiae* which has been led to the biosynthesis of artemisinin precursors, amorphadiene and artemisinic acid (Chemler & Koffas 2008; Zhang et al. 2011). There are also numerous reports on the heterologous production of stilbenes in *E. coli* and *S. cerevisiae* (Zhang et al. 2006; Katsuyama et al. 2007). Stilbenes are classified as phenylpropanoids and due to their antioxidant activities, have attracted great attention (Chemler & Koffas 2008). Although there are great advances in the heterologous production of natural products, there still exist numerous challenges. The main limitation regarding the heterologous production of valuable natural products is the lack of their fully characterized metabolic pathways and therefore only a small number of natural products are currently produced by this production route (Wilson & Roberts 2012).

1.2.3 Plant cell culture and limitations

Since a long time, there is a great interest to produce natural products by plant cell cultures. Some natural compounds of great medicinal importance, even such complex structures like paclitaxel, are produced by undifferentiated cell cultures. However, some compounds like morphine, vinblastine or many terpenoids, e.g. cardenolides, are not found in unorganized cell cultures. Low yields and high production costs are amongst those factors which have been led that the commercial success of this research remains limited. However, there is a combination of different approaches in order to increase the product yields in cell culture systems. Traditional methods attempt to select high producing plants for initiating their desired cultures and from the best cultures, high producing cell lines are isolated. Media optimization in favor of growth and productivity would be pursued next (Wink 2010). Furthermore, elicitation is also used to increase secondary metabolite accumulation in plant cell cultures (Bourgaud et al. 2001). Due to the link between many secondary metabolites and plant defense, applying different chemical or physical stresses can manipulate the cellular metabolism towards the production of the desired product. Therefore, elicitors are widely used in medicinal plant cell culture to increase the production of target compounds. Jasmonic acid (JA) and its derivatives which are involved in plant growth and development are commonly applied as elicitors in cell culture (Roberts 2007; Wink 2010; Gaosheng & Jingming 2012; Wilson & Roberts 2012). Due to great progress in molecular biological methods and our knowledge concerning the biosynthetic pathways and the regulatory control mechanisms, increase or decrease of product yields by genetic manipulation has been feasible (Wink 2010). Applying metabolic engineering in recent years has been opened up new perspectives for improving the yields in plant cell culture. Establishment of efficient transformation protocols and identification of metabolic pathways and rate limiting enzymes involved has been made the way smooth for metabolic engineering of plant cell culture (Wilson & Roberts 2012).

Despite all efforts during last years, the commercial success of plant cell culture as a source of natural product is still very limited. One reason might be the fact that different cell types have to interact for metabolite maturation, which is poorly mimicked in suspension cell lines. In other words, production of several natural products in suspension cultures is not possible and they could be produced only in specific organs. It reveals this fact that a certain level of differentiation is required for the expression of the desired biosynthetic pathway (Wink 2010). This is confirmed by several reports on the involvement of different cell types in the biosynthesis of different classes of chemical compounds such as alkaloids (Nakajima & Hashimoto 1999; Ziegler & Facchini 2008). For instance, biosynthesis of vindoline which is a chemical precursor for the microtubule-disruptive drug, vinblastine, involves at least two distinct cell types and requires the intercellular translocation of pathway intermediates (St-Pierre et al. 1999). Therefore, compartmentation and transport of products and intermediates are crucial for metabolite maturation.

Another limiting factor regarding the production of natural compounds in cell culture is that these compounds often tend to remain intracellular. If a metabolite remains intracellular, it is usually necessary to kill the cells in order to extract the chemicals. By disruption of biomass, downstream processing costs would be increased and also further use of biomass would not be feasible anymore. Although there are different permeabilization methods which force the cells to excrete their chemicals into the culture medium (Bourgaud et al. 2001) most of them are not applicable for all cell types (Brodelius 1988; Park & Martinez 1992).

1.3 Scope of this study

Plant cell cultures certainly represent a reliable source for natural compounds production. There are some limitations regarding low yield, undifferentiated state, genetic variability, lack of compartmentation and maintenance of metabolite intracellular. Most efforts have been

invested in the discovering different approaches to increase the final product yields. However, almost no attention has been devoted to overcome difficulties regarding the lack of compartmentation in cell culture systems. This can partially explain why attempts to produce some natural compounds by cell culture technology have been failed. Therefore, in this dissertation, the necessity of the presence of different cell types for production of natural compounds in cell cultures will be examined and discussed. Furthermore, the nanosecond pulse electric field (nsPEFs) approach will be investigated as an alternative method to improve efficiency for product recovery of metabolites in cell cultures.

1.3.1 How can we mimic the interaction of metabolically different cell types found in plant tissues using a cell culture system?

The importance of the presence of different cell types for the metabolite maturation and especially production of some valuable compounds was discussed in details. It was explained how the necessity of compartmentalization has been caused limitation for the production of some valuable natural compounds which make natural harvesting the only possible production route so far. However, investing in natural sources to meet the increasing demands of these compounds does not seem a logical way. The plant cell culture system as an alternative production route has also the same limitation for production of some natural compounds due to the lack of different cell types in the culture. Therefore, it is of great importance to find out a way for the interaction of different cell types in a cell culture system. To test this idea, a complex target compound with an almost well characterized metabolic pathway and a good knowledge of its gene networking was required.

In this study, nornicotine is used as a case study to demonstrate the impact of metabolic partitioning. This nicotine metabolite has acquired great interest because of its efficacy against

the Alzheimer disease (Dickerson & Janda 2003). The nitrosylated product of nornicotine and other main alkaloids of tobacco are considered as potent carcinogens. However, these problematic derivatives are mostly formed during the tobacco curing process (Cai et al. 2016). Synthesis of the alkaloid nornicotine provides a striking example of metabolic partitioning in plants: Nornicotine synthesis occurs predominantly in leaves, whereas the nornicotine precursor, nicotine, is synthesised in the roots and subsequently transported via the xylem to leaves and the other aerial parts of the plant (Hashimoto & Yamada 1994; Baldwin 2001; Shoji et al. 2008). Nicotine is primarily stored in the vacuole of the cells and acts as a defense mechanism against herbivores (Steppuhn et al. 2004; Dewey & Xie 2013). In a final step, the nicotine imported into the leaf is demethylated to nornicotine through an oxidative process catalyzed by an enzyme belonging to the cytochrome P450 family of monooxygenases (Hao & Yeoman 1998; Siminszky et al. 2005; Lewis et al. 2008). Different from other alkaloid biosynthesis enzymes, nicotine N-demethylase (NND) occurs exclusively in leaf tissue rather than in the root, with high levels of activity during leaf senescence (Dewey & Xie 2013).

This pronounced metabolic partitioning on the level of different cell types is accompanied by a prominent compartmentalization of different metabolic steps within the cell (Figure 1.1): The pyrrolidine moiety of nicotine derives from the symmetric diamine putrescine. N-methylation of putrescine by putrescine N-methyltransferase (PMT) produces N-methylputrescine. This product is then deaminated oxidatively by N-methylputrescine oxidase (MPO) to form 4-methylaminobutanal. Spontaneous cyclisation of 4-methylaminobutanal produces N-methylpyrrolinium cation (N-methyl-Δ1-pyrrolinium). MPO belongs to a subclass of diamine oxidases which are dependent on copper and topaquinone for activity and was found to colocalize with the peroxisome in *Nicotiana benthamiana* leaf epidermal cells (Heim et al. 2007; Katoh et al. 2007; Naconsie et al. 2014). Nicotine is then formed through coupling of N-methylpyrrolinium cation and a non-identified intermediate from nicotinic acid (Hashimoto &

Yamada 1994). Nicotinic acid formation is carried out through the salvage pathway of nicotinamide adenine dinucleotide (NAD), which is derived from aspartic acid (Katoh et al. 2006). This aspartate derived pathway is coupled to the pyridine nucleotide cycle by quinolinate phosphoribosyltransferase (QPT). Quinolinate-dependent synthesis of nicotinic acid mononucleotide (NAMN) apparently can occur in both, cytosolic and mitochondrial, compartments (Sinclair et al. 2000; Noctor et al. 2006). There is good evidence that QPT is targeted to plastids as well (Katoh et al. 2006). Subsequently, the nicotinic acid precursor is thought to be first reduced, then decarboxylated and eventually coupled to the N-methyl-Δ1-pyrrolinium substrate by an unknown mechanism. However, the possible involvement of additional intermediates in these reactions has remained ambiguous. Recent studies indicate a possible involvement of two further gene products (A622 and BBL) during the final stages of nicotine biosynthesis. Although the N-terminal region of BBL enzymes contains putative vacuolar sorting determinants (Kajikawa et al. 2011) which qualifies these enzymes as nicotine synthases, the molecular nature of the final enzyme and its corresponding pyridine substrate are still unclear (Shoji & Hashimoto 2008; DeBoer et al. 2009; Kajikawa et al. 2011).

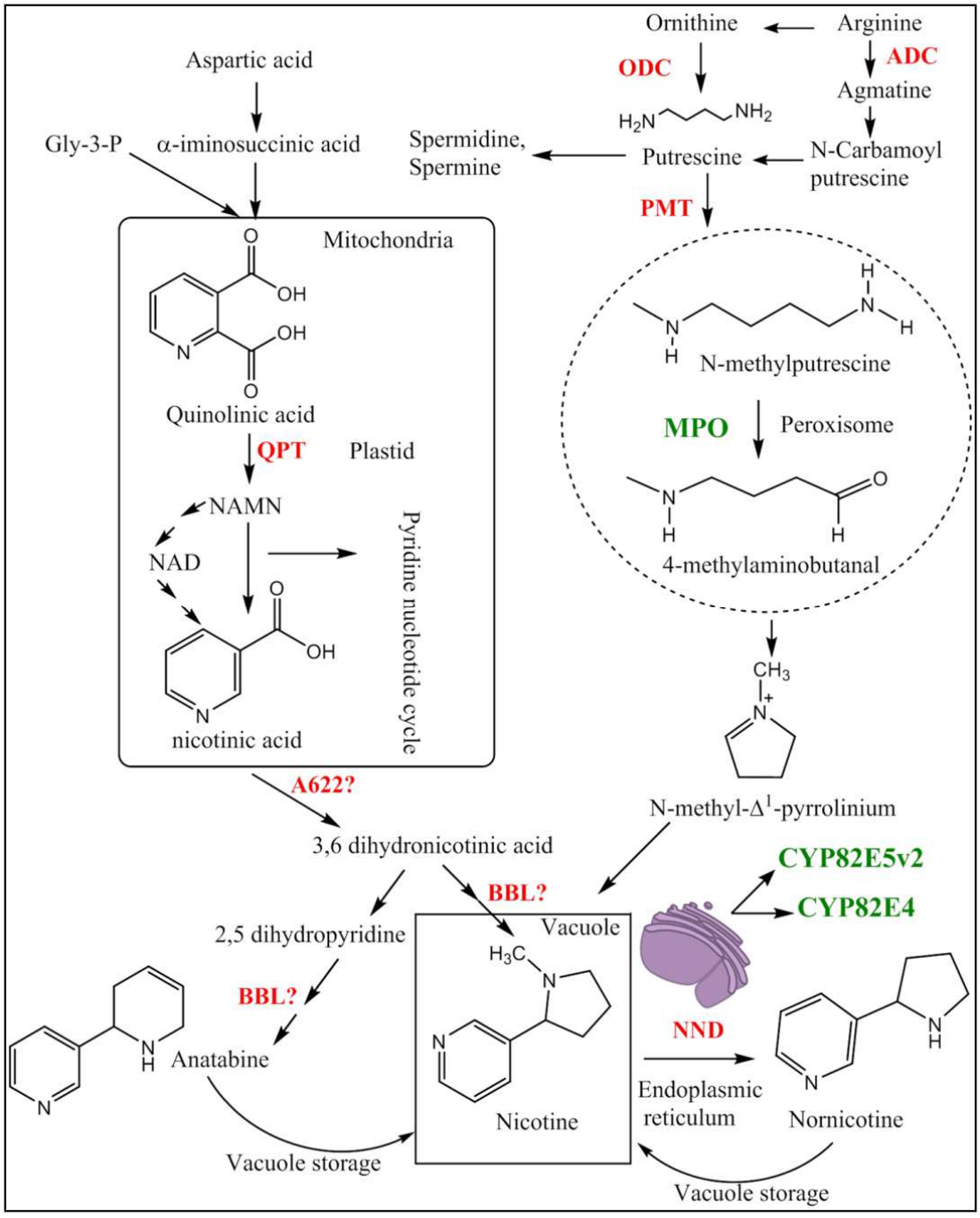


Figure 1.1 Subcellular compartmentalization of nicotine / nornicotine biosynthetic enzymes - modified from (Dewey & Xie 2013). A622: isoflavone reductase-like protein; ADC: arginine decarboxylase; BBL: berberine bridge enzyme-like; MPO: N-methylputrescine oxidase; NND: nicotine N-demethylase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate

phosphoribosyltransferase. Genes that have been overexpressed in the present study are shown in green (From Rajabi et al., 2017).

The basic strategy in this study was to experimentally mimic the situation in a plant tissue by coupling two different cell types: The supernatant generated by a donor cell type is added to a metabolically different receiver cell type. Since the biosynthetic pathway for nornicotine is relatively well understood and most of the enzymes involved in the production of this metabolite are already known (Dewey & Xie 2013), it was possible to generate two different metabolic modules based on genetic engineering of the alkaloid biosynthetic pathway in the tobacco BY-2 model. Since the nicotine alkaloids synthesis is functionally linked with the response to herbivores, it is induced by wounding. Wound signaling is mainly conveyed by jasmonic acid. Therefore, jasmonic acid can be used as an elicitor to activate nicotine alkaloids synthesis (Hashimoto & Yamada 1994; Goossens et al. 2003; Steppuhn et al. 2004; Shoji & Hashimoto 2008). It has been further demonstrated, how modular combination of these transgenic cell strains yields synergy that cannot be achieved by each of the two cell lines alone.

1.3.2 Do cells release their stored natural products in response to nanosecond pulsed electric fields (nsPEFs) treatment?

Plant secondary metabolites are mainly produced intracellular and this is considered as a drawback for downstream processing of a specific natural product. When a natural compound can pass through cell barriers, the purification procedure would be easier and more cost-efficient. Furthermore, continuous release of metabolites from inner organelles such as vacuoles can significantly decrease product inhibition and therefore improve productivity (Chattopadhyay et al. 2002). It is therefore advantageous to take out the products to the culture

medium before the purification of the desired product takes place. Till now, different approaches have been tested for permeabilization of cultivated plant cells resulting in the release of intracellularly stored products. However, the crucial point is that these approaches should not disturb cell growth and viability to enable constant harvesting of products from continuous cultures (Brodelius et al. 1988).

Electroporation is one of the approaches which have been applied in food science mostly for irreversible plasma membrane permeabilization in terms of improvement of extraction yields of biomolecules (e.g. colorants and antioxidants) and fruit juices (Galindo 2016). Classical electroporation uses long pulses (in the range of milliseconds or microseconds) with less intense electric fields to produce hydrophilic pores in the plasma membrane. When cells are exposed to these electric fields, the charged polar head of phospholipids changes direction and thus force the whole phospholipid to change its orientation leading to an increase of cell membrane permeability (Chopinet & Rols 2014; Bai et al. 2017). There are also reports on the use of electroporation for the release of the betacyanin from freely suspended cells of *Chenopodium rubrum* and the release of berberine from freely suspended cells of *Thalietrum rugosum*. However, from those experiments, it appears that it is not possible to release secondary product efficiently without irreversible damage to the cells (Brodelius et al. 1988). Cell membrane permeabilization may be achieved without affecting cell viability through strict control of the electric pulse parameters (Galindo 2016).

Nanosecond pulsed electric fields (nsPEFs) is an improved form of traditional electroporation which applies electric pulses with higher intensity but shorter duration (in the nanosecond range). The mechanism of membrane permeabilization in both is similar, since it is caused by rearrangement of phospholipid molecules in the membrane. However, the main difference is the selective effect of nsPEFs on internal membranes. Simultaneous applying of electric fields

with high intensities and short durations (nanoseconds) can affect also the membrane of inner organelles (Frey et al. 2006; White et al. 2011). Therefore, for efficient downstream recovery of the products in this work, a nanosecond pulsed electric fields (nsPEFs) strategy was tested. To maintain cell viability and allow for the further use of biomass, cells were exposed to high field strength (5-20 $kV.cm^{-1}$) and extremely short duration (in the nanosecond range). This treatment will induce a transient increase of membrane permeability and therefore allows for a mild form of electroporation without affecting cell viability.

2 Materials and Methods

2.1 Plant material, cell culture and elicitation

Plants of *Nicotiana tabacum* cv 'Bright Yellow 2' (voucher KIT 8579), *Nicotiana paniculata* (voucher KIT 0056), *Nicotiana rustica* (voucher KIT 6534), and *Nicotiana tomentosiformis* (voucher KIT 1367) were provided by the Botanical Garden of the Karlsruhe Institute of Technology. Leaves from fully developed plants were collected from these accessions to determine the content of specific alkaloids. The cell strain BY-2 (*Nicotiana tabacum* L. cv Bright Yellow 2 (Nagata et al. 1992) was cultivated in liquid medium containing 4.3 g l^{-1} Murashige and Skoog salts (Duchefa, http://www.duchefa.com), 30 g l^{-1} sucrose, 200 mg l^{-1} KH_2PO_4, 100 mg l^{-1} inositol, 1 mg l^{-1} thiamine, and 0.2 mg l^{-1} (0.9 µM) 2,4-D, pH 5.8. The cells were subcultivated weekly, inoculating 1.0–1.5 ml of stationary cells into fresh medium (30 ml) in 100 ml Erlenmeyer flasks. The cells were incubated at 26 °C under constant shaking on a KS260 basic orbital shaker (IKA Labortechnik, http://www.ika.de) at 150 rpm. Every three weeks, stock calli were subcultured on media solidified with 0.8% (w/v) agar (Roth, http://www.carlroth.com). Suspension cultures and calli of the transgenic strains *Ntab*MPO1ox, *Ntab*CYP82E5*v2*ox and *Ntom*CYP82E4ox were cultivated in the same media as non-transformed wild-type cultures (BY-2 WT), but supplemented with 50 mg l^{-1} kanamycin. Jasmonic acid dissolved in ethanol (OlChemIm, http://www.olchemim.cz/) was used as elicitor and was added to the culture medium to a final concentration of 10 µM or 100 µM. Addition of an equivalent volume of ethanol alone served as solvent control. If not stated otherwise, the samples for alkaloid analysis were collected 3 days after subcultivation.

2.2 Constructs

2.2.1 RNA extraction and cDNA synthesis

2 milliliters of cycling BY-2 WT (3 d after subcultivation, 100 mg of cells) were pipetted onto filter paper to remove the liquid medium. The cells were transferred with a spatula into a 2 ml reaction tube, immediately frozen in liquid nitrogen, and ground with a 5 mm steel bead in a TissueLyser (Qiagen, http://www.qiagen.com). Total RNA was extracted using an RNeasy Plant Mini Kit (Sigma, http://www.sigmaaldrich.com). Optional on-column digestion of genomic DNA was performed with RNase free DNAse I (Sigma) according to the manufacturer's instructions. Purity and integrity of the RNA preparation were checked by electrophoresis. RNA was transcribed into cDNA using the SuperScript® II Reverse Transcriptase (https://www.lifetechnologies.com) with 100 ng of RNA as template.

2.2.1 Cloning procedure

Plasmids for stable and transient transformation of BY-2 WT cells were constructed via Gateway®-Cloning (Invitrogen, http://www.invitrogen.com). The cDNA transcripts encoding *Ntab*MPO1 (Katoh et al. 2007), *Ntab*CYP82E5*v2* (Gavilano & Siminszky 2007) and *Ntom*CYP82E4 (Gavilano et al. 2007) were amplified via PCR (for the sequences encoding the genes of interest and PCR program, see Appendix 5.1, p. 91 and 5.2, p. 93, respectively) using oligonucleotide primers with Gateway®-specific flanks (Appendix 5.3, p. 95). The size of the amplicon was verified by electrophoresis, and amplicons were purified via the NucleoSpin® Extract II kit (Machery-Nagel, http://www.mn-net.com) according to the manufacturer's instructions. The resulting full-length cDNA of *Ntab*MPO1 was inserted into the binary vector pK7WGF2 (Karimi et al. 2002) producing a fusion, where GFP is N-terminal. For

*Ntab*CYP82E5*v2* and *Ntom*CYP82E4, the binary vector pK7FWG2 (Karimi et al. 2002) was used yielding a fusion, where GFP is C-terminal. All inserts were under control of the constitutive CaMV 35S promoter. The sequence of the fusion construct was verified by restriction digest and sequencing (GATC, http://www.gatc-biotech.com). A complete overview of all constructs generated from this study can be found in Appendix 5.4, p. 95.

2.3 Transformation and establishment of stable transgenic tobacco BY-2 cells

2.3.1 Biolistic, transient expression

Gold particles (1.5-3.0 µm; Sigma-Aldrich, http://www.sigmaaldrich.com) were coated with the corresponding plasmid DNA according to the standard manual of Bio-Rad with the following modifications. 12.5 µl (1.5 mg) of gold suspension particles were coated with 1 µg of plasmid-DNA and dispersed throughout the macrocarriers (Bio-Rad, http://www.bio-rad.com/). 800 µl of 3-day-old transformed and non-transformed tobacco BY-2 cells were settled for 5 min in a 2 ml Eppendorf reaction tube. After that, 300 µl of the supernatant was removed. The remaining 500 µl of cells were resuspended and then evenly disseminated onto the PetriSlides™ (Millipore, Billerica, USA) in which 1.5 ml of the above mentioned solid medium for BY-2 calli were inlaid in advance. Both the loaded PetriSlides™ and macrocarriers were transferred into the chamber of the custom-made particle gun according to Finer *et al.* (1992) and bombarded three times at a pressure of 1.5 bar in a vacuum chamber at -0.8 bar. After the bombardment, the cells were first incubated for 16-24 h at 26 °C in darkness and then observed under fluorescence microscope.

2.3.2 Agrobacterium-mediated, stable expression

Stable cell lines overexpressing *Ntab*MPO1, *Ntab*CYP82E5*v2* and *Ntom*CYP82E4 were obtained through a method developed by Buschmann et al. (2011) with several modifications for better performance. 1.5 ml of 7-day-old BY-2 WT cells instead of 1 ml was used during subcultivation and kept for 3 days under the same conditions as normal suspension cell cultures. Afterwards, 3 flasks each containing 31.5 ml suspension cell culture were pooled together and washed twice with 200 ml of washing media (4.3 g l^{-1} Murashige and Skoog salts; (Duchefa Biochemie, https://www.duchefa-biochemie.com/), 10 g l^{-1} sucrose, pH 5.8) each time. The washing steps were performed using a scientific Nalgene® filter holder (Thermo Fischer Scientific) combined with a nylon mesh with pores of a diameter of 70 µm. The washed cells were then resuspended in 15-18 ml of washing medium yielding a 5- to 6- fold concentrated cell suspension. 6 ml of these concentrated cell suspension was mixed with a cell suspension of *Agrobacterium tumefaciens* transformants prepared as follows.

100 µl electro-competent *A. tumefaciens* (strain LBA 4404; Invitrogen Corporation, Paisley, UK) was thawed on ice and incubated with 100 ng binary expression vectors containing the genes of interests on ice for another 20 min. Following that, the mixture was transferred into an electroporation cuvette with 2 mm electrode gap (Peqlab, Erlangen, Germany) pre-cooled on the ice and exposed to electric pulses of 2.5 kV, 200 Ω for 5 ms (Gene Pulser Xcell™ electroporator, Bio-Rad, http://www.bio-rad.com/). After incubation, the bacteria were spread onto solid LB (Lennox Broth, Roth, https://www.carlroth.com) agar medium containing antibiotics (100 µg ml^{-1} rifampicin, 300 µg ml^{-1} streptomycin and 100 µg ml^{-1} spectinomycin) and incubate for 3 days at 28 °C in the dark. One of the single colonies was inoculated into 5 ml LB liquid medium supplied with the same selective antibiotics and incubated at 28 °C agitated vigorously overnight. The OD_{600} of the overnight culture was determined and a certain amount of the overnight culture was inoculated into 5 ml of fresh LB-medium (without

antibiotics) to reach an OD_{600} of 0.15. After approximately 5 h of growth, 6 ml of the transformed *A. tumefaciens* bacteria were harvested at an OD_{600} of 0.8 by centrifugation at 8000 g using the Heraeus Pico 17 Centrifuge (Thermo Fischer Scientific) for 7 min in a 50 ml falcon tube at 28 °C. The bacteria were then resuspended in 180 µl washing medium by mixing vigorously using a bench-top vortexer (Bender & Hobein Zurich, Switzerland) till the suspension becomes homogeneous.

The prepared BY-2 cells were mixed with the homogenized bacteria suspension and incubated on an orbital shaker at a 30° angle above the horizontal at 100 rpm for 5 min till fully mixed. Subsequently, this mixture was dropped with sterile oblique cut tips onto petri dishes inlaid with washing medium solidified with 0.5 % (w/v) Phytagel™ (Sigma-Aldrich) without any antibiotics on which a single layer of sterile filter paper was placed in advance. Those plates were sealed with parafilm and incubated at 22 °C in the dark instead of 27 °C as recommended in the original publication. 3 to 4 days later, the cell plaques together with the filter paper were transferred on to MS agar plates supplied with 300 µg ml^{-1} cefotaxime together with 100 µg ml^{-1} kanamycin and incubated at 26 °C in the dark. After approximately 3 weeks incubation, the appeared calli were transferred onto fresh MS agar plates (with corresponding antibiotics and cefotaxime) for further growth and a suspension culture was then established from the calli after enough of them had grown into appropriate sizes.

2.4 Localisation study of the constructs

For the localization study of *Ntab*MPO1, the peroxisomal mCherry marker PTS1-mCherry (Ching et al. 2012) was transiently introduced by particle bombardment into the background of the *Ntab*MPO1-GFP ox line expressing the enzyme as fusion with GFP. The localization study of *Ntab*CYP82E5*v2* and *Ntom*CYP82E4 was performed by staining the related overexpressing

cell lines with 1 µM ER-Tracker™ Red (glibenclamide BODIPY® TR, Thermo Fisher Scientific) according to the manufacturer's instructions.

2.5 Precursor feeding, combination and co-cultivation experiments

The transformed and non-transformed BY-2 cell suspension cultures were fed with the nornicotine precursor nicotine (Sigma-Aldrich). Nicotine was added to the culture medium at a final concentration of 15 µg ml^{-1}. For combination experiments, medium from *Ntab*MPO1ox or WT cell lines was collected at day 3 after subcultivation, when cells had reached the maximal proliferation activity. This conditioned supernatant (around 25 ml) was mixed with the same volume of fresh medium and 1.5 ml of stationary *Ntom*CYP82E4 overexpressor cells were then inoculated into 30 ml of this mixture in the same way as during standard subcultivation. The conditioned medium was separated from the cells by sterile filtration with autoclaved nalgene devices (Nalgene, http://www.nalgene.com) using a nylon mesh of 40 µm pore width (Nick et al. 2000). For the co-cultivation experiment, an inoculum of 3 ml consisting of 50% from *Ntab*MPO1 and 50% of *Ntom*CYP82E4 cells in 60 ml of fresh medium were used. After 3 days of culture, both cells and medium were analyzed for the abundance of alkaloids.

2.6 Determination of transcript abundance

The abundance of CYPs transcripts was quantified by real-time qPCR analysis. Quality and integrity of extracted RNA were analyzed using spectrophotometry and agarose gel electrophoresis. First-strand cDNA synthesis was carried out from 1 µg total RNA as described above. Primers for real time PCR were designed using the Primer3 software (http://primer3.ut.ee/). The specificity of the amplification was verified by melting curve analysis

and gel electrophoresis, efficiency was determined by analysis of serial cDNA dilution curves. qPCR analysis was carried out in 20 µl reactions containing in final a concentration of 200 nM of each primer, 200 nM of each dNTP, 1X GoTaq colorless buffer, 2.5 mM $MgCl_2$, 0.5 U GoTaq polymerase (Promega, https://www.promega.de/), 1x SYBR green I (Thermo Fisher Scientific), and 1 µl of a cDNA template diluted tenfold (Gutjahr et al. 2008). Each experiment was repeated in three biological replicates, and the mean fold change was calculated and plotted along with corresponding standard deviation values. The cycling conditions comprised 3 min polymerase activation at 95 °C, followed by 40 cycles of strand separation at 95 °C for 15 s, annealing and synthesis at 60 °C for 40 s. Each assay was performed in triplicate. The relative expression of each gene was calculated with the delta delta C_t method (Livak & Schmittgen 2001) using L25 ribosomal protein and elongation factor 1α (EF-1 α) as endogenous controls for normalization (Schmidt & Delaney 2010).

2.7 Alkaloid extraction

Nicotine alkaloids were extracted according to techniques developed by Häkkinen and his colleagues (Häkkinen et al., 2004) with some modifications: To extract tobacco alkaloids, one gram fresh weight of BY-2 cells was dispersed in 2 ml of water. The mixture was basified with 3 ml 3.3% NH_4OH. To release the cell content, cells were lysed by ultrasonication for 2 minutes by means of a high-efficiency ultrasound device (UP 100H, Hielscher, https://www.hielscher.com) pulsed with 0.5 s intervals using an amplitude of 100%. The lysate was spun down for 15 min at 2100 ×g (Z 383 K, Hermle KG, https://www.hermle.de), and the supernatant was collected and extracted with 10 ml dichloromethane. The mixture was incubated for 30 min at ambient temperature on an orbital shaker (150 rpm). Subsequently, the polar dichloromethane layer was separated and collected through a 50-ml separation

funnel. In order to improve the efficiency of extraction, this step was repeated. Precipitated proteins were separated by centrifugation from the collected polar phase at 2100 ×g for 15 min. In the next step, the clear lower phase was concentrated by a rotary evaporator (Büchi® rotary evaporator Model R-205, http://www.buchi.com) under a reduced pressure of 550 millibars and a temperature of 40 °C. After complete evaporation of dichloromethane, the extract was dissolved in 500 µl of 80% (v/v) methanol for HPLC analysis.

2.8 Separation of alkaloids by High-Performance Liquid Chromatography

The Agilent-1200-Series HPLC system equipped with a diode array detector (G1315D), Agilent ChemStation software and a Phenomenex Gemini-NX 5µ C18 110A 150 mm x 4.6 mm column (Phenomenex, http://www.phenomenex.com/) was used at a column temperature of 35 °C and a flow rate of 1.0 ml min^{-1}. The injection volume was 20 µl for cell extracts and 30 µl for medium extracts, peaks were quantified at 260 nm. UV spectra were collected over the wavelength range from 200 nm to 700 nm. Eluent A contained 10% acetonitrile in 20 mM ammonium formate adjusted to pH 8.7, and eluent B consisted of 100% acetonitrile. A gradient program was employed composed of a sequence of linear gradients with an initial step of 100% A to 80% A and 20% B over the first 10 min, followed by a second gradient to 10% A and 90% B over the next 10 min, and a final step to 100% B from 21 min after injection till the end of the run at 30 min after injection (Trehy et al. 2011). The reference alkaloids nicotine, anatabine, anatalline and nornicotine were purchased from Sigma Aldrich (Munich, Germany) and used for sample spiking to verify the identified peaks (Figure 2.1).

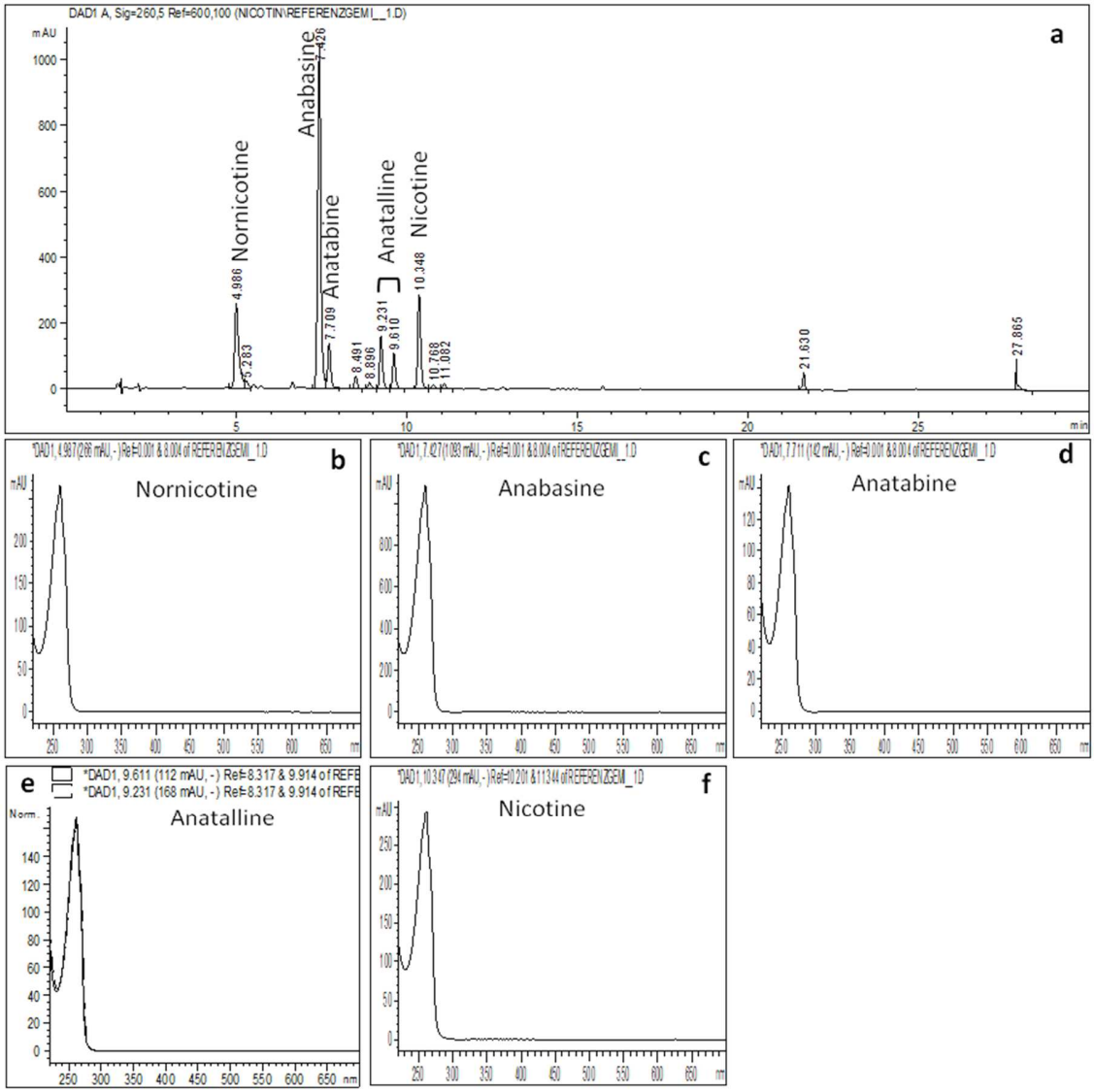


Figure 2.1 High-performance liquid chromatography (HPLC) profile of the reference mixture of nicotinic alkaloids **(**a**)**. Diode array detection (HPLC-DAD; 260 nm) chromatogram of pure standards nornicotine **(b)**, anabasine **(c)**, anatabine **(d)**, anatalline (two isomeric forms) **(e)**, and nicotine **(f)** (From Rajabi et al., 2017).

2.9 Quantitative HPLC analysis of Nicotine alkaloids

To calibrate the instrument response, 10 µl of the standard solution of known concentration of 10, 20, 40, 60, 80 and 100 µg ml^{-1} were injected to the HPLC. A calibration curve based on the

amount and response data (peak area) was obtained for nicotine, anatabine, anatalline and nornicotine separately. A line of best fit (regression line) was used to join the points of the curve obtained. From the calibration curve, a regression analysis yield an equation which describes the line of best fit through the data points, with the form: $y = mx + c$, Where:

y = the peak area

m = the slope of the regression line

c = the intercept of the regression line with the y-axis

Dilution factors were taken into account for the calculation of the final analyte concentration when required. The "correlation coefficient" which is the square root of the regression coefficient indicated that all data points are well fitted to a straight line. There was no large positive or negative number for the intercept of the regression equation which indicates precision and accuracy in sample preparation and analysis. All the calibration curves can be found in Appendix 5.5, p. 97.

2.10 Phenotyping of transgenic cell lines

The mitotic index (MI) of tobacco BY-2 cell suspension cells was determined following fixation with Carnoy fixative and staining with the nuclear dye Höchst 33258 (2'-(4-hydroxyphenyl)5-(4-methyl-1-piperazinyl)-2,5'-bi(1H-benzimidazole)-trihydrochloride, Sigma), as described in Maisch and Nick (2007). Cells were observed and captured using an AxioImager Z1 microscope (https://www.zeiss.de). The images were analyzed using the AxioVision (Rel. 4.8.2) software (Zeiss GmbH). To gain more details of the transformed cells, an Axio Observer Z1 microscope (Zeiss GmbH) in combination with a 63 × /1.44 DIC oil objective and the 488 nm and 561 nm emission lines of the Ar-Kr laser as well as a spinning-disc device (YOKOGAWA CSU-X1 5000) was used. Mitotic indices were determined as the relative

frequency of mitotic cells out of a sample of 500 cells scored for each data point. Cell length and width were determined from the central section of the cells using the length function of the AxioVision software according to Maisch and Nick (2007). Each data point represents the average and standard error from 500 individual cells from three independent experimental series. Cell viability was analyzed by the Evans Blue dye exclusion test (Gaff & Okong'o-Ogola 1971). Aliquots (0.5 ml) from each sample were stained with 0.4% (w/v) Evans Blue solution (Sigma-Aldrich) at a ratio of 1:100 (v/v). After incubation for 3 min, the frequency of the unstained (viable) cells was determined as well as the cell number per ml using a hematocytometer (Fuchs-Rosenthal) under bright-field illumination. For each individual sample, 1000 cells were scored. Division synchrony, MI, cell length and width were observed to be unaffected by kanamycin selection as verified by comparison with negative controls cultivated in the absence of the antibiotics. The average length of the cell cycle was estimated from the time course of cell density estimated by a hematocytometer (Fuchs-Rosenthal), using an exponential model for proliferation ($N_t=N_0 \cdot e^{kt}$ with N_t cell density at time point t, N_0 cell density at inoculation, and k the time constant). In order to set the reference, the starting number (N_0) was quantified just after subcultivation.

2.11 Nanosecond pulsed electric fields (nsPEFs)

2.11.1 Basic experimental setup for nsPEFs treatment

The BY-2 cell suspensions were treated in the treatment chamber, shown in Figure 2.2. The chamber was constructed for continuous flow treatment of biomass suspensions and manufactured at the Institute of Pulsed Power and Microwave Technology (Karlsruhe Institute of Technology, Germany). The details about the pulsed generator system have been reported in previous works of Eing et al. 2009 and Goettel et al. 2013 and can be found in Figure 2.3.

Thirty ml of cells were treated with nsPEFs in a treatment chamber. The custom-made treatment chamber consisted of two stainless steel electrodes with a diameter of 60 mm, oriented in parallel and separated by a gap integrated into a transparent polycarbonate housing. The cell suspensions passed the treatment chamber from the bottom via sterilized silica rubber tubing driven by a peristaltic pump (Ismatec Ism 834C, Switzerland) at a constant flow rate of 5.3 ml min^{-1}. After passing the outlet at the top of the chamber, the suspension was collected in an empty, sterilized 100-ml Erlenmeyer flask sealed by a silicone plug. In this work, the transmission line pulse generator delivered rectangular pulses with a voltage amplitude of 2 kV, corresponding to an electric field strength of 5 kV cm^{-1}. The distance between the electrodes is 4 mm and the treatment volume is 2 ml. The detailed experiment set up for nsPEFs treatment is shown in Figure 2.4.

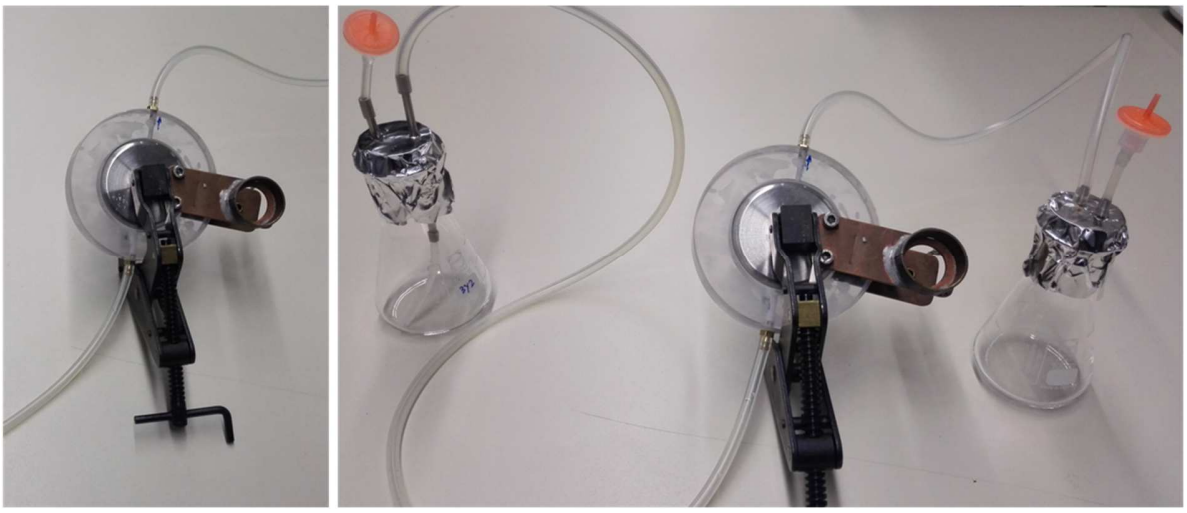


Figure 2.2 Flow-cell for nsPEFs treatment of BY-2 cell suspensions; volume 2 ml; gap between electrodes 4 mm.

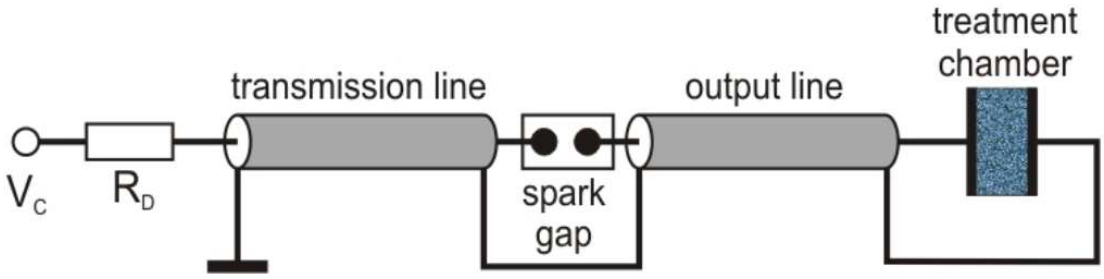


Figure 2.3 Schematic of the transmission line generator for nsPEFs (Eing et al. 2009).

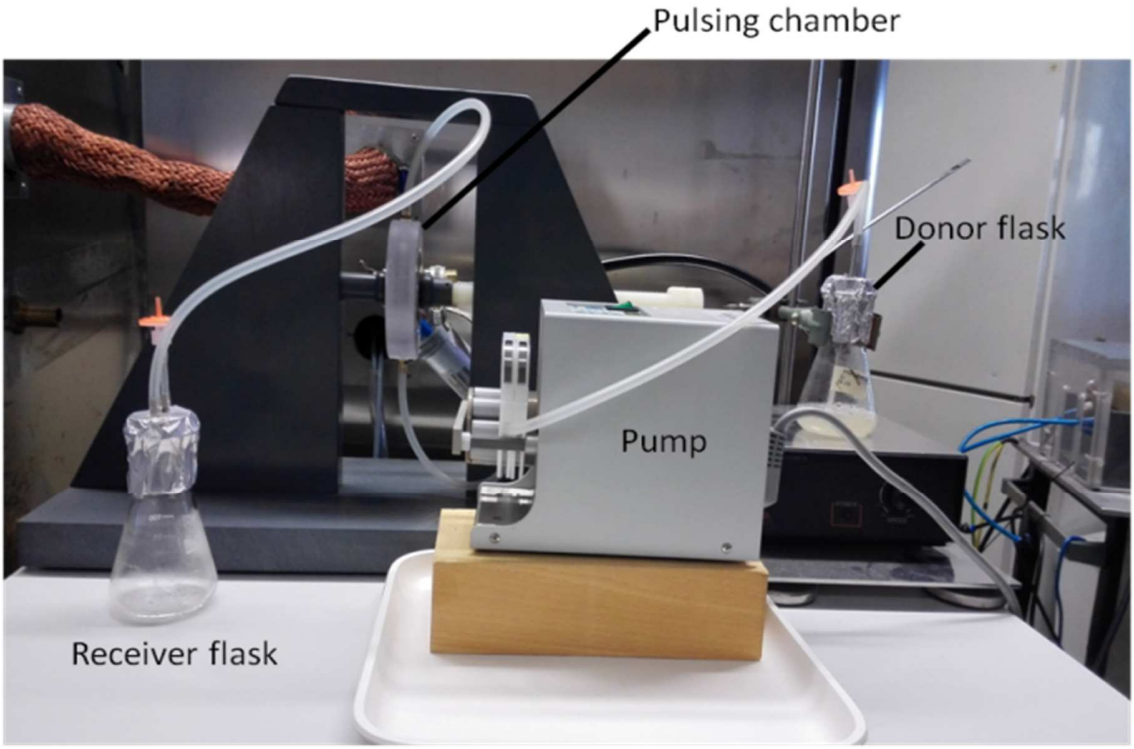


Figure 2.4 Experimental setup; device for continuous flow nsPEF treatment. The cell suspension is pumped from the donor flask, the suspension is then passing through the pulsing chamber where cells experience the nsPEFs treatment and are collected in the receiver flask.

2.11.2 Experiment setup and nsPEFs parameters

To investigate whether nsPEFs can stimulate secretion of the desired alkaloids without affecting cell viability and cell growth, tobacco BY-2 cells were used. Non-transformed cells (wild type), *Ntab*MPO and *Ntom*CYP82E4 overexpression cell lines were pulsed with parameter sets defined as pulsing indices (PI) 1-3 for a general overview of the response to nsPEFs. For further investigations, PI 3 was chosen as optimal parameters set.

Table 2.1 shows the applied parameter sets for these indices. Pulsing index 0 represents control treatment with no nsPEF application. When many samples had to be treated, the experiment was repeated sequentially and the cells were kept on an orbital shaker within an incubator at 25 °C in dark at 150 rpm prior to sample preparation to ensure that potential effects of environmental factors were as constant as possible. Both short-term (over the first 2 hours after the nsPEFs treatment), and long-term (over 24 hours after the nsPEFs treatment) responses were investigated. To follow the short term responses, 3 days old cell suspensions were pulsed and cell viability, intracellular and secreted alkaloids were measured immediately afterward. In order to observe the long-term responses to nsPEFs treatment, pulsed samples were transferred back to the incubator in their normal condition and both parameters of viability and alkaloid contents were quantified at 24 hours after nsPEFs treatment. As a negative control, the suspension was cultivated and treated the same way, just omitting the application of nsPEFs.

Due to the location of the pulse generator at the KIT Campus North, the cell culture had to be transported back between KIT Campus South and North by the shuttle bus. To protect the cultures from cooling, they were carried in a polystyrene foam box during the transport that lasted half an hour before and after pulse treatment, respectively. Due to the season (late autumn and winter), the temperature during transport was certainly lower than 25 °C.

Pulsing Index [PI]	Field strength [kV cm^{-1}]	Pulsed duration [ns]	Conductivity [mS cm^{-1}]	Flow rate [ml s^{-1}]	Frequency [Hz]	Pulse number	Imput energy [J g^{-1}]
0	-	-	-	-	-	-	-
1	5	25	4	0.0892	2	45	0.112
2	5	50	4	0.0892	0.25	6	0.667
3	**5**	**100**	**4**	**0.0892**	**0.5**	**11**	**0.112**

Table 2.1 Pulsing parameters including field strength, pulse duration, conductivity, flow rate, frequency, number of pulses and electric strength applied in the experiments using WT, *Ntab*MPO and *Ntom*CYP overexpressor BY-2 cell lines. These parameter sets were taken for a general overview on the response to nsPEFs. For further investigations, PI 3 was taken.

3 Results

In this chapter, the results from this work will be presented in two main sections as established in the beginning. The first part deals with the production of metabolically different cell types by the use of metabolic engineering and genetic engineering approaches which can be categorized into three parts. Firstly, the generation of stable transgenic cell lines and microscopic studies examining the transgenic cell lines. Secondly, quantitative phenotyping of overexpressed cell lines to detect potential side effects of the overexpression by using viability, mitotic index, cell elongation (in the sense of proportionality, assessed as ratio of cell length over width), and cycling time as parameters. Lastly, metabolic studies analyzing the effect of the overexpression on nicotine alkaloid contents of the transgenic lines compared to the wild type BY-2 cells. Following that, the metabolic interaction of the different plant-cell based metabolic modules was studied using the so called conditioned medium and co-cultivation strategies. The second section investigates an alternative strategy called nanosecond pulsed electric fields (nsPEFs) for efficient downstream recovery of the products accumulated within the cells. This chapter finally ends with a brief summary of the findings from this work.

3.1 Definition of molecular targets for stimulated nornicotine synthesis

In order to design a strategy for metabolic engineering of nornicotine, this work focused on MPO1 and CYP82E as rate-limiting enzymes determining the production of nicotine and nornicotine, respectively (Figure 1.1). Nicotine biosynthesis requires an oxidative deamination of N-methylputrescine, catalyzed by N-methyl putrescine oxidase (MPO1) [GenBank: AB289456.1] (Katoh et al. 2007). The conversion of nicotine to nornicotine involves *N*-

demethylation of nicotine, catalyzed by different isotypes of a cytochrome P_{450} enzyme (Siminszky et al. 2005; Gavilano & Siminszky 2007; Cai et al. 2012). Two alleles of this gene, CYP82E, were tested: *Ntab*CYP82E5*v2* [GenBank: EU182719.1] is found in green leaves of *Nicotiana tabacum*, a species with low nornicotine levels, whereas *Ntom*CYP82E4 [GenBank: EF042307.1] originates from *Nicotiana tomentosiformis*, a strong converter of nicotine to nornicotine (Gavilano & Siminszky 2007; Dewey & Xie 2013).

3.2 Overexpression of MPO1 promotes nicotine accumulation but does not lead to nornicotine

Since low expression of MPO1 is the main reason for the deficiency of tobacco BY-2 cells to synthesize nicotine efficiently, overexpression of MPO1 represents a strategy to boost nicotine formation (Shoji & Hashimoto 2008b). To stimulate the production of nicotine, the native tobacco BY-2 MPO1 cDNA was expressed in fusion with GFP as a reporter under the control of the cauliflower mosaic virus (CaMV) 35S promoter in tobacco BY-2 cells using transformation via *Agrobacterium tumefaciens* using kanamycin as a selection marker. The resulting *Ntab*MPO1-GFPox cell line exhibited punctuated signals suggesting that the GFP fusion of this enzyme was located in small organelles. To clarify the subcellular localization of MPO1, a peroxisome-targeted mCherry marker (PTS1-Cherry) (Ching et al. 2012) was transiently introduced into the *Ntab*MPO1-GFPox cell line. Since MPO1 was shown to be targeted to peroxisomes in epidermal pavement cells of *Nicotiana benthamiana* (Naconsie et al. 2014), the peroxisomal mCherry marker PTS1-mCherry (Ching et al. 2012) was transiently introduced by particle bombardment into the background of the *Ntab*MPO1-GFP ox line expressing the enzyme as fusion with GFP. The tight colocalization of the mCherry and the

GFP signal (Figure 3.1) provides evidence for a correct localization of the overexpressed MPO1.

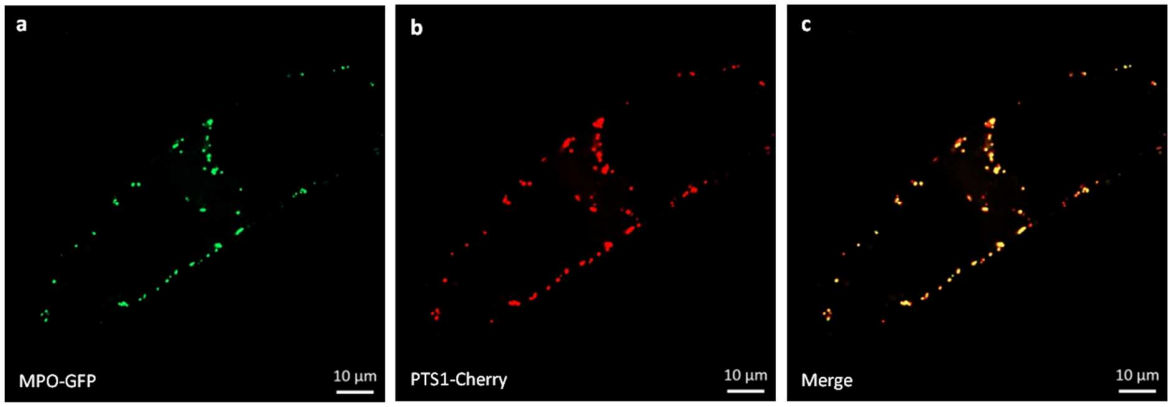


Figure 3.1 Colocalization of NtabMPO1-GFP and of PTS1-mCherry in BY-2 cells overexpressing NtabMPO1-GFP showed peroxisome localization of NtabMPO1. NtabMPO-GFP fluorescence **(a)** PTS1-mCherry fluorescence **(b)** and merged fluorescence of a and b **(c)** Orange color indicates areas where the images overlap and where the two markers colocalize (Bars = 10 µm) (From Rajabi et al., 2017).

To detect potential side effects of the overexpression, the BY-2 MPO1-GFP ox line was monitored by quantitative phenotyping, using viability, mitotic index, cell elongation (in the sense of proportionality, assessed as ratio of cell length over width), and cycling time as parameters. The phenotyping results did not detect any significant difference, neither in cell viability, nor with respect to mitotic index, or cell elongation compared with non-transformed BY-2 cells (Figure 3.2 a, b, c). Since mitotic index cannot only increase as consequence of stimulated proliferation but also by delayed or arrested progression through mitosis, the duration of the cell cycle was quantified in addition. The doubling time can be inferred from the time course of cell density based on the model of exponential growth (Figure 3.2 d, e). From this approach, a longer cell cycle was inferred for the *Ntab*MPO1-GFP ox line compared to the

non-transformed BY-2 cells (WT). This delay in the *Ntab*MPO1 overexpressing line (from 28.0 h in the WT to 32.9 h in the overexpressor) was minor, however.

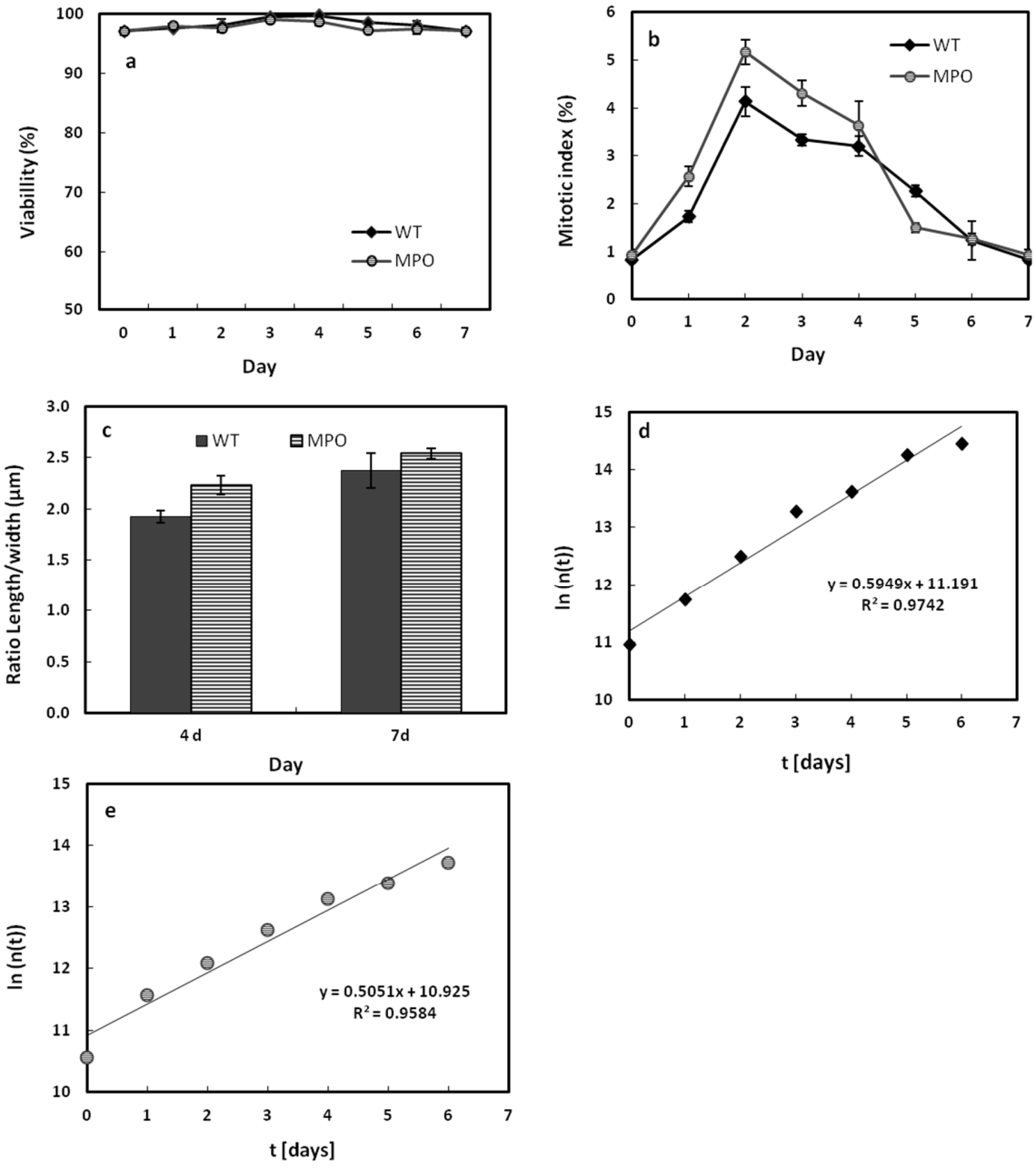


Figure 3.2 Phenotyping and alkaloid accumulation patterns for BY-2 cells overexpressing *Ntab*MPO1 compared to non-transformed BY-2 cells (WT). **(a)** cell viability (mean of n=1000), **(b)** mitotic index

(mean of n=1000), **(c)** cell elongation as ratio of cell length over cell width at day 4 and 7 (mean of n=500), **(d, e)** from the time course of cell density a cell cycle duration of 27.96 h for the non-transformed BY-2 cells **(d)** and of 32.9 h for BY-2 cells overexpressing *Ntab*MPO1 **(e)** can be inferred. All experimental data are derived from three independent experimental series; error bars = SE (From Rajabi et al., 2017).

Despite the negligible impact of the overexpressed MPO on cellular physiology, the alkaloid profile was substantially changed after elicitation with jasmonic acid (Figure 3.3).

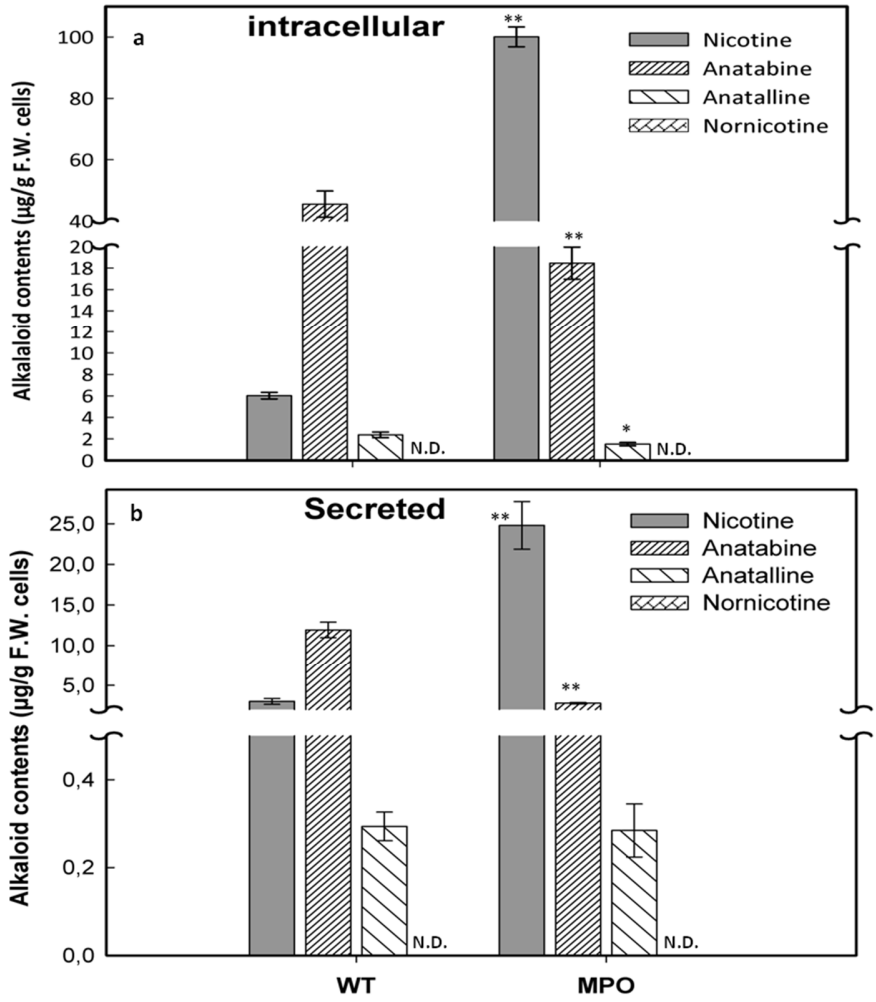


Figure 3.3 Alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing MPO after 3 days of culture in presence of 100 µM jasmonic acid either intracellularly **(a)** or secreted to

the medium **(b)**. The levels of nornicotine were below detection limit (indicated by N.D.). Note the difference in scales between **(a)** and **(b)**. For the alkaloid measurement, mean and SE are shown from six independent experimental series. Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

Preparatory experiments had shown that elicitations with 10 µM of jasmonic acid (JA) were only inducing a modest accumulation of alkaloids over the experimental period of 3 days (Figure 3.4). However, by increasing the concentrations to 100 µM, the levels of alkaloids could be increased by a factor of 4 in case of nicotine, and more than 20 in case of anatabine (Figure 3.3 a) compared to the treatment with 10 µM JA (Figure 3.4). Under this optimised elicitation protocol, it was observed that the intracellular content of nicotine was more than 15 times higher in the MPO overexpressor compared to the non-transformed wild type (Figure 3.3 a). In contrast, the levels of anatabine (produced by the concurrent branch of the pathway) were decreased by around 3 times in the MPO overexpressor. Only residual levels of anatalline were measured, and the levels of nornicotine remained below the detection limit. When the secreted alkaloids were analyzed (Figure 3.3 b), again a strong stimulation of nicotine and anatabine secretion was found in the MPO overexpressor and WT cells, respectively, whereas the anatalline was comparable to the wild type. The general abundance of secreted alkaloids was around four times lower than those present within the cells indicating that only around 20% of the induced alkaloids were secreted.

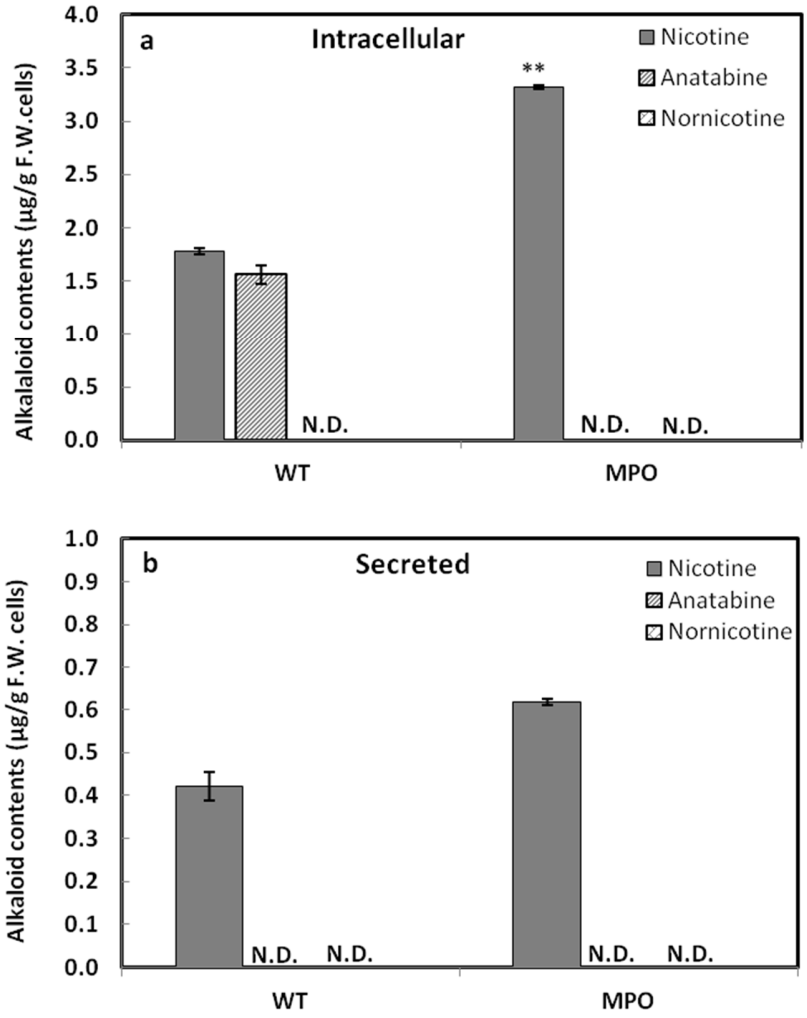


Figure 3.4 Alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing *Ntab*MPO1 (MPO) after 3 days of culture in presence of 10 µM jasmonic acid either intracellularly (**a**) or secreted to the medium (**b**). The levels of nornicotine and in some case anatabine were below detection limit (indicated by N.D.). Note the difference in scales between **(a)** and **(b)**. For the alkaloid measurement, mean and SE are shown from six independent experimental series. Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

3.3 Overexpression of the nicotine demethylase *Ntab*CYP82E5*v2* does not lead to nornicotine

Nornicotine is principally synthesized by enzymatic nicotine *N*-demethylation, catalyzed by nicotine *N*-demethylase (NND), and mostly accumulates in the senescing leaves of specific tobacco species, so called converter species of the genus *Nicotiana*. However, low levels (2–5% of total alkaloid) of nornicotine can also be detected in green leaves of non-converter plants (Siminszky et al. 2005). The enzyme underlying NND has been proposed to belong to the CYP82E clade of cytochrome P_{450} proteins. The residual NND activity in the green leaves found in the non-converter plant *N. tabacum* has been linked with *Ntab*CYP82E5*v2* (Siminszky et al. 2005; Gavilano & Siminszky 2007; Cai et al. 2012).

Therefore, a transformed BY-2 cell line overexpressing the native enzyme, *Ntab*CYP82E5*v2*, was produced, which was originated from BY-2 in fusion with GFP under control of the CaMV 35S promoter. The GFP signal was organized in a reticulate pattern. To verify, whether this pattern was caused by a localization in the endoplasmic reticulum, a rhodamine labeled version of the ER tracker was used and a tight colocalization of both signals was observed (Figure 3.5).

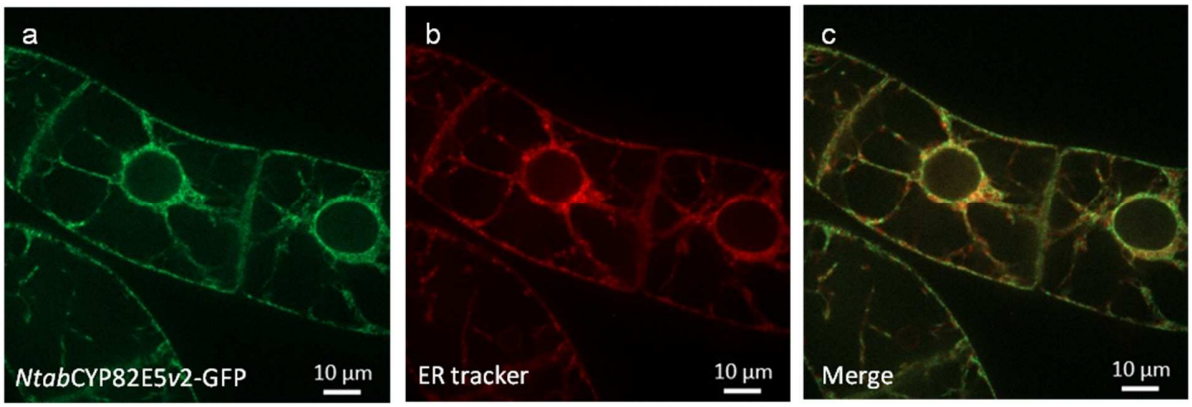


Figure 3.5 Colocalization of *Ntab*CYP82E5*v2*-GFP and of rhodamine-conjugated ER-tracker in BY-2 cells overexpressing *Ntab*CYP82E5*v2*-GFP showed endoplasmic reticulum localization of *Ntab*CYP82E5*v2*. **(a)** *Ntab*CYP82E5*v2*-GFP signal, **(b)** rhodamine-conjugated ER-tracker, **(c)** merged signal of both channels showing the tight colocalization of the nicotine demethylase *Ntab*CYP82E5*v2* GFP with the endoplasmic reticulum. (Bars = 10 µm).

It was further investigated, whether the overexpression of *Ntab*CYP82E5v2-GFP would alter cellular physiology. But neither cell viability nor cell proportionality showed any significant differences between *Ntab*CYP82E5*v2*ox and the WT cells (Figure 3.6). Only a minor increase in the progression of the mitotic index accompanied by a small acceleration of the cell cycle by around 5% could be noted.

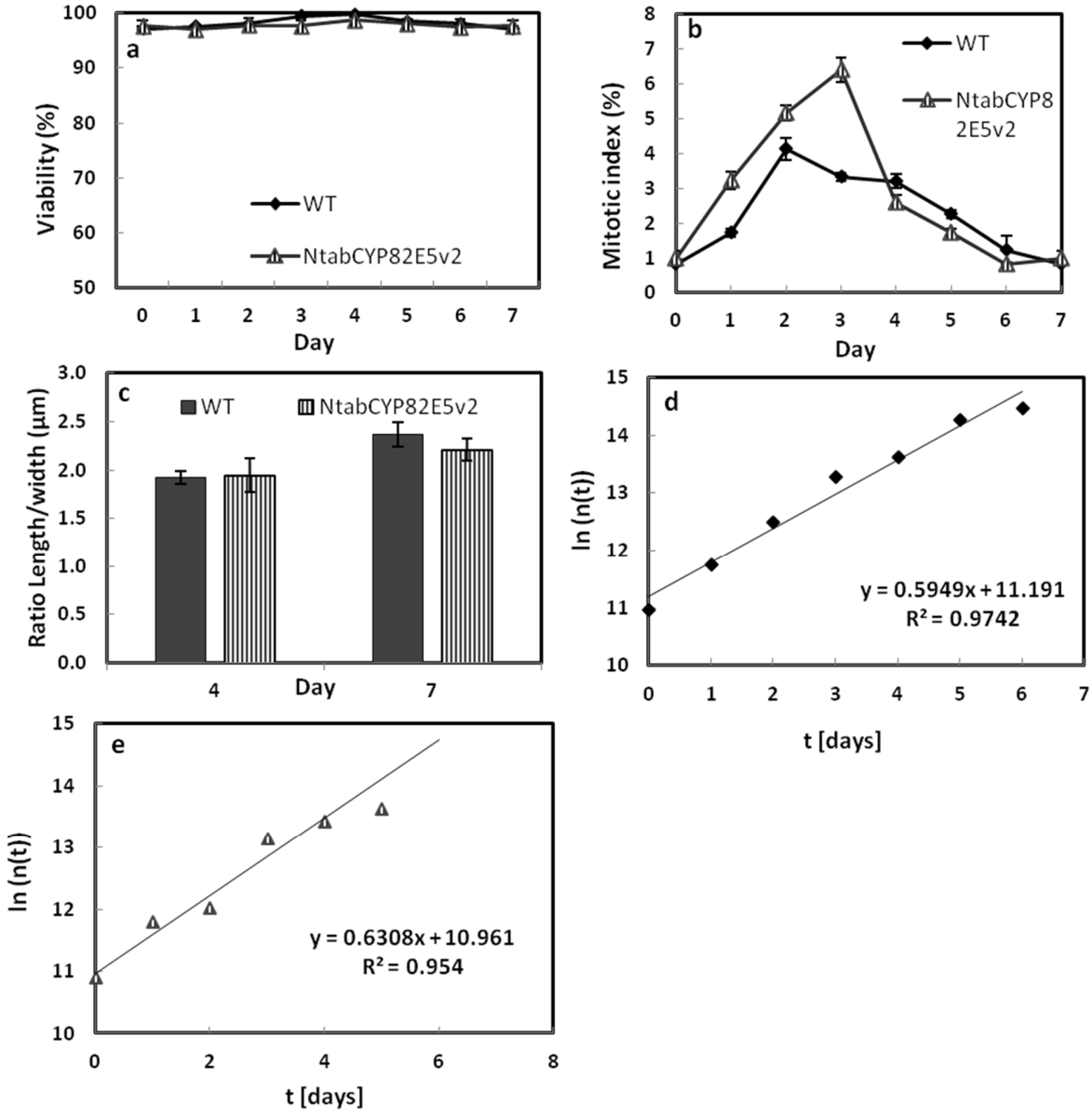


Figure 3.6 Phenotyping of BY-2 cells overexpressing *Ntab*CYP82E5v2 compared to non-transformed BY-2 cells (WT). **(a)** Cell viability (mean of n = 1000), **(b)** Mitotic index (mean of n = 1000), **(c)** Cell elongation as ratio of cell length over cell width at day 4 and 7 (mean of n = 500), **(d, e)** From the time course of cell density a cell cycle duration of 28.0 h for the non-transformed BY-2 cells **(d)** and of 26.4 h for BY-2 cells overexpressing *Ntab*CYP82E5v2 **(e)** can be inferred. All experimental data are derived from three independent experimental series; error bars = SE (From Rajabi et al., 2017).

Although the overexpressed nicotine demethylase *Ntab*CYP82E5*v2* did not show any impact on cellular physiology, the alkaloid profile was substantially changed after elicitation with 100 µM of JA (Figure 3.7): Although there were no significant differences in intracellular alkaloids between nicotine demethylase overexpressor and the non-transformed wild type (Figure 3.7 a), significantly less nicotine and anatabine were secreted by nicotine demethylase overexpressor cells (Figure 3.7 b). However, neither the intracellular nor the secreted nicotine reached the levels found in the MPO1 overexpressor line (compare with Figure 3.3, p. 39). The effect was even more pronounced for anatabine, where the intracellular steady-state levels were almost the same in the nicotine demethylase overexpressor compared to the wild type, but nevertheless significantly lower amounts were secreted. Against the expectation, the overexpression of *Ntab*CYP82E5*v2* did not yield any detectable nornicotine, neither in intracellular nor secreted form (Figure 3.7). Again, preparatory experiments were conducted with 10 µM of JA (Figure 3.8) yielding a different pattern compared to 100 µM JA, and generally much lower induction of alkaloids. Both, intracellular and secreted nicotine and anatabine levels were significantly higher in the nicotine demethylase overexpressor compared to the wild type. One detail was interesting, however: The intracellular levels of nicotine and anatabine were already significantly elevated over those seen in the wild type at these low concentrations of the elicitor, whereas for 100 µM JA the wild type had the same (nicotine) or higher (anatabine) levels. This indicates a higher sensitivity of alkaloid synthesis in the nicotine demethylase overexpressor. Similar to the findings for the MPO1 overexpressor, the general abundance of secreted nicotine was about 4 times lower than those present within the cells and for anatabine around one order of magnitude lower than those present within the cells indicating that a maximum around 10% of the induced alkaloids were secreted.

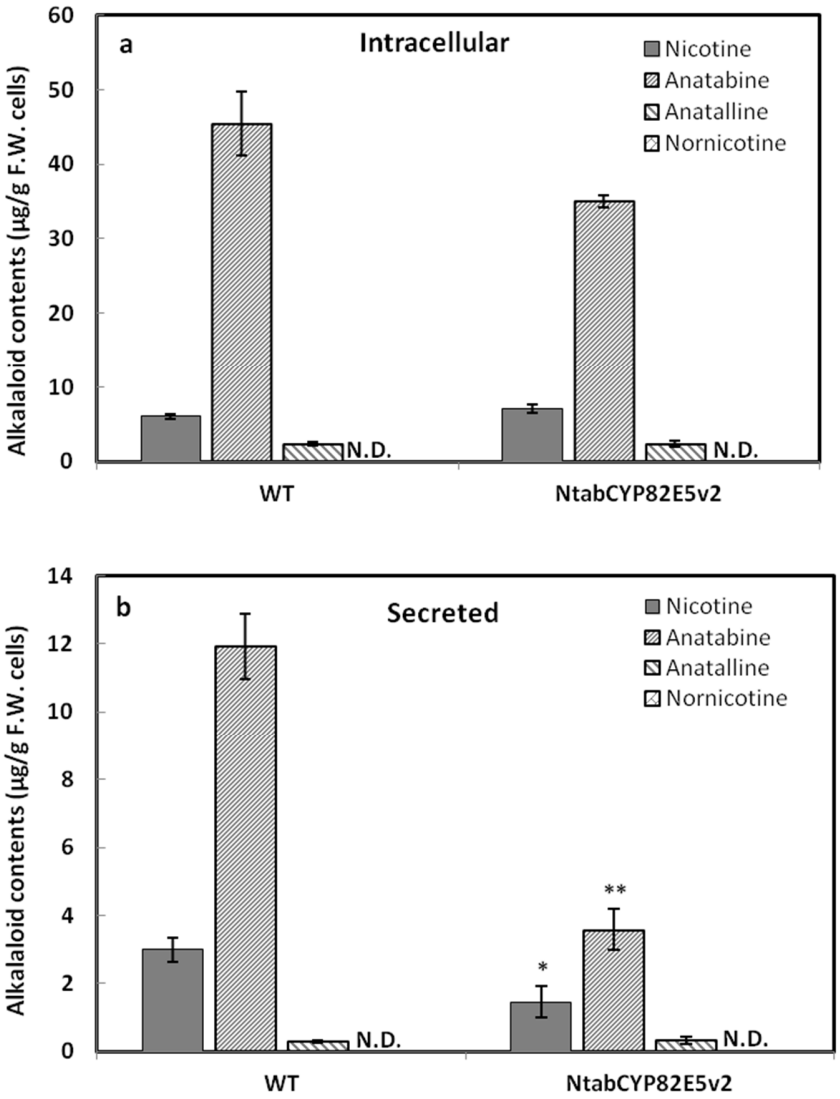


Figure 3.7 Alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing *Ntab*CYP82E5*v2* after 3 days of culture in presence of 100 µM jasmonic acid either intracellularly **(a)** or secreted to the medium **(b)**. The levels of nornicotine were below detection limit (indicated by N.D.). Note the difference in scales between **(a)** and **(b)** (the level of secreted alkaloids is in some case around tenfold lower). For the alkaloid measurement, mean and SE are shown from six independent experimental series. Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

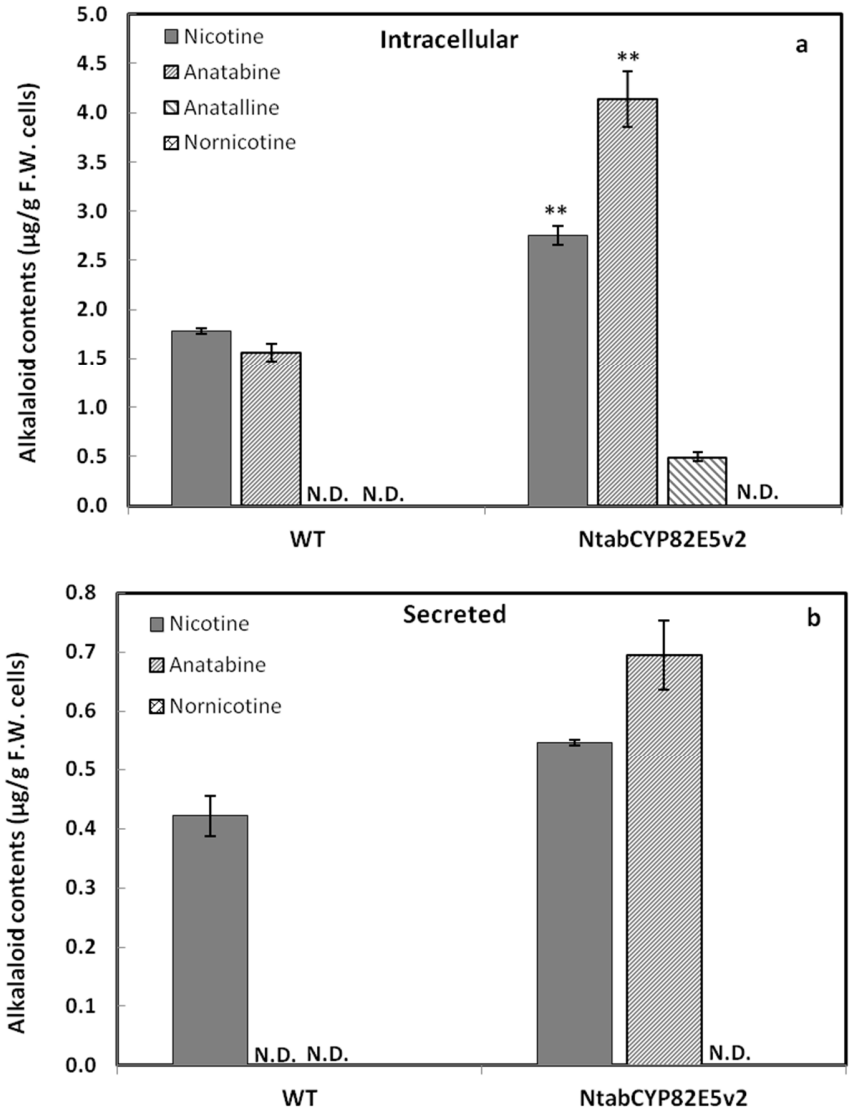


Figure 3.8 Alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing *Ntab*CYP82E5*v2* after 3 days of culture in presence of 10 µM jasmonic acid either intracellularly **(a)** or secreted to the medium **(b)**. The levels of nornicotine and in some cases anatabine and anatalline were below detection limit (indicated by N.D.). Note the difference in scales between **(a)** and **(b)**. For the alkaloid measurement, mean and SE are shown from six independent experimental series. Significant differences to the non transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively.

3.4 Overexpression of a nicotine demethylase from a converter species leads to nornicotine

Since the overexpression of the native BY-2 nicotine demethylase *Ntab*CYP82E5*v2* failed to generate nornicotine, it was of great importance to compare different genotypes of *Nicotiana* for their ability to accumulate nornicotine in senescent leaves (Figure 3.9). Whereas the cultivar *N. tabacum* cv. Bright Yellow 2, the background for the BY-2 cell line, accumulated high levels of nicotine, the leaves were almost void of nornicotine. A similar pattern was observed for the species *N. rustica*. In contrast, *N. paniculata* and *N. tomentosiformis* were found to produce relatively high levels of nornicotine. In case of *N. tomentosiformis*, most of the nicotine was converted to nornicotine. This indicates that this species harbors a very efficient version of nicotine demethylase.

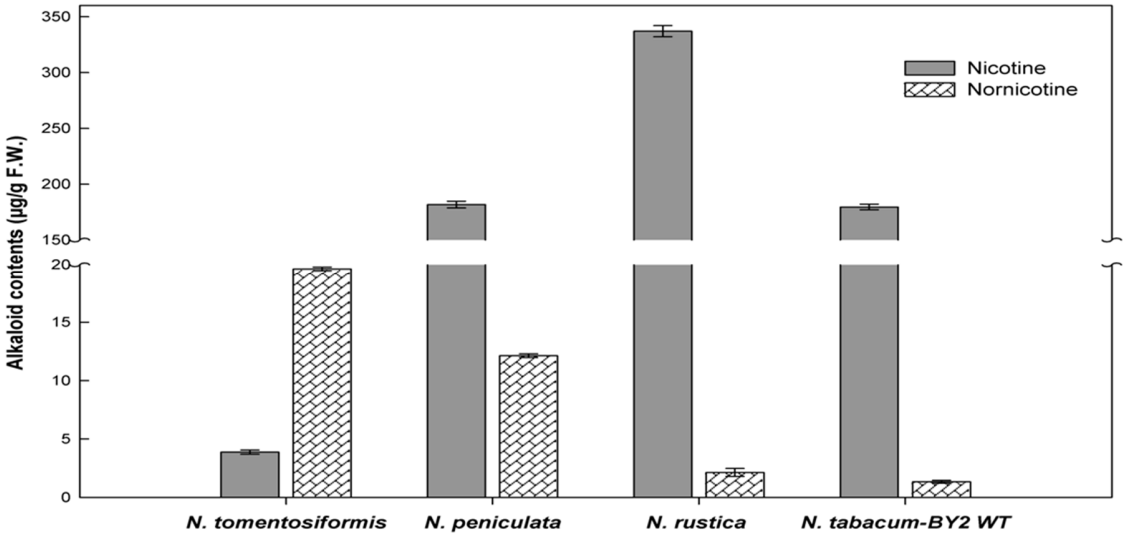


Figure 3.9 Nicotine and nornicotine contents in senescent leaves of different *Nicotiana* species. The low ratio of nornicotine to nicotine in *N. tabacum* and the high level of nornicotine compared to nicotine in *N. tomentosiformis* are evident. Error bars represent SE from three independent experiments (From Rajabi et al., 2017).

For this reason, the nornicotine demethylase *Ntom*CYP82E4 from this converter species was cloned and this gene overexpressed in fusion with GFP in BY-2 cells to test, whether this cell line would be able to produce nornicotine. Similar to the native BY-2 nicotine demethylase *Ntab*CYP82E5*v2*, the *Ntom*CYP82E4-GFP fusion protein was localized in the endoplasmic reticulum (Figure 3.10) and the physiology of this overexpressor line was completely normal with respect to viability and cell proportionality (Figure 3.11). In addition, similar to the *Ntab*CYP82E5*v2*-GFP overexpressor, the cell cycle was accelerated slightly (by around 5%).

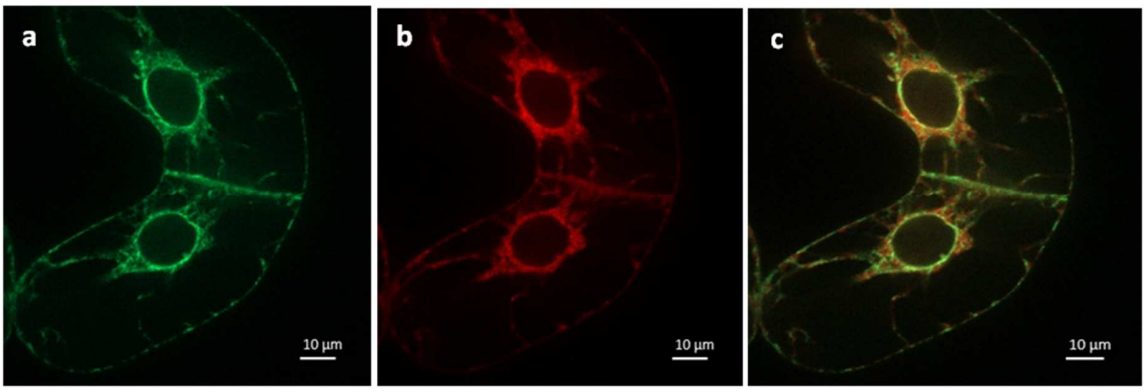


Figure 3.10 Localization study of overexpressed *Ntom*CYP82E4-GFP. **(**a**)** *Ntom*CYP82E4-GFP signal, **(**b**)** Rhodamine-conjugated ER-tracker, **(**c**)** merged signal of both channels showing the tight colocalization of the nicotine demethylase *Ntom*CYP82E4-GFP with the endoplasmic reticulum (From Rajabi et al., 2017).

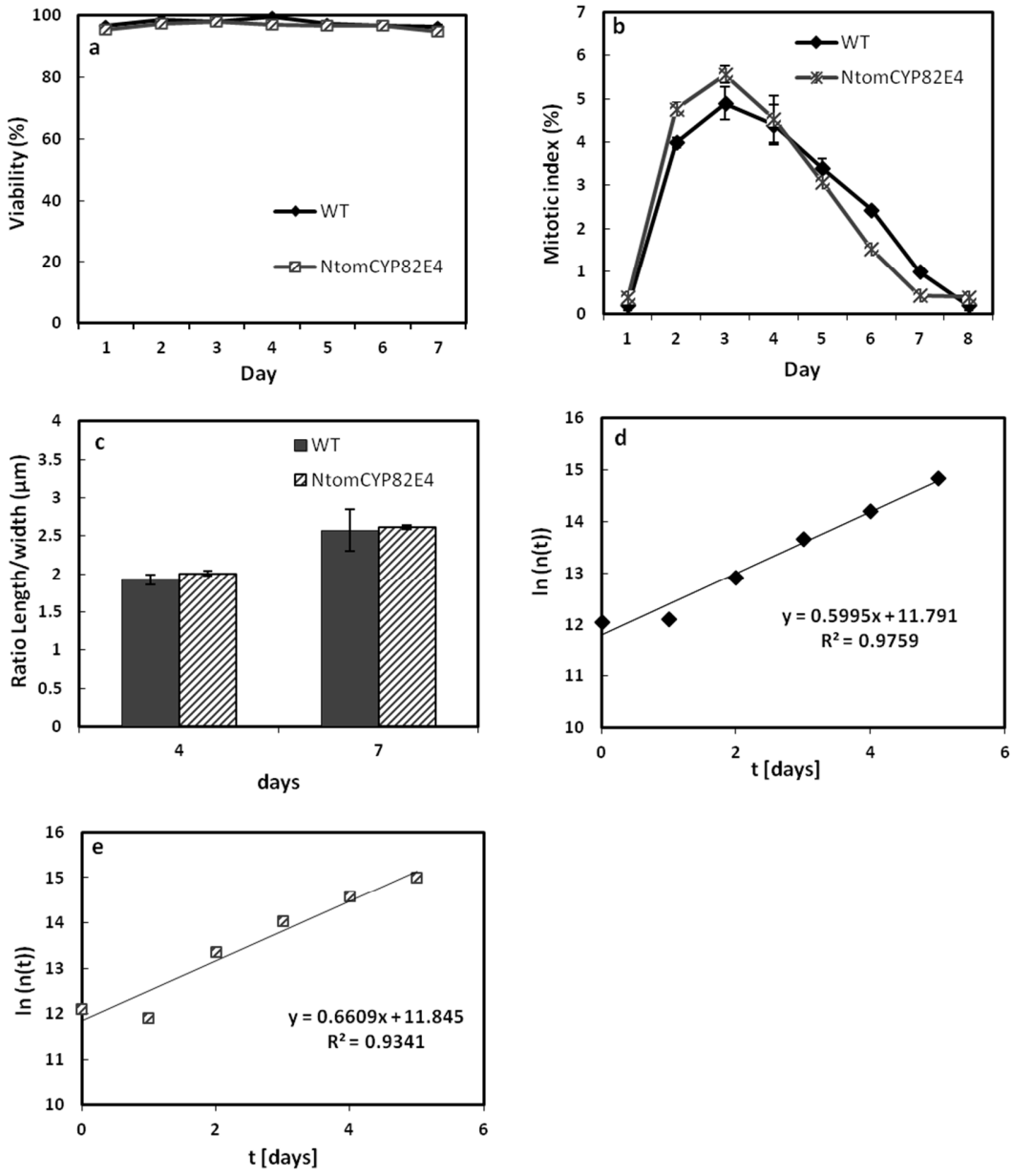


Figure 3.11 Physiological impact of overexpressed *Ntom*CYP82E4-GFP. **(**a) Cell viability (mean of n = 1000), **(b)** Mitotic index (mean of n = 1000), **(c)** Cell elongation as ratio of cell length over cell width at day 4 and 7 (mean of n = 500), **(d, e)** From the time course of cell density a cell cycle duration of 27.2 h for the non-transformed BY-2 cells **(d)** and of 25.2 h for BY-2 cells overexpressing *Ntom*CYP82E4 **(e)** can be inferred. All experimental data are derived from three independent experimental series; error bars = SE (From Rajabi et al., 2017).

When the alkaloid accumulation in response to 100 µM of JA was investigated in this line overexpressing the nicotine demethylase from a converter species, nornicotine was observed for the first time (Figure 3.12 a). However, the level of nornicotine was still modest, and it seemed to be sequestered in the cells, since no secreted nornicotine could be detected (Figure 3.12 b). Compared to the non-transformed wild type, the nicotine demethylase overexpressor accumulated less nicotine. This reduced intracellular accumulation was accompanied by a significant decrease in the fraction of secreted nicotine and anatabine.

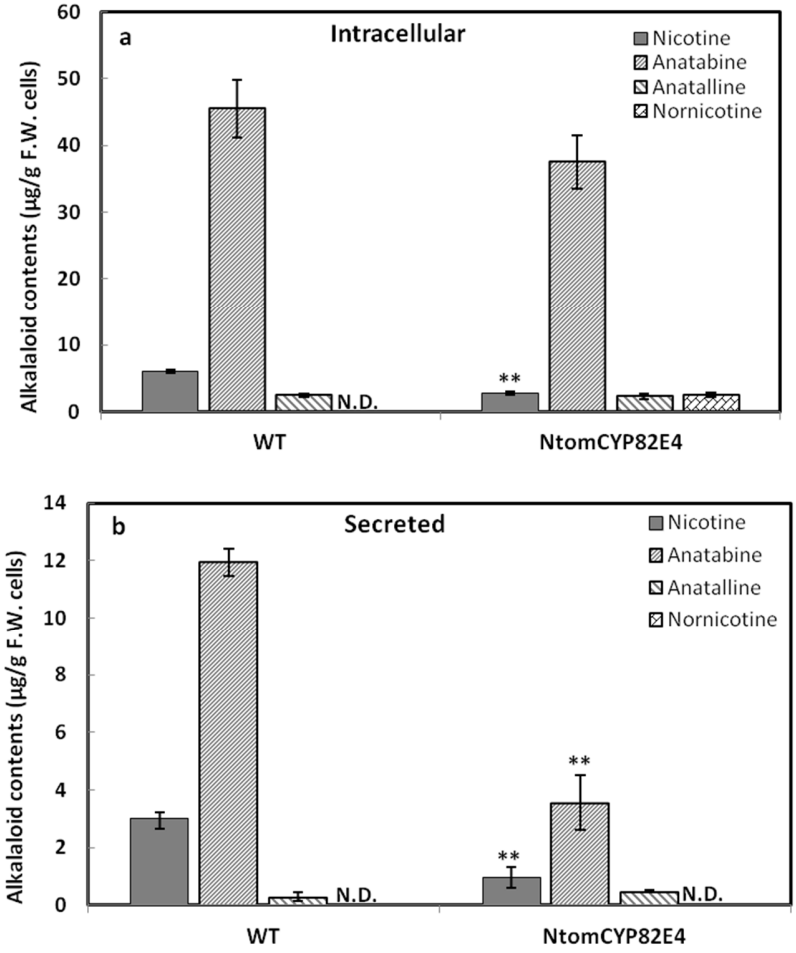


Figure 3.12 Alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing *Nom*CYP82E4 after 3 days of culture in presence of 100 µM jasmonic acid either intracellularly **(a)** or secreted to the medium **(b)**. The level of nornicotine below detection limit is indicated by non-detectable

(N.D.). Note the difference in scales between **(a)** and **(b)** (the level of secreted alkaloids is in some case around tenfold lower). For the alkaloid measurement, mean and SE are shown from six independent experimental series. Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

When intracellular nornicotine was tested in response to 10 µM of JA (Figure 3.13), both nornicotine and nicotine were found to be already induced by this low concentration of the elicitor. In contrast, anatabine was not detected. This indicates a higher sensitivity of alkaloid synthesis for overexpression of the converter version nicotine demethylase. But in contrast to the non-converter version of nicotine demethylase (Figure 3.8), this increase in JA sensitivity seems to be limited to the nicotine branch of the pathway.

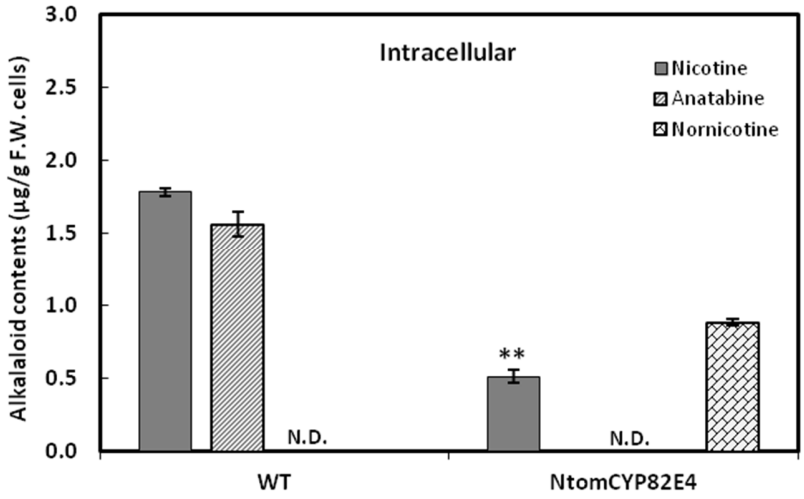


Figure 3.13 Intracellular alkaloid profiles measured in non-transformed BY-2 cells (WT) and cells overexpressing *Ntom*CYP82E4 after 3 days of culture in presence of 10 µM jasmonic acid. For the alkaloid measurement, mean and SE are shown from six independent experimental series. Non-detectable alkaloids are indicated by (N.D.). Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively.

3.5 Feeding of low concentrations of nicotine can release hidden potential for nornicotine accumulation in cell culture

Even with the nicotine demethylase version from the converter species *N. tomentosiformis*, the accumulation of nornicotine remained relatively modest (Figure 3.12, p. 51). The yield of this reaction might be limited by the availability of substrate for this overexpressed enzyme. Alternatively, it might be limited by regulatory factors that depend on the presence of the substrate. To address this question, the precursor nicotine was fed (Figure 3.14). However, the quantity of nicotine was kept low (15 µg ml^{-1}). This should avoid that the added nicotine would simply overrun the accumulation of the nicotine produced by the cells themselves. This approach was efficient in stimulating the accumulation of intracellular nicotine and nornicotine even in non-transformed wild type cells. This increase of nicotine and nornicotine was about two orders of magnitude above the quantity of the added nicotine, i.e. it could not be explained in terms of addition or direct conversion of the added substrate. For the line overexpressing the GFP fusion of the non-converter nicotine methylase *Ntab*CYP82E5*v2*, the levels of nornicotine were doubled over the levels found in the nicotine-supplemented wild type, and this could be further doubled by feeding nicotine to the line overexpressing the converter nicotine methylase *Ntom*CYP82E4 in fusion with GFP. Here, the ratio of nicotine conversion was so high that the levels of nornicotine even exceeded those of nicotine, simulating the situation in the senescent leaves of this species (Figure 3.9, p. 48).

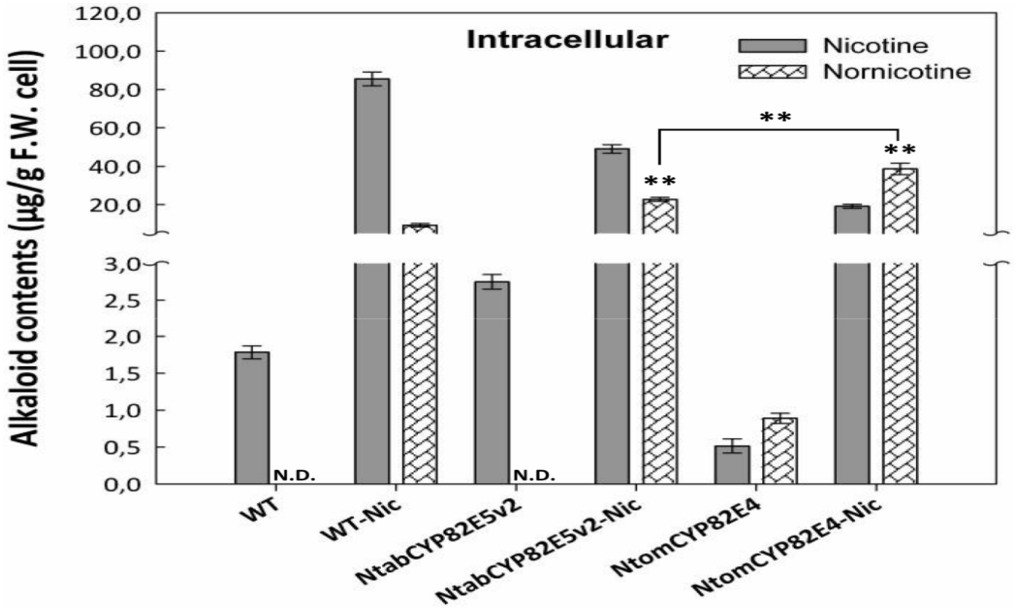


Figure 3.14 Alkaloid accumulation in non-transformed BY-2 cells (WT) compared to cells overexpressing *Ntab*CYP82E5*v2* and *Ntom*CYP82E4, respectively, after feeding with 15 µg ml^{-1} of pure nicotine (Nic). The level of nornicotine below detection limit is indicated by non-detectable (N.D.). Error bars represent SE from 3 independent experimental series. Significant differences in nornicotine production to the WT treated with nicotine and also between two CYP overexpressing cell lines in a Student's t-test are indicated by two asterisks ($P < 0.01$) (From Rajabi et al., 2017).

The observed significant increase of intracellular nornicotine in the treated samples might have been caused by gene activation of one of the endogenous nicotine demethylases (i.e. *NtabCYP82E5v2*, or *NtabCYP82E4*). However, the steady-state level of both transcripts was reduced after elicitation by jasmonic acid and was not stimulated by nicotine feeding, neither in presence nor in the absence of jasmonic acid (Figure 3.15). Also in the MPO1ox line, these transcripts were downregulated by jasmonic acid. Under control conditions, the CYPs genes were expressed in the MPO overexpressor at significantly lower levels as compared to wild type cells. These experiments show that there exists a considerable potential for the accumulation of nornicotine even in the non-transformed wild type. This sleeping potential can be released by adding the precursor nicotine.

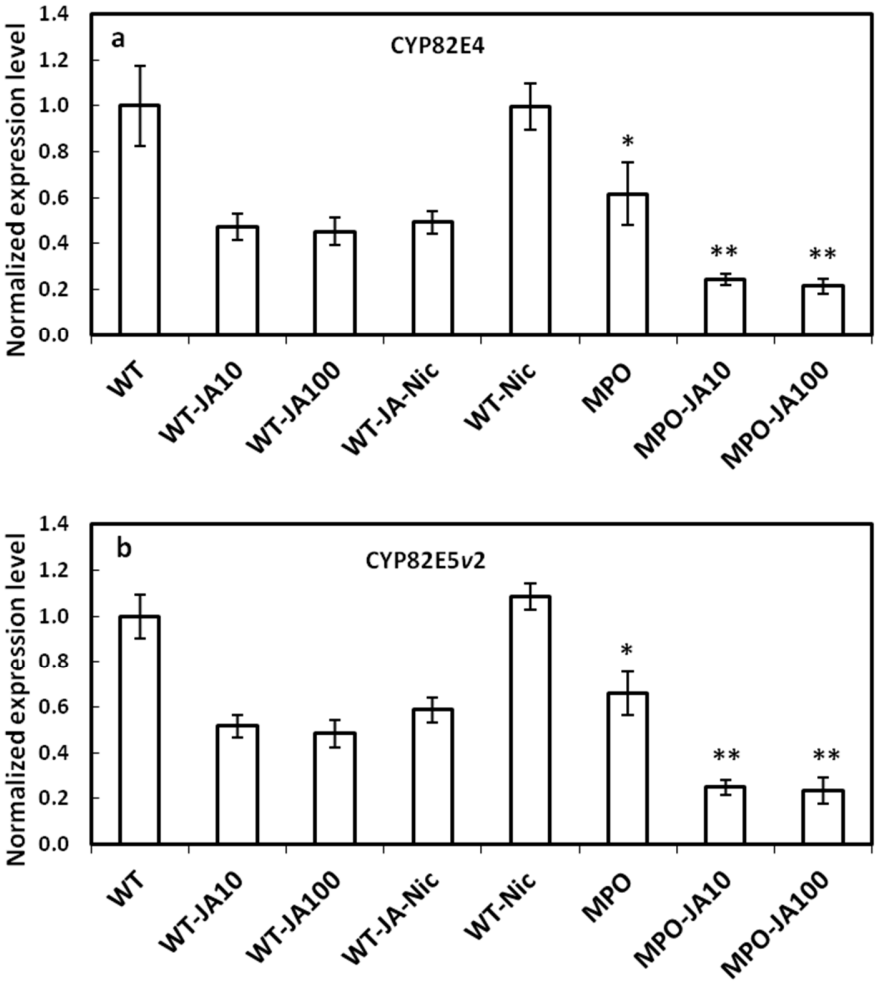


Figure 3.15 Steady state levels of the *Ntab*CYP82E4 **(a)** and the *Ntab*CYP82E5*v2* **(b)** transcripts in non-transformed BY-2 cells (WT) and MPO overexpressor line elicited with jasmonic acid at a final concentration of 10 µM and 100 µM and without elicitation as well as WT cells treated with 15 µg ml^{-1} nicotine and jasmonic acid at the same time and treated with nicotine alone normalized to L25 ribosomal protein and elongation factor 1α (EF-1 α) as internal standards. Error bars indicate SE from three independent experimental series. Significant differences of *Ntab*MPO1 (elicited and non-elicited) to the WT (elicited and non-elicited) assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

3.6 Conditioned medium from BY-2 cells can stimulate alkaloid synthesis

In the natural context, nicotine is synthesized in the roots and transported into the leaves, where it is converted into nornicotine. In the aforementioned precursor feeding experiment, this situation was mimicked by confronting the *Ntom*CYP82E4-GFP overexpressor cells with nicotine. To develop this simulation of the situation *in planta* one step further, it was asked, whether a similar effect might be produced by generating the precursor in a natural way, i.e. by secretion from elicited cells. This would provide a condition, where different metabolic modules can interact with each other. Similar to the situation in the plant, cells with different metabolic capacity would be coupled together in a way that one cell type would produce and secrete the compound which can act directly as a precursor for the other cell type. Therefore, combination experiments were designed to investigate the regulatory or metabolic interaction of different cell types. To mimic the interaction found between the nicotine secreting cells of the root and the nicotine converting cells of the senescent leaf, conditioned medium from either non-transformed wild type or the MPO1 overexpressor which contains the precursor signal (secreted nicotine) were used. Then, the *Ntom*CYP82E4-GFP overexpressor was cultivated in this conditioned medium.

For this experiment, the medium of wild-type cells (Figure 3.16, M_1) as control and *Ntab*MPO1-overexpressing cells (Figure 3.16, M_2) was collected at day 3 and mixed with fresh medium separately. Thirty ml of these conditioned media were used for subcultivation of cells overexpressing *Ntom*CYP82E4-GFP. After 3 days, the alkaloid level of *Ntom*CYP82E4 cells cultivated in the conditioned medium (M_1 and M_2) were analyzed and compared with that of unconditioned medium (M_0). The *Ntom*CYP82E4 cells were elicited with JA at a final concentration of 100 µM. Accumulation of the target compound nornicotine was measured and

a significant stimulation in *Ntom*CYP82E4 cells cultivated in conditioned medium M_2 was observed (Figure 3.16). It was recognized that the treatment with the conditioned medium M_1 in *Ntom*CYP82E4 cells did not affect the intracellular nicotine and nornicotine production, but significantly stimulated the accumulation of intracellular anatabine (Figure 3.16). In contrast, when the conditioned medium was collected from the MPO1 overexpressor line (M_2), the induction of intracellular nicotine was significantly enhanced over that observed for cultivation in unconditioned medium (M_0). Strikingly, there was also a strong stimulation in the accumulation of nornicotine in response to conditioned medium M_2 reaching around half of the levels achieved in the nicotine feeding experiment (Figure 3.14, p. 54). In contrast, conditioned medium M_1, which contains very low amounts of secreted nicotine (about 100 ng ml^{-1}), was not efficient in stimulating nornicotine accumulation. Cultivation in the conditioned medium did not impair viability as verified by the Evans Blue dye exclusion test (Figure 3.17).

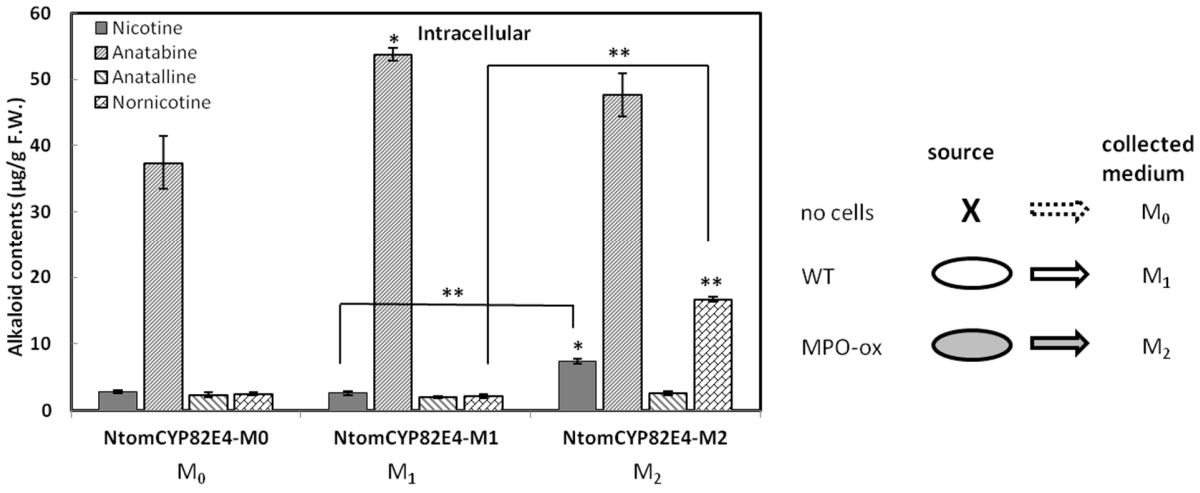


Figure 3.16 Profile of intracellular alkaloids accumulated in the *Ntom*CYP82E4 overexpressor in response to conditioned medium. Stimulation of intracellular alkaloid accumulation by conditioned medium collected from non-transformed wild type (M_1) or cells overexpressing MPO1 (M_2). Unconditioned medium (M_0) was used as negative control. Alkaloid synthesis was elicited by 100 µM of jasmonic acid for three days. Error bars indicate SE from three independent experimental series.

Significant differences to the non-transformed WT cells assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

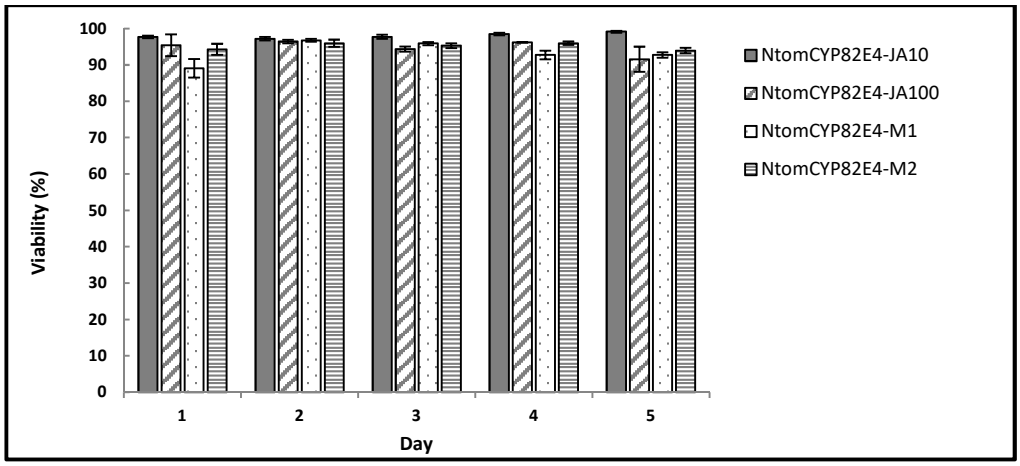


Figure 3.17 Viability of BY-2 cells overexpressing *Ntom*CYP82E4 elicited with jasmonic acid at a final concentration of 10 µM and 100 µM, and when cultivated in conditioned medium of non-transformed (M_1) and *Ntab*MPO1 overexpressing cells (M_2) (n = 500). Data are derived from three independent experimental series. Error bars represent SE (From Rajabi et al., 2017).

3.7 Co-cultivation of different cell types can efficiently release the metabolic potential for nornicotine synthesis

In the search for more efficient strategies to release the silent potential of the *Ntom*CYP82E4 overexpressor for nornicotine accumulation, co-cultivation was tested. In case that non-secreted factors present on the surface of one cell type would stimulate the metabolic potential of the recipient cell, this would not be mimicked by a transfer of conditioned medium. Since all overexpressor lines were generated using the same selection marker (KanR), such a co-cultivation strategy was feasible. Equal volumes of the *Ntab*MPO1 overexpressor and *Ntom*CYP82E4 overexpressors in the double volume of fresh medium were cultivated and the nornicotine contents in cells were measured after 3 days following elicitation with JA at a final concentration of 100 µM. The results (Figure 3.18) show a strong increase in nornicotine

accumulation, which exceeded the accumulation reached by cultivation in conditioned medium M_2 from the MPO1 overexpressor (Figure 3.16, M_2 p. 57). This stimulation of nornicotine synthesis was accompanied by a concomitant decrease in steady-state levels of nicotine (Figure 3.19). This indicates that non-soluble factors on the cell surface can stimulate the potential of the target cell (overexpressing *Ntom*CYP82E4) to convert nicotine to nornicotine.

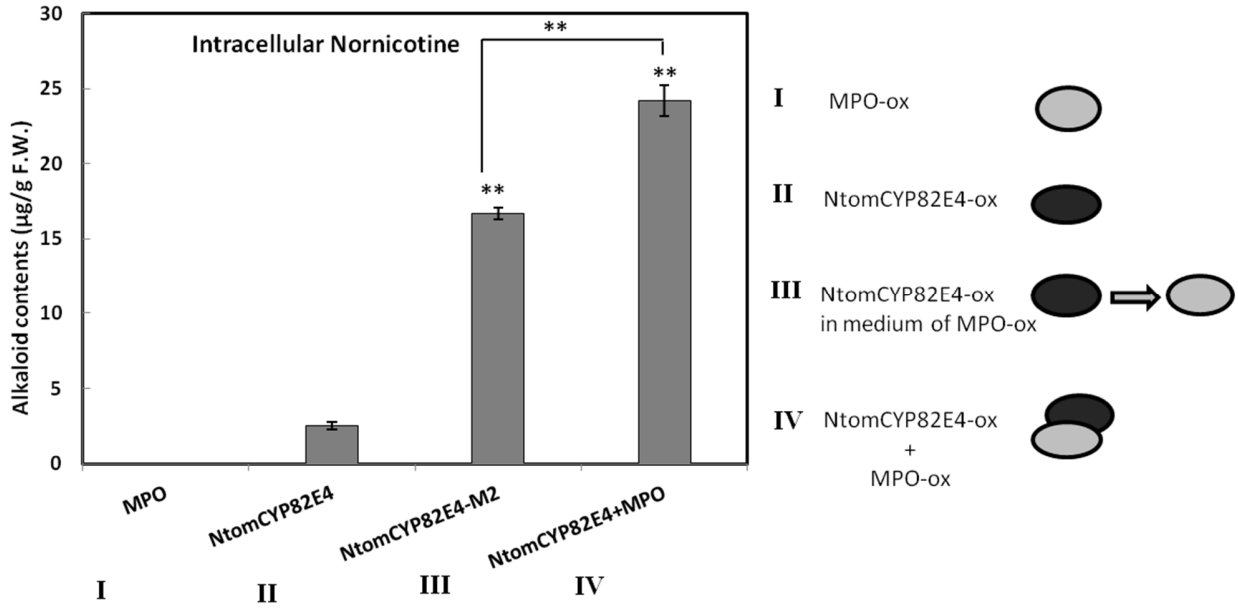


Figure 3.18 Nornicotine contents in BY-2 cells overexpressing *Ntab*MPO1 (**I**) and cells overexpressing *Ntom*CYP82E4 (**II)** compared to *Ntom*CYP82E4 cells cultivated in a mixture composed of the medium of three days old *Ntab*MPO1 cell line (M_2) (**III)** and when co-cultivated together with *Ntab*MPO1 cells (**IV)**. Error bars represent SE (n=3). Significant differences to the *Ntom*CYP82E4 in a Student's t-test are indicated by two asterisks ($P < 0.01$) (From Rajabi et al., 2017).

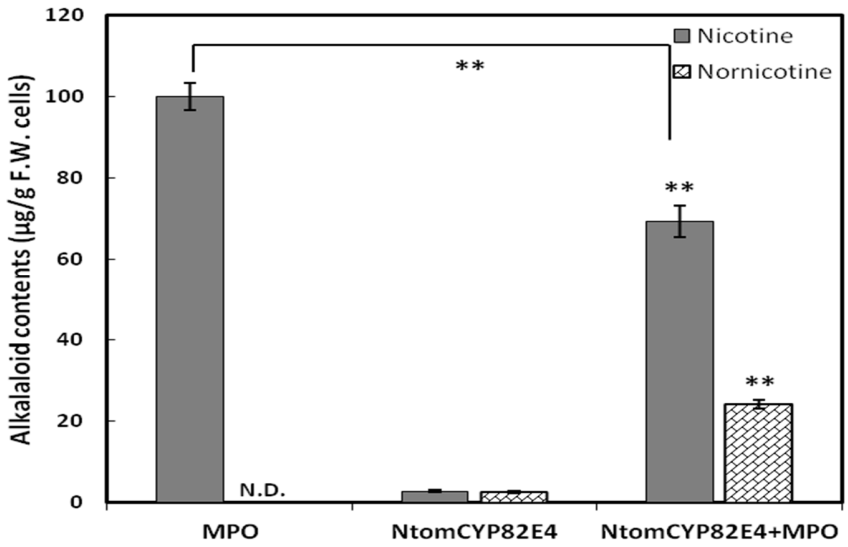


Figure 3.19 Nicotine and nornicotine contents in BY-2 cells overexpressing *Ntom*CYP82E4 co-cultured together with *Ntab*MPO1 compared to *Ntab*MPO1 and *Ntom*CYP82E4 cell lines. The nornicotine level in MPO line was below detection limit (indicated by N.D.) Error bars represent SE (n = 3). Significant differences to the *Ntom*CYP82E4 and *Ntab*MPO1 assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively (From Rajabi et al., 2017).

3.8 nsPEFs treatment of BY-2 cells

Accumulation of natural products within cells is a common phenomenon observed for the most of valuable compounds in plants. This is considered as a limiting factor in downstream processing which makes the whole procedure more costly. Nicotine alkaloids are not excluded since they are also not secreted into the culture medium in sufficient amounts. Therefore, the nanosecond pulsed electric fields (nsPEFs) strategy was used as an alternative to release the stored nicotine alkaloids from cells without disturbing the viability of the cells.

3.8.1 Setting parameters for nsPEFs

In order to find the optimal pulse conditions for electropermeabilization of BY-2 cells, the influence of specific treatment energy input and pulse duration was investigated (Table 2.1, p. 34). Electromanipulation is able to cause various reversible but also irreversible changes to the different compartments of plant cells. A cell line with reversible changes would be able to regenerate by cell division within few days while cells with irreversible damages face cell death. Field strength, pulse duration, and number of pulses are amongst critical factors determining the intensity of the damage. In fact, these three factors are the main determinants of the total energy level exposed to the cells. For this reason, a set of different pulsing indices were tested to treat BY2 cells and viability and alkaloids contents were further measured (data not shown). According to our results, pulsing index 3 (PI3, Table 2.1, p. 34) which resulted in a higher degree of membrane permeabilization and therefore a stronger release of intracellular alkaloids into the extracellular medium with the minimum effect on the cell viability was chosen for further analysis.

3.8.2 Effect of nsPEFs on alkaloid secretion

Since the co-cultivation strategy leads to a strong accumulation of nornicotine in the *Ntom*CYP82E4 cell line, we used the same co-cultivated cell suspension sample for nsPEFs treatment. For this reason, equal volumes of the *Ntab*MPO1 overexpressor and *Ntom*CYP82E4 overexpressors were cultivated in the double volume of fresh medium elicited with JA at a final concentration of 100 µM. Three days after cultivation the cells were pulsed with a frequency of 4 Hz and the pulse number of 11 per cell. Pulses were generated in durations of 100 ns with a final input energy of 100 $J.kg^{-1}$ (Table 2.1, p. 34).

Alkaloid measurement immediately after nsPEFs treatment (Figure 3.20) showed a strong increase in the level of nicotine and anatabine secretion into the culture medium followed by a decrease of relative intracellular alkaloids. However, nornicotine secretion remained very low compared to other alkaloids. Although very low amounts of secreted nornicotine were detected in this experiment it seems that these parameters were not efficient enough to stimulate nornicotine secretion (Figure 3.20 b).

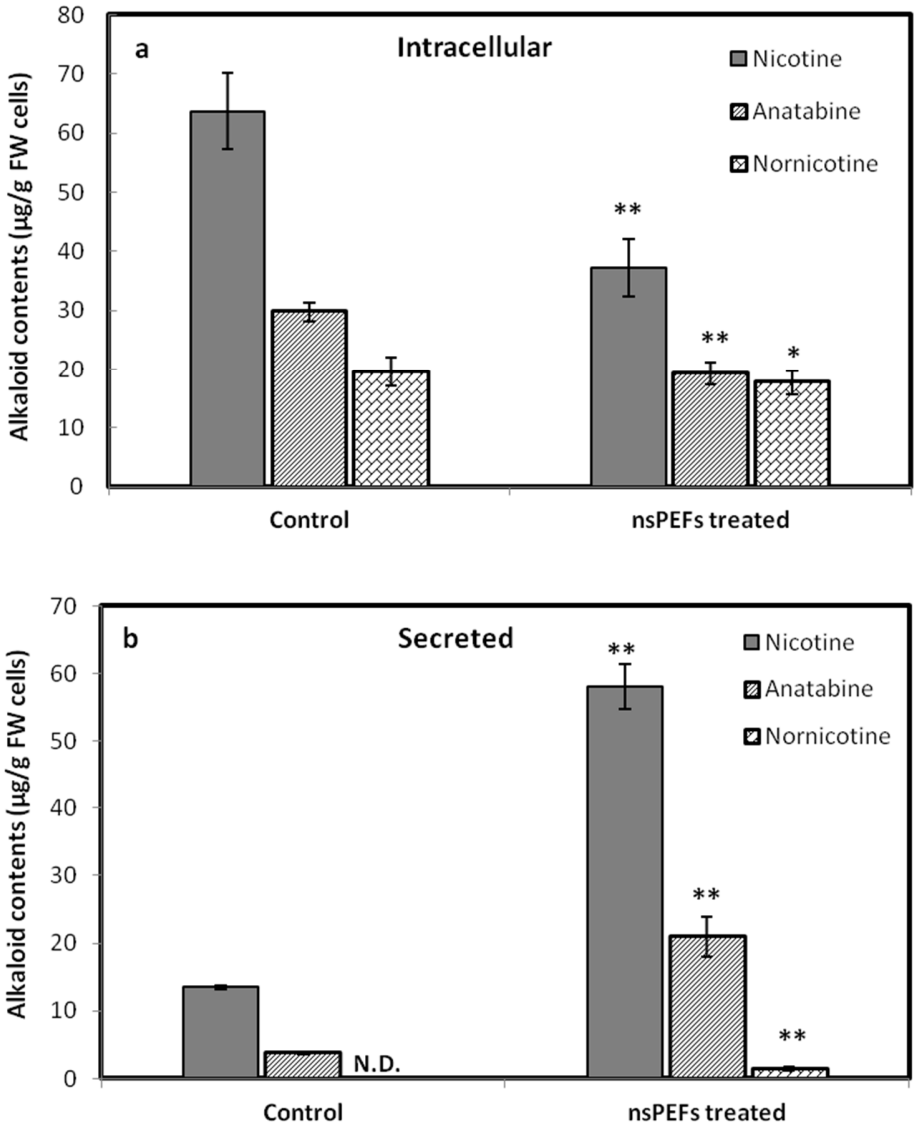


Figure 3.20 Alkaloid content of cells overexpressing *Ntom*CYP82E4 co-cultivated together with *Ntab*MPO1 in response to nsPEFs treatment (PI3, see Table 2.1, p. 34). **(a)** Intracellular alkaloid level in nsPEFs treated cells shows a significant decrease compared to non-nsPEFs treated cells (control). **(b)** Secreted alkaloid level in nsPEFs treated cells shows a significant increase compared to non-nsPEFs treated cells. Level of nornicotine below detection limit is indicated by non-detectable (N.D.). Error bars represent SE (n = 3). Significant differences to the non-treated cells (control) assessed by a Student's t-test are indicated by * ($P < 0.05$) or ** ($P < 0.01$), respectively.

The most important information of the experiment is that the difference of untreated to treated cells in the number of dead cells immediately after nsPEFs treatment is generally 8% higher (Figure 3.21). This modest increase in mortality as verified by the Evans Blue dye exclusion test cannot justify the enhancement of alkaloid secretion, which means that this phenomenon was not due to cell death. The intracellular alkaloid level was measured at 24 hours after nsPEFs treatment, which reached a higher content than that of the control (Figure 3.22 a). However, no significant effect was observed regarding secreted alkaloids compared with the control (Figure 3.22 b). As shown in Figure 3.21 the difference of treated to untreated cells in mortality dropped to 6% after 24 hours (day 4), thus proving that the damage caused by the selected pulse index (PI3) was reversible.

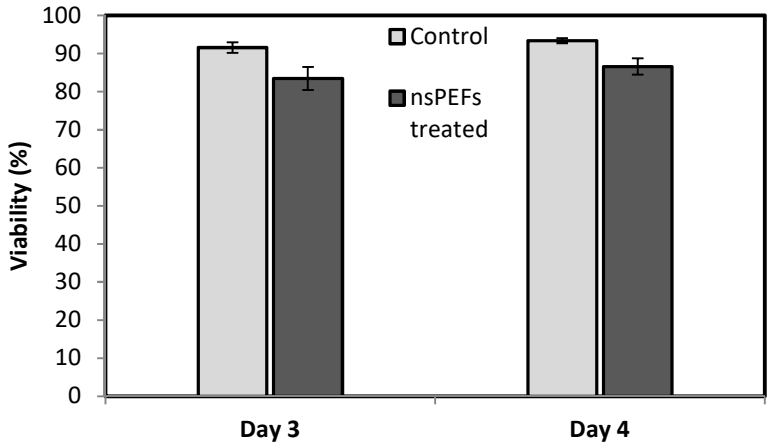


Figure 3.21 Viability of nsPEFs treated cells compared to untreated cells (control) immediately (cells were 3 days old when treated with nsPEFs) and 24 hours (day 4) after treatment (n = 1000). Data are derived from three independent experimental series. Error bars represent SE.

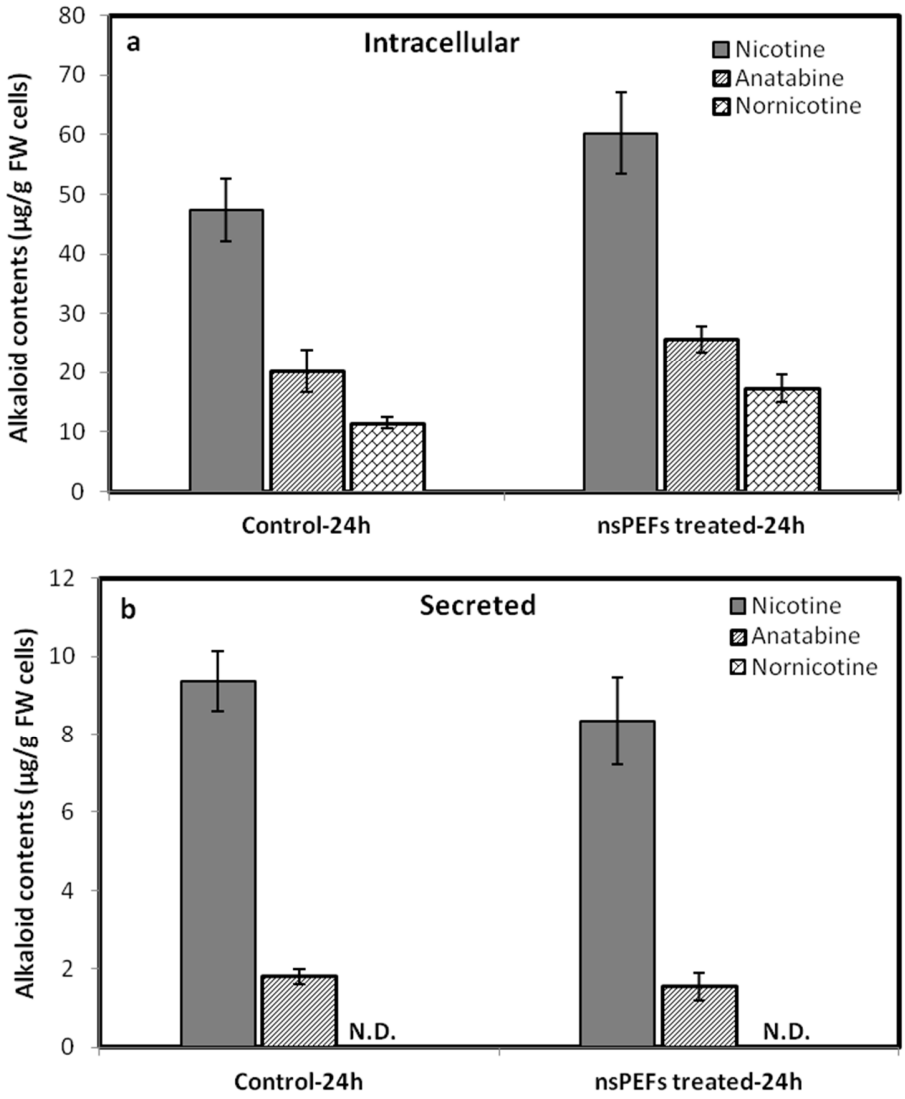


Figure 3.22 Alkaloid content of cells overexpressing *Ntom*CYP82E4 co-cultivated together with *Ntab*MPO1 at 24 hours after nsPEFs treatment (PI3) **(a)** Intracellular alkaloid level in nsPEFs treated cells show a significant increase compared to non-nsPEFs treated cells (control). **(b)** Secreted alkaloid level in nsPEFs treated cells shows no difference compared to non-nsPEFs treated cells. Level of nornicotine below detection limit is indicated by non-detectable (N.D.). Error bars represent SE (n = 3).

3.9 Summary

Plants produce secondary metabolites for their own benefits mainly to protect themselves against herbivores and pathogens. However, the importance of these valuable compounds for humans has been revealed since ancient times. Therefore, production of these compounds through different routes and dealing with limitations related to each route has been always of great importance. In this work, nicotine alkaloids metabolism of tobacco were used to show the necessity of metabolic partitioning into different cell types for metabolite maturation and high product yields, one of the factors which has limited the success of plant cell fermentation. This study stimulates the interaction found between the nicotine secreting cells of the root and nicotine-converting cells of the senescent leaf to generate the target compound nornicotine in the model cell line tobacco BY-2. When *Ntab*MPO1 was overexpressed in tobacco BY-2 cells, accumulation and secretion of nornicotine precursor was stimulated. However, no nornicotine was detected in this cell line. By engineering of another cell line overexpressing the key enzyme *Ntom*CYP82E4 from a converter species, nornicotine synthesis was triggered, but only to a minor extent. This low yield of nornicotine in the nicotine demethylase version from a converter species might be limited by the availability of the substrate for this overexpressed enzyme. To address this question, the precursor nicotine was fed in a low quantity (15 µg ml^{-1}), which stimulated the accumulation of intracellular nicotine and nornicotine even in non-transformed wild type cells. The steady-state level of endogenous nicotine demethylases (*Ntab*CYP82E5v2 or *Ntab*CYP82E4) was measured to investigate their role in the significant increase of intracellular nornicotine in treated samples. However, the steady-state level of both transcripts was not stimulated by nicotine feeding. Furthermore, combination experiments were designed to answer this question whether a similar effect might be produced by generating the precursor nicotine in a natural way. Similar to the situation in the plant, the two generated cell lines with different metabolic capacity were coupled together in a way that one

cell type would produce and secrete the compound which can act directly as a precursor for the other cell type. Combination experiments showed that co-cultivation of two cell types is more efficient to release the silent potential of the *Ntom*CYP82E4 overexpressor for nornicotine accumulation compared to just feeding the conditioned medium collected from the same cells. Furthermore, exposure of the cells to the nanosecond pulsed electric fields with a special parameter set (PI 3), improved secretion of some nicotine alkaloids into the culture medium significantly while maintaining viability of the cells.

4 Discussion

By the use of molecular tools, the regulation of secondary metabolism has strongly accelerated the understanding of the biosynthetic pathways for natural products (Jimenez-Garcia et al. 2013). In order to achieve high yields for the final products, combinations of elicitor treatment, precursor feeding and metabolic engineering have been successfully employed. However, many target molecules are still not accessible, due to their chemical complexity or due to metabolic constraints. Still, most plant secondary metabolites of commercial use such as the anti-cancer agents vinblastine and camptothecin are produced from the extraction of plants because better options are not available (Wilson & Roberts 2012). Metabolic engineering can be used in several ways to improve yields. To increase the carbon flux towards the desired product requires a detailed knowledge of the biosynthetic pathways involved. The most important step in metabolic engineering is to determine the rate-limiting enzymes as primary targets for genetic engineering. However, other factors such as regulatory aspects like feedback mechanisms and compartmentalization must be considered as well (Verpoorte et al. 1999; Sato et al. 2001; Capell & Christou 2004). In the current study, nicotine alkaloids were exemplarily used to demonstrate the importance of metabolic compartmentalization into different cell types and tissue (roots versus leaves). Therefore, the main aim of the present work is to provide a condition in which metabolically different cell types could interact in order to increase the final product.

The current work was successful in generating two cell lines, where one line, *Ntab*MPO1, produces the precursor for the other line, *Ntom*CYP82E4. In all experiments, several transformant lines have been used with quantitatively the same results. Furthermore, different strategies have been tested to connect these two metabolic modules. The results from this work suggest that although metabolic engineering was effective in case of *Ntab*MPO1 to improve the yield for the precursor nicotine, additional factors still may become limiting. For

example, in the current work, the target compound was nornicotine, but cells overexpressing the nicotine demethylase which were engineered to produce this target compound, failed to accumulate nornicotine. By feeding these cells with the precursor, nicotine, this bottleneck could be removed suggesting substrate availability and compartmentalization as two limiting factors for the production of the final product by these engineered cells. Although precursor feeding is an alternative way to improve productivity, it is not cost-effective in most cases (Mehrotra et al. 2015). As an alternative, a strategy was designed, where the precursor was supplied "in a natural way" by secretion from one metabolic module. This strategy to combine different metabolic modules was successful to release metabolic synergies that could not be produced by each of the modules alone.

4.1 Nornicotine accumulation is controlled by nicotine

BY-2 cells overexpressing a key enzyme responsible for the conversion of nicotine to nornicotine in *Nicotiana tabacum* (*Ntab*CYP82E5*v2*) were not able to produce nornicotine (Figure 3.7, p. 46). However, the overexpression of a rate-limiting enzyme (*Ntom*CYP82E4) from the converter plant *Nicotiana tomentosiformis* leads to nornicotine accumulation although in minor amounts (Figure 3.12 a, p. 51). The low level of nornicotine production in BY-2 cells overexpressing nicotine demethylase might be caused by limited abundance of the precursor nicotine. In fact, feeding a low concentration of nicotine as precursor led to a high accumulation of nornicotine in both CYP overexpressing cell lines as well as in BY-2 wild type cells (Figure 3.14, p. 54). The potential for nornicotine biosynthesis by non-transformed BY-2 cells in presence of the precursor nicotine indicates that nornicotine accumulation is first controlled by nicotine and then by the abundance and enzymatic efficiency of the nicotine demethylase. However, alternative possibilities exist. For instance, the exogenously fed nicotine might be

more readily available to the nornicotine demethylase because it enters through the cytosol. To address this, it would be necessary to discriminate endogenous and exogenous nicotine, for instance by using radioactively labeled nicotine. Irrespective of the intracellular compartmentalization, one can conclude that the sleeping potential for nornicotine accumulation is released by adding nicotine.

4.2 Nicotine acts as a signal, not as a substrate in controlling nornicotine accumulation

A closer look at this precursor feeding experiment revealed, however, that the situation differs from simple substrate limitation. The concentrations of nicotine added were low (15 µg ml^{-1}), but caused an alkaloid production in both non-transformed and transformed BY-2 cells that was much higher (Figure 3.14, p. 54). This is valid also for the synthesis of nicotine itself: When the non-transformed BY-2 cells were treated with this low concentration of nicotine, they produced more than 80 µg g^{-1} fresh weight of cells nicotine and, in addition, a considerable amount of nornicotine compared to non-treated cells. A similar stimulation with even higher intracellular nornicotine accumulation could be generated in the overexpressor lines. This increase in accumulated nicotine alkaloids cannot be explained by a model, where the exogenous nicotine is simply imported and subsequently converted to nornicotine. The discrepancy between the exogenous precursor and accumulated product rather suggest a regulatory role for nicotine. In other words, nicotine is not only acting as a precursor but also as a signal able to stimulate the activity of the nicotine demethylase. To understand the mechanism behind this regulatory effect, the expression level of endogenous key genes involved in nornicotine biosynthesis (CYP82E4 and CYP82E5*v*2) in response to that low level of nicotine treatment were further measured in both non-transformed wild type and MPO

overexpressor line (Figure 3.15, p. 55). The results (Figure 3.15) clearly show that the stimulation of nornicotine accumulation cannot be explained by transcriptional activation, but must either act at the level of compartmentalization or enzymatic activity, i.e. by posttranslational mechanisms. In other words, nicotine could have an effect on the capacity of cells to translate mRNA encoding nicotine demethylase enzymes. A further argument against a potential stimulation of nornicotine demethylase transcripts by nicotine is given by the observation that these transcripts are reduced in the MPO1 overexpressor, which accumulates and secretes more nicotine (Figure 3.15).

4.3 Nicotine is not the only signal controlling nornicotine accumulation

Nicotiana tomentosiformis is one of the ancestral species of *Nicotiana tabacum* with a high ability to convert nicotine to nornicotine linked with a specific version of nicotine demethylase, *Ntom*CYP82E4. The ability to accumulate nornicotine already in the green, non-senescent leaf is a characteristic feature that discriminates *N. tomentosiformis* from *N. tabacum* (Murad et al. 2002; Gavilano et al. 2007; Yukawa et al. 2006). This work was able to release this metabolic potential in the *Ntom*CYP82E4 overexpressor by feeding nicotine (Figure 3.14, p. 54). Although this strategy requires only small amounts of the precursor, because nicotine seems to act as a signal rather than as a substrate, this approach is still costly, which holds true for precursor feeding in general (Mehrotra et al. 2015). In the search for cost-effective alternatives, a strategy was tested, where the nicotine would come from the conditioned medium of other cells. Nicotine is the main precursor for nornicotine biosynthesis and its biosynthesis in *Nicotiana* species requires an oxidative deamination of N-methylputrescine, catalyzed by N-methyl putrescine oxidase (MPO1) (Katoh et al. 2007; Shoji & Hashimoto 2008). The intracellular and

secreted nicotine in tobacco BY-2 cells is very low compared to anatabine because MPO1 is hardly expressed in these cells (Shoji & Hashimoto 2008). Therefore, a transgenic line overexpressing MPO1 was used, because these cells were able, after elicitation to produce and secret nicotine in a relatively high amount (Figure 3.3, p. 39).

This strategy was successful: Using the medium from elicited BY-2 cells overexpressing *Ntab*MPO1, an eightfold increase of nornicotine accumulation in the *Ntom*CYP82E4 overexpressor was achieved (Figure 3.16, p. 57). This finding raised the question, whether this stimulation was caused by the secreted nicotine in the conditioned medium M_2. However, when the concentration of nicotine in this conditioned medium is estimated based on the data shown in Figure 3.3b (p. 39), it is found to be very low (only 500 ng ml^{-1} compared to the 15 µg ml^{-1} used in the nicotine feeding experiment). Thus, although the secreted nicotine concentration in the conditioned medium M_2 is around 30 times lower than the nicotine used in the feeding experiment (Figure 3.14, p. 54), the nornicotine accumulation induced by the conditioned medium M_2 reached half of the values achieved by feeding of a much higher concentration of exogenous nicotine. This means that the stimulation of nornicotine accumulation by the conditioned medium M_2 cannot be explained by the traces of nicotine present in this medium, but must be triggered by a different positive regulator. Since the viability of cells cultivated in conditioned medium M_1 or M_2 was completely normal, precluding a scenario that nornicotine accumulation was triggered in consequence of senescence as response to stress (Figure 3.17, p. 58).

Co-cultivation of *Ntab*MPO1 and *Ntom*CYP82E4 transgenic lines together was another logical alternative and additional method to provide an environment for cell interaction. Co-culture of root and shoot has been used previously to improve production of tropane alkaloids in *Atropa belladonna* and *Duboisia leichhardtii* × *D. myoporoides* hybrid plants (Subroto et al. 1995). The significant increase in nornicotine production by *Ntom*CYP82E4 when co-cultivated with

*Ntab*MPO1 cells demonstrated that the physical presence of cells was superior to just feeding the conditioned medium collected from the same cells (Figure 3.18, p. 59). At the current state, this result is surprising, and there are four scenarios that should be pursued in the future: Non-diffusible signals of the extracellular matrix might be involved in metabolic regulation, and efficient exchange of these signals would require physical contact of the two cell types. Alternatively, there might be reciprocal signaling from the cells accumulating nornicotine (*Ntom*CYP82E4) on those that produce nicotine (*Ntab*MPO1), and this reciprocal signaling stimulates the production of the positive regulator secreted by the precursor cells (*Ntab*MPO1). There might be also an unstable signal produced by *Ntab*MPO cells stimulating the production of nornicotine by *Ntom*CYP82E4 cells which is already degraded in the condition medium M_2. The last scenario considers the difference in timing: In the M_2 conditioned medium experiment, the *Ntom*CYP82E4 cells are exposed to the unknown factor secreted by the *Ntab*MPO cells from the very beginning. In the co-cultivation experiment, the *Ntab*MPO cells have first to accumulate and secrete this factor, such that the *Ntom*CYP82E4 cells will first proliferate in the absence or under only low levels of this factor. Only with time, the *Ntom*CYP82E4 cells are exposed to the higher levels present in the M_2 conditioned medium. The conditioned medium might not only contain compounds that stimulate the nornicotine synthesis, but also compounds that slow down cell division. This scenario is also indicated by the finding that the cell cycle in the *Ntab*MPO1 line is a bit slower than in the wild type, which might be due to such secreted factors accumulating in the medium (Figure 3.2 e, p. 38).

4.4 nsPEFs trigger secretion as well as biosynthesis of alkaloids

Plant secondary metabolites are normally accumulated within the cells and therefore improving product secretion into the culture medium will certainly decrease production costs associated

with plant cell culture systems (Chattopadhyay et al. 2002; Wilson & Roberts 2012). Nicotine alkaloids are not excluded from this principle and they are mainly remained intracellular with as little as 20% of the product released to the extracellular medium. This was even worst for nornicotine since this alkaloid was not detected in the culture medium. Hence, this study was interested in permeabilizing the plant cell membranes in a reversible manner with economically viable and eco-friendly techniques. It was also of great importance that the chosen approach could be applicable to a variety of cell types. In recent years many attempts have been made to permeabilize the plant cell membranes and several extraction techniques have been proposed. To obtain the more efficient method for industrial production, the pulsed electric field extraction technique has attracted attention as a potential technology (Patras et al. 2017). There have been many reports on varying effects of pulsed electric fields treatment on biological systems. These effects are varying from food decontamination to delivery of macromolecules including genetic materials (Chang et al. 1992; Aronsson et al. 2005; Sepulveda et al. 2005) and from treatment of cancer to extraction of cellular contents (Brodelius et al. 1988; Beebe et al. 2002; Sack et al. 2005), and so on. Since most of the studies were done in the range of irreversible membrane permeabilization, this work focused on the study of the nsPEFs effects on reversible permeabilization of cell membranes with the aim of enhancing product recovery.

Treating the nicotine alkaloid accumulating cells with pulsing index 3 (PI3, Table 2.1, p. 34) resulted in a strong release of intracellular nicotine and anatabine into the extracellular medium with the minimum effect on cell viability. When *Ntab*MPO overexpressor cells were co-cultivated together with the *Ntom*CYP82E4 cells, nicotine constituted the major alkaloid (64 µg g^{-1} FW) accumulated in the cells. This was followed by anatabine and nornicotine with 30 µg g^{-1} FW and 20 µg g^{-1} FW respectively (Figure 3.20, p. 63). Compared to the control, when these cells were exposed to the nsPEFs treatment, about 92% of intracellular nicotine, 68% of anatabine and 7.5% of nornicotine were secreted into the culture medium. Despite the large

amounts of nicotine and anatabine secretion to the culture medium, a high amount of these alkaloids were detected within the nsPEFs treated cells. The intracellular nicotine level in treated cells reached almost half of the values detected in untreated cells, indicating that nsPEFs have stimulated alkaloids production. Since the viability of these cells was completely normal, precluding a scenario that this stimulation in alkaloid production and secretion was triggered in consequence of cell death (Figure 3.21, p. 64).

When the long-term response to nsPEFs was investigated at 24 hours after treatment, a meaningful enhancement of alkaloid production was observed. Though these alkaloids were mainly accumulated intracellular and were not secreted to the culture medium. In other words, nsPEFs treatment induced only a transient increase in nicotine alkaloid secretion (Figure 3.22, p. 64) which indicates a transient cell permeabilization. Under normal cultivation conditions, nicotine alkaloids in tobacco BY-2 cells were produced during exponential growth phase and reach their highest level at day 3. A gradual decrease in both intracellular and secreted alkaloids was observed in the following days. A high intracellular alkaloid level in cells treated with nsPEFs after 24 hours indicate that nsPEFs could work as an external stimulus to influence cellular secondary metabolism. The synthesis of nicotine alkaloids in plants is usually associated with plant defense response to herbivores and environmental stimuli. These findings suggest that nsPEFs, as an external stimulus or stress, can be considered as an abiotic elicitor which can induce a long-lasting stress signal. Apparently, nsPEFs initiate a cascade of coordinately regulated events including changes in membrane potential and ion fluxes, induction of oxidative burst, activation of defense-related genes and finally synthesis of secondary metabolites. A similar elicitor-like effect of pulsed electric field has been suggested by Ye et al. (2004), which could induce plant defense response via oxidative burst and stimulate secondary metabolites accumulation in suspension cultures of *Taxus chinensis*.

In regard to the scarce level of secreted nornicotine in response to nsPEFs, two scenarios exist which should be pursued in the future: The first possibility could be the different

subcellular compartmentation of nicotine alkaloids, which leads to unequal effects of nsPEFs on relative compartments and therefore a weaker permeabilization of nornicotine containing organelles. There are already reports on nicotine storage in the vacuole of the cells but the nornicotine accumulation place has been remained unknown (Dewey & Xie 2013). The second hypothesis is a possible enhancement of enzyme reactions may occur as an immediate effect of nsPEFs, which leads to degradation of nornicotine to some extent. Nevertheless, the relative participation of each hypothesis could be determined with some modifications in the set of parameters of nsPEFs.

Conclusion

Despite more than three decades of efforts, plant cell cultures have led to very few commercial successes for the production of valuable secondary compounds. Amongst several shortcomings of plant cell cultures, the role of metabolic interaction of different cell types on metabolite maturation has attracted little attention. Therefore, the aim of this research work was to provide a condition in which different cell types could interact in order to increase the final product. In this work, nicotine alkaloids were used to demonstrate the importance of metabolic compartmentalization into different cell types and tissue (roots versus leaves). For this reason and based on the biosynthetic pathway of nornicotine, two cell lines were generated, where one line, *Ntab*MPO1, produces the precursor for the other line, *Ntom*CYP82E4 which accumulate a scarce amount of nornicotine by itself. Different approaches were applied to connect these two metabolic modules in order to overcome aforementioned obstacle related to cell culture systems. In the first strategy, the *Ntom*CYP82E4 overexpressor was cultivated in the conditioned medium from the *Ntab*MPO1 overexpressor which contains the precursor signal (secreted nicotine). Remarkably, there was a strong stimulation in the accumulation of nornicotine in response to conditioned medium. However, in case that non-secreted factors present on the surface of one cell type would stimulate the metabolic potential of the recipient cell, this would not be mimicked by a transfer of conditioned medium. Therefore in the second strategy, equal volumes of the *Ntab*MPO1 and *Ntom*CYP82E4 overexpressors were cultivated together in fresh medium and resulted in a strong increase in nornicotine accumulation, which exceeded the accumulation reached by cultivation in conditioned medium from the MPO1 overexpressor. This indicates that non-soluble factors on the cell surface can stimulate the potential of the target cell (overexpressing *Ntom*CYP82E4) to convert nicotine to nornicotine.

Another issue that has been addressed in this study was the ongoing debate as to whether nsPEFs could improve recovery of valuable plant products from cell culture systems. Secretion of nicotine alkaloids to the culture medium in response to nsPEFs treatment demonstrated that nsPEFs treatment can be optimized to enhance the release of intracellular metabolites without harmful effects on cell viability. In addition, accumulation of these metabolites even at 24 hours after nsPEFs treatment indicates that nsPEFs can elicit defense responses and stimulate secondary metabolite production. These results suggest that nsPEFs can be used as a novel abiotic elicitor to improve secondary metabolite production in plant cell cultures. However, more research is necessary to discover the optimum nsPEFs parameter set in a cell culture system, which may lead to the highly efficient production of intracellular secondary metabolites with their continuous biosynthesis and release into the medium, which is beneficial to industrial application.

Outlook

The present work suggests a strategy to technically mimic the cooperation of different cell types in a plant tissue in order to release hidden metabolic potentials and obtain valuable secondary metabolites that otherwise would not be produced in cell culture. Compared to alternative strategies, such as multiplex transformation, the combination strategy used in the current work has the advantage that it can be easily extended and adapted in a modular manner leading to a high degree of versatility. Future work will be dedicated to identify the unknown regulatory factors in the conditioned medium. Moreover, the strategy might be further improved by integrating temporal dynamics to address cell proliferation and metabolic activity differently. For this purpose, microfluidic strategies (Maisch et al. 2016) will be used as a platform to integrate different plant-cell based metabolic modules. These findings suggest that change in basic parameters of nsPEFs would significantly affect secretion of different chemical compounds. It is, thus, necessary to find out a standardized protocol for the optimal recovery of the desired product nornicotine and to accomplish this goal, the underlying molecular processes need to be understood.

Reference

Ajikumar, P.K., Xiao, W.-H., Tyo, K.E.J., Wang, Y., Simeon, F., Leonard, E., Mucha, O., Phon, T.H., Pfeifer B. & Stephanopoulos, G. (2010). Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science* **330**, 70–74.

Amann, M., Wanner, G. & Zenk, M.H. (1986). Intracellular compartmentation of two enzymes of berberine biosynthesis in plant cell cultures. *Planta* **167**, 310–320.

Anarat-Cappillino, G. & Sattely, E.S. (2014). The chemical logic of plant natural product biosynthesis. *Curr Opin Plant Biol* **19**, 51–58.

Aronsson, K., Ulf, R. & Borch, E. (2005). Inactivation of *Escherichia coli*, *Listeria innocua* and *Saccharomyces cerevisiae* in relation to membrane permeabilization and subsequent leakage of intracellular compounds due to pulsed electric field processing. *Int J Food Microbiol* **99**, 19–32.

Bai, F., Gusbeth, C., Frey, W. & Nick, P. (2017). Nanosecond pulsed electric fields trigger cell differentiation in *Chlamydomonas reinhardtii*. *Biochim Biophys Acta, Biomembr* **1859**, 651–661.

Baldwin, I.T. (2001). An ecologically motivated analysis of plant-herbivore interactions in native tobacco. *Plant Physiol* **127**, 1449–1458.

Barbulova, A., Apone, F. & Colucci, G. (2014). Plant cell cultures as source of cosmetic active ingredients. *Cosmetics* **1**, 94–104.

Beebe, S.J., Fox, P.M., Rec, L.J., Somers, K., Stark, R.H. & Schoenbach, K. H. (2002). Nanosecond Pulsed Electric Field (nsPEF) Effects on Cells and Tissues : Apoptosis Induction and Tumor Growth Inhibition. *IEEE Trans Plasma Sci* **30**, 286–292.

Besumbes, Ó., Sauret-Güeto, S., Phillips, M.A., Imperial, S., Rodríguez-Concepción, M. & Boronat, A. (2004). Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of taxol. *Biotech Bioen*g **88**, 168–175.

Bourgaud, F., Gravot, A., Milesi, S. & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Sci* **161**, 839–851.

Brodelius, P. (1988). Permeabilization of plant cells for release of intracellularly stored products : viability studies. *Appl Microbiol Biotechnol* **27**, 561–566.

Brodelius, P.E., Funk, C. & Shillito, R.D. (1988). Permeabilization of cultivated plant cells by electroporation for release of intracellularly stored secondary products. *Plant Cell Rep* **7**, 186–188.

Buschmann, H., Green, P., Sambade, A., Doonan, J.H. & Lloyd, C.W. (2011). Cytoskeletal dynamics in interphase, mitosis and cytokinesis analysed through *Agrobacterium*-mediated transient transformation of tobacco BY-2 cells. *New Phytol* **190**, 258–267.

Cai, B., Ji, H., Fannin, F.F. & Bush, L.P. (2016). Contribution of nicotine and nornicotine toward the production of N′-Nitrosonornicotine in air-cured tobacco (*Nicotiana tabacum*). *J Nat Prod* **79**, 754–759.

Cai, B., Siminszky, B., Chappell, J., Dewey, R.E. & Bush, L. P. (2012). Enantioselective demethylation of nicotine as a mechanism for variable nornicotine composition in tobacco leaf Leaf. *J Biol Chem* **287**, 42804–42811.

Cai, Z., Kastell, A., Knorr, D. & Smetanska, I. (2012). Exudation: An expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. *Plant Cell Rep* **31**, 461–477.

Capell, T. & Christou, P. (2004). Progress in plant metabolic engineering. *Curr Opin Plant Biol* **15**, 148–154.

Chang D.C., Saunders J.A., Chassy, B.M. & Sowers, A.E. (1992). Guide to electroporation and electrofusion. *Academic Press, Inc*.

Chattopadhyay, S., Farkya, S., Srivastava, A.K. & Bisaria, V.S. (2002). Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. *Biotechnol Bioprocess Eng* **7**, 138–149.

Chemler, J.A. & Koffas, M.A. (2008). Metabolic engineering for plant natural product biosynthesis in microbes. *Curr Opin Plant Biol* **19**, 597–605.

Ching, S.L.K., Gidda, S.K., Rochon, A., van Cauwenberghe, O.R., Shelp, B.J. & Mullen, R.T. (2012). Glyoxylate Reductase isoform 1 is localized in the cytosol and not peroxisomes in plant cells. *J Integr Plant Biol* **54**, 152–168.

Chopinet, L. & Rols, M.P. (2014). Nanosecond electric pulses: A mini-review of the present state of the art. *Bioelectrochemistry* **103**, 2–6.

Cipollini, M.L., Levey, D.J., Naturalist, T.A. & Sep, N. (1997). Secondary metabolites of fleshy vertebrate-dispersed fruits: Adaptive hypotheses and implications for seed dispersal. *Am Nat* **150**, 346–372.

Covello, P. S. (2008). Making artemisinin. *Phytochemistry* **69**, 2881–2885.

Sepulveda, D.R., Go´ngora-Nieto, M.M., San-Martin, M.F. & Barbosa-Ca´novas G.V. (2005). Influence of treatment temperature on the inactivation of Listeria innocua by pulsed electric fields. *Lebensm Wiss Technol* 38, 167–172.

DeBoer, K.D., Lye, J.C., Aitken, C.D., Su, A.K.K. & Hamill, J.D. (2009). The A622 gene in *Nicotiana glauca* (tree tobacco): Evidence for a functional role in pyridine alkaloid synthesis. *Plant Mol Biol* **69**(3), 299–312.

Dewey, R.E. & Xie, J. (2013). Molecular genetics of alkaloid biosynthesis in *Nicotiana tabacum*. *Phytochemistry* **94**, 10–27.

Dickerson, T.J. & Janda, K.D. (2003). Glycation of the amyloid beta-protein by a nicotine metabolite: A fortuitous chemical dynamic between smoking and alzheimer's disease. *Proc Natl Acad Sci U S A* **100**, 8182–7.

Eing, C.J., Bonnet, S., Pacher, M., Puchta, H. & Frey, W. (2009). Effects of nanosecond pulsed electric field exposure on *Arabidopsis thaliana*. *IEEE Trans Dielectr Electr Insul* **16** 1322–1328.

Facchini, P. & De Luca, V. (2008). *Opium poppy* and Madagascar periwinkle : model non-model systems to investigate alkaloid biosynthesis in plants. *Plant J* **54**, 763–784.

Finer, J.J., Vain, P., Jones, M.W. & Mcmullen, M.D. (1992). Development of the particle inflow gun for DNA delivery to plant cells. *Plant Cell Rep* **11**, 323–328.

Frey, W., White, J.A., Price, R.O., Blackmore, P.F., Joshi, R.P., Nuccitelli, R., Beebe, S.J., Schoenbach, K.H. & Kolb, J.F. (2006). Plasma membrane voltage changes during nanosecond pulsed electric field exposure. *Biophys J* **90**, 3608–3615.

Gaff, D.F. & Okong'o-Ogola, O. (1971). The use of non-permeating pigments for testing the survival of cells. *J Exp Bot* **22**, 756–758.

Galindo, F.G. (2016). Responses of plant cells and tissues to pulsed electric field treatments. *Handbook of Electroporation*, 1–15.

Gaosheng, H. & Jingming, J. (2012). Production of useful secondary metabolites through regulation of biosynthetic pathway in cell and tissue suspension culture of medicinal plants. *Recent Adv Plant Vitr Cult*, 197–210.

Gavilano, L.B., Coleman, N.P., Bowen, S.W. & Siminszky, B. (2007). Functional analysis of nicotine demethylase genes reveals insights into the evolution of modern tobacco. *J Biol Chem* **282**, 249–256.

Gavilano, L.B. & Siminszky, B. (2007). Isolation and characterization of the cytochrome P450 gene CYP82E5*v2* that mediates nicotine to nornicotine conversion in the green leaves of tobacco. *Plant Cell Physiol* 48, 1567–1574.

Georgiev, M.I., Weber, J. & Macluk, A. (2009). Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl Microbiol Biotechnol* **83**, 809–823.

Gerardy, R. & Zenk, M.H. (1992). Formation of salutaridine from (R)-reticuline by a membrane bound cytochrome P450 enzyme from *Papaver somniferum*. *Phytochemistry* **32**, 79–86.

Goettel, M., Eing, C., Gusbeth, C., Straessner, R. & Frey, W. (2013). Pulsed electric field assisted extraction of intracellular valuables from microalgae. *Algal Res* **2**, 401–408.

Goossens, A., Häkkinen, S.T., Laakso, I., Seppänen-Laakso, T., Biondi, S., De Sutter, V., Lammertyn, F., Nuutila, A.M., Söderlund, H., Zabeau, M., Inze, D. & Oksman-Caldentey, K.M. (2003). A functional genomics approach toward the understanding of secondary metabolism in plant cells. *Proc Natl Acad Sci U S A* **100**, 8595–8600.

Gutjahr, C., Banba, M., Croset, V., An, K., Miyao, A., An, G., Hirochika, H., Imaizumi-Anraku, H. & Paszkowski, U. (2008). Arbuscular Mycorrhiza-specific signaling in rice transcends the common symbiosis signaling pathway. *Plant Cell* **20**, 2989–3005.

Häkkinen, S.T., Rischer, H., Laakso, I., Maaheimo, H., Seppänen-Laakso, T. & Oksman-Caldentey K.M. (2004). Anatalline and other methyl jasmonate-inducible nicotine alkaloids from *Nicotiana tabacum* cv. By-2 cell cultures. *Planta Med* **70**, 936-941.

Han, J., Pang, X., Liao, B., Yao, H., Song, J. & Chen, S. (2016). An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Sci Rep* **6**, 18723.

Hao, D. & Yeoman, M.M. (1998). Evidence in favour of an oxidative *N*-demethylation of nicotine to nornicotine in tobacco cell cultures. *J Plant Physiol* **152**, 420–426.

Hapala, I. (1997). Breaking the barrier: methods for reversible permeabilization of cellular membranes. *Crit Rev Biotechnol* **17**, 105–122.

Hashimoto, T. & Yamada, Y. (1994). Alkaloid biogenesis: molecular aspects. *Annu Rev Plant Physiol Plant Mol Biol* **45**, 257–285.

Heim, W.G., Sykes, K.A., Hildreth, S.B., Sun, J., Lu, R.H. & Jelesko, J.G. (2007). Cloning and characterization of a *Nicotiana tabacum* methylputrescine oxidase transcript. *Phytochemistry* **68**, 454–463.

Ishikawa, H., Colby, D.A., Seto, S., Va, P., Tam, A., Kakei, H., Rayl, T.J., Hwang, I. & Boger, D.L. (2010). Total synthesis of vinblastine, vincristine, related natural products, and key structural analogues. *J Am Chem Soc* **131**, 4904–4916.

Jimenez-Garcia, S.N., Vazquez-Cruz, M.A., Guevara-Gonzalez, R.G., Torres-Pacheco, I., Cruz-Hernandez, A. & Feregrino-Perez, A.A. (2013). Current approaches for enhanced expression of secondary metabolites as bioactive compounds in plants for agronomic and human health purposes - A review. *Pol J Food Nutr Sci* **63**, 67–78.

Kajikawa, M., Shoji, T., Kato, A. & Hashimoto, T. (2011). Vacuole-localized berberine bridge enzyme-like proteins are required for a late step of nicotine biosynthesis in tobacco. *Plant Physiol* **155**, 2010–2022.

Karimi, M., Inzé, D. & Depicker, A. (2002). GATEWAYTM vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci* **7**, 193–195.

Katoh, A., Shoji, T. & Hashimoto, T. (2007). Molecular cloning of N-methylputrescine oxidase from tobacco. *Plant Cell Physiol* **48**, 550–554.

Katoh, A., Uenohara, K., Akita, M. & Hashimoto, T. (2006). Early steps in the biosynthesis of NAD in *Arabidopsis* start with aspartate and occur in the plastid. *Plant Physiol* **141,** 851–857.

Katsuyama, Y., Funa, N., Miyahisa, I. & Horinouchi, S. (2007). Synthesis of unnatural flavonoids and stilbenes by exploiting the plant biosynthetic pathway in *Escherichia coli*. *Chem Biol* **14**, 613–621.

Knorr, D. & Angersbach, A. (1998). Impact of high-intensity electric field pulses on plant membrane permeabilization. *Trends Food Sci Technol* **9**, 185–191.

Kovacs, K., Zhang, L., Linforth, R.S.T., Whittaker, B., Hayes, C.J. & Fray, R.G. (2007). Redirection of carotenoid metabolism for the efficient production of taxadiene [taxa-4(5),11(12)-diene] in transgenic tomato fruit. *Transgenic Res* **16**, 121–126.

Lewis, R.S., Jack, A.M., Morris, J.W., Robert, V.J.M., Gavilano, L.B., Siminszky, B., Bush, L.P., Hayes, A.J. & Dewey, R.E. (2008). RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. *Plant Biotech J* **6**, 346–354.

Livak, K.J. & Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408

Lopez-Meyer, M., Nessler, C.L. & McKnight, T.D. (1994). Sites of accumulation of the antitumor alkaloid camptothecin in *Camptotheca acuminata*. *Planta Med* **60**, 558–560.

Maisch, J., Kreppenhofer, K., Büchler, S., Merle, C., Sobich, S., Görling, B., Luy, B., Ahrens, R., Guber, A.E. & Nick, P. (2016). Time-resolved NMR metabolomics of plant cells based on a microfluidic chip. *J Plant Physiol* **200**, 28–34.

Maisch, J. & Nick, P. (2007). Actin is involved in auxin-dependent patterning. *Plant Physiol* **143**, 1695–1704.

Mann, J. (1992). Murder, magic and medicine. *Oxford University Press*, *London*.

Mehrotra, S., Srivastava, V., Rahman, L.U. & Kukreja, A.K. (2015). Hairy root biotechnology—indicative timeline to understand missing links and future outlook. *Protoplasma* **252**, 1189–1201.

Murad, L., Yoong Lim, K., Christopodulou, V., Matyasek, R., Lichtenstein, C.P., Kovarik, A. & Leitch, A.R. (2002). The origin of tobacco's T genome is traced to a particular lineage within *Nicotiana tomentosiformis* (Solanaceae). *Am J Bot* **89**, 921–928.

Murata, J., Roepke, J., Gordon, H. & De Luca, V. (2008). The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. *Plant Cell* **20**, 524–542.

Naconsie, M., Kato, K., Shoji, T. & Hashimoto, T. (2014). Molecular evolution of N-methylputrescine oxidase in tobacco. *Plant Cell Physiol* **55**, 436–444.

Nagata, T., Nemoto, Y. & Hasezawa, S. (1992). Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. *Int Rev Cytol* **132**, 1–30.

Nakajima, K. & Hashimoto, T. (1999). Two tropinone reductases, that catalyze opposite stereospecific reductions in tropane alkaloid biosynthesis, are localized in plant root with different cell-specific patterns. *Plant Cell Physiol* **40**, 1099–1107.

Nick, P., Heuing, A. & Ehmann, B. (2000). Plant chaperonins: a role in microtubule-dependent wall formation? *Protoplasma* **211**, 234–244.

Nicolaou, K.C., Yang, Z., Liu, J.J., Ueno, H., Nantermet, P.G., Guy, R.K., Claiborne, C.F., Renaud, J., Couladouros, E.A., Paulvannan, K. & Sorens, E.J. (1994). Total synthesis of taxol. *Nature* **367**, 630–634.

Noctor, G., Queval, G. & Gakière, B. (2006). NAD(P) synthesis and pyridine nucleotide cycling in plants and their potential importance in stress conditions. *J Exp Bot* **57**, 1603–1620.

Odell, L.R., Skopec, J. & McCluskey, A. (2008). Isolation and identification of unique marker compounds from the Tasmanian poppy *Papaver somniferum* N. Implications for the identification of illicit heroin of Tasmanian origin. *Forensic Sci Int* **175**, 202–208.

Osbourn, A.E. & Lanzotti, V. (2009). Plant-derived Natural Product; Synthesis, Function, and Application. *Springer, New York*.

Pais, M. S.S., Mavituna, F. & Novais, J.M. (1988). Plant Cell Biotechnology. *Springer, Berlin, Heidelberg.*

Park, C. & Martinez, B.C. (1992). Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by dimethyl sulfoxide (DMSO) while preserving cell viability and growth. *Biotechnol Bioeng* **40**, 459–464.

Patras, A., Choudhary, P. & Rawson, A. (2017). Recovery of Primary and Secondary Plant Metabolites by Pulsed Electric Field Treatment. *Handbook of Electroporation,* 1–21.

Thimmaraju, R., Bhagyalakshmi, N., Narayan, M.S. & Ravishankar, G.A. (2003). Kinetics of pigment release from hairy root cultures of *Beta vulgaris* under the influence of pH, sonication, temperature and oxygen stress. *Process Biochem* **38**, 1069–1076.

Roberts, M.F. & Wink, M. (1998). Alkaloids: Biochemistry, ecological functions and medical applications. *Plenum Press*, *New York*.

Roberts, S.C. (2007). Production and engineering of terpenoids in plant cell culture. *Nat Chem Biol* **3**, 387–395.

Sack, M., Schultheiss, C. & Bluhm, H. (2005). Triggered Marx Generators for the Industrial-Scale electroporation of sugar beets, *IEEE Trans Ind Appl* **41**, 707–714.

Sato, F., Hashimoto, T., Hachiya, A., Tamura, K., Choi, K.B., Morishige, T., Fujimoto, H. & Yamada, Y. (2001). Metabolic engineering of plant alkaloid biosynthesis. *Proc Natl Acad Sci U S A* **98**, 367–372.

Schmidt, G.W. & Delaney, S.K. (2010). Stable internal reference genes for normalization of real-time RT-PCR in tobacco (*Nicotiana tabacum*) during development and abiotic stress. *Mol Genet Genomics* **283**, 233–241.

Schwab, W., Davidovich-Rikanati, R. & Lewinsohn, E. (2008). Biosynthesis of plant-derived flavor compounds. *Plant J* **54**, 712–732.

Shoji, T. & Hashimoto, T. (2008). Why does anatabine, but not nicotine, accumulate in jasmonate-elicited cultured tobacco BY-2 cells? *Plant Cell Physiol* **49**, 1209–1216.

Shoji, T., Inai, K., Yazaki, Y., Sato, Y., Takase, H., Shitan, N., Yazaki, K., Goto, Y., Toyooka, K., Matsuoka, K. & Hashimoto, T. (2008). Multidrug and toxic compound extrusion-type transporters implicated in vacuolar sequestration of nicotine in tobacco roots. *Plant Physiol* **149**, 708–718.

Siminszky, B., Gavilano, L., Bowen, S.W. & Dewey, R.E. (2005). Conversion of nicotine to nornicotine in *Nicotiana tabacum* is mediated by CYP82E4, a cytochrome P450 monooxygenase. *Proc Natl Acad Sci U S A* **102**, 14919–14924.

Sinclair, S.J., Murphy, K.J., Birch, C.D. & Hamill, J.D. (2000). Molecular characterization of quinolinate phosphoribosyltransferase (QPRTase) in *Nicotiana*. *Plant Mol Biol* **44**, 603–617.

St-Pierre, B., Vazquez-Flota, F. & De Luca V. (1999). Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. *Plant Cell* **11**, 887–900.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R. & Baldwin, I. T. (2004). Nicotine's defensive function in nature. *PLoS Biol* **2**, 1074–1080.

Subroto, M.A., Kwok, K.H., Hamill, J.D. & Doran, P. M. (1995). Co-culture of genetically transformed roots and shoots for synthesis, translocation, and biotransformation of secondary metabolites. *Biotechnol* **49**, 481–494.

Trehy, M.L., Ye, W., Hadwiger, M.E., Moore, T.W., Allgire, J.F., Woodruff, J.T., Ahadi, S.S., Black, J.C. & Westenberger, B.J. (2011). Analysis of electronic cigarette cartridges, refill solutions, and smoke for nicotine and nicotine related impurities. *J Liq Chrom Relat Tech* **34,** 1442–1458.

Verpoorte, R., Van Der Hejden, R., Ten Hoopen, H.J.G. & Memelink, J. (1999). Metabolic engineering of plant secondary metabolite pathways for the production of fine chemicals. *Biotechnol Lett* **21**, 467–479.

White, J.A., Pliquett, U. & Blackmore, P.F. (2011). Plasma membrane charging of Jurkat cells by nanosecond pulsed electric fields. *Eur Biophys J* **40**, 947–957.

Wilson, S.A. & Roberts, S.C. (2012). Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotech J* **10**, 249–268.

Wink, M. (1988). Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor Appl Gen* **75**, 225–33.

Wink, M. (2000). Interference of alkaloids with neuroreceptors and ion channels, in Bioactive Natural Products. Atta-Ur-Rahman (Ed.), *Studies in Natural Products Chemistry* **21**, 3–122.

Wink, M. (2007). Molecular modes of action of cytotoxic alkaloids: From DNA intercalation, spindle poisoning, topoisomerase inhibition to apoptosis and multiple drug resistance. *Alkaloids Chem Biol* **64**, 1-47.

Wink, M. (2010). Functions and biotechnology of plant secondary metabolites. *Annual Plant Reviews* **39**.

Ye, H., Huang, L.L., Chen, S.D. & Zhong, J.J. (2004). Pulsed electric field stimulates plant secondary metabolism in suspension cultures of *Taxus chinensis*. *Biotechnol Bioeng* **88**, 788-795.

Yukawa, M., Tsudzuki, T. & Sugiura, M. (2006). The chloroplast genome of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*: Complete sequencing confirms that the *Nicotiana sylvestris* progenitor is the maternal genome donor of *Nicotiana tabacum*. *Mol Genet Genomics* **275**, 367–373.

Zhang, H., Boghigian, B.A., Armando, J, & Pfeifer, B.A. (2011). Methods and options for the heterologous production of complex natural products. *Nat Prod Rep* **28**, 125–151.

Zhang, Y., Li, S.Z., Li, J., Pan, X., Cahoon, R.E., Jaworski, J.G., Wang, X., Jez, J.M., Chen, F. & Yu, O. (2006). Using unnatural protein fusions to engineer resveratrol biosynthesis in yeast and mammalian cells. *J Am Chem Soc* **128**, 13030–13031.

Ziegler, J. & Facchini, P.J. (2008). Alkaloid biosynthesis: metabolism and trafficking. *Annu. Rev. Plant Biol* **59**, 735–769.

5 Appendix

5.1 Coding sequences of genes under investigation

5.1.1 *Ntab*MPO (790 AA GenBank: BAF49519.1)

MATTKQKVTAPSPSPSSSTASCCPSTSILRREATAAIAVVGDGLQNWTNIPSVDEKQKKTAS
SALASLPTTEPLSTNTSTKGIQIMTRAQTCHPLDPLSAAEISVAVATVRAAGETPEVRDGMRF
IEVVLVEPDKSVVALADAYFFPPFQSSLMPRTKGGSQIPTKLPPRRARLIVYNKKTNETSIWIV
ELNEVHAAARGGHHRGKVIASNVVPDVQPPIDAQEYAECEAVVKSYPPFRDAMRRRGIDDL
DLVMVDPWCVGYHSEADAPSRRLAKPLVFCRTESDCPMENGYARPVEGIYVLVDVQNMKII
EFEDRKLVPLPPVDPLRNYTAGETRGGVDRSDVKPLHIIQPEGPSFRISGNYVEWQKWNFRI
GFTPREGLVIHSVAYLDGSRGRRPIAHRLSFVEMVVPYGDPNDPHYRKNAFDAGEDGLGK
NAHSLKRGCDCLGYIKYFDAHFTNFTGGVETTENCVCLHEEDHGMLWKHQDWRTGLAEVR
RSRRLTVSFVCTVANYEYAFYWHFYQDGKIEAEVKLTGILSLGALQPGEYRKYGTTILPGLY
APVHQHFFVARMNMAVDCKPGEAHNQVVEVNVKVEEPGKENVHNNAFYAEETLLRSELQA
MRDCDPFSARHWIVRNTRTVNRTGQLTGYKLVPGPNCLPLAGPEAKFLRRAAFLKHNLWV
TQYAPGEDFPGGEFPNQNPRVGEGLASWVKQDRPLEESDIVLWYIFGITHVPRLEDWPVM
PVEHIGFVLQPHGYFNCSPAVDVPPPFACDSESRDSDVTETSVAKSTATSLLAKL

5.1.2 *Ntab*CYP82E5*v*2 (517 AA GenBank:ABX56037.1)

MVSPVEAIVGLVTLTLLFYFLWPKKFQIPSKPLPPKIPGGWPVIGHLFYFDDDGDDRPLARKL
GDLADKYGPVFTFRLGLPLVLVVSSYEAVKDCFSTNDAIFSNRPAFLYGEYLGYSNAMLFLT
KYGPYWRKNRKLVIQEVLSASRLEKLKHVRFGKIQTSIKSLYTRIDGNSSTINLTDWLEELNF
GLIVKMIAGKNYESGKGDEQVERFRKAFKDFIILSMEFVLWDAFPIPLFKWVDFQGHVKAMK
RTFKDIDSVFQNWLEEHVKKREKMEVNAQGNEQDFIDVVLSKMSNEYLDEGYSRDTVIKAT

VFSLVLDAADTVALHMNWGMALLINNQHALKKAQEEIDKKVGKERWVEESDIKDLVYLQAIV
KEVLRLYPPGPLLVPHENVEDCVVSGYHIPKGTRLFANVMKLQRDPKLWSNPDKFDPERFF
ADDIDYRGQHYEFIPFGSGRRSCPGMTYALQVEHLTIAHLIQGFNYKTPNDEPLDMKEGAGL
TIRKVNPVEVTITARLAPELY

5.1.3 *Ntom*CYP82E4 (517 AA GenBank: ABM46920.1)

MLSPIEAIVGLVTFTFLFYFLWTKKSQKPSKPLPPKIPGGWPVIGHLFHFNDDGDDRPLARKL
GDLADKYGPVFTFRLGLPLVLVVSSYEAVKDCFSTNDAIFSNRPAFLYGDYLGYNNAMLFLA
NYGPYWRKNRKLVIQEVLSASRLEKFKHVRFARIQASIKNLYTRIDGNSSTINLTDWLEELNF
GLIVKMIAGKNYESGKGDEQVERFKKAFKDFMILSMEFVLWDAFPIPLFKWVDFQGHVKAM
KRTFKDIDSVFQNWLEEHINKREKMEVNAEGNEQDFIDVVLSKMSNEYLGEGYSRDTVIKAT
VFSLVLDAADTVALHINWGMALLINNQKALTKAQEEIDTKVGKDRWVEESDIKDLVYLQAIVK
EVLRLYPPGPLLVPHENVEDCVVSGYHIPKGTRLFANVMKLQRDPKLWSDPDTFDPERFIAT
DIDFRGQYYKYIPFGSGRRSCPGMTYALQVEHLTMAHLIQGFNYRTPNDEPLDMKEGAGITI
RKVNPVELIIAPRLAPELY

5.2 PCR reaction for Gateway® cloning

Amplification of *Ntab*MPO and *Ntab*CYP82E5*v2* from BY-2 cDNA and *Ntom*CYP82E4 from *N. tomentosiformis* leave cDNA

Ingredients	Volume in µl
Template DNA (cDNA)	1
Forward primer (10 µM)	2
N or C- terminal Reverse primer (10 µM)	2
dNTPs (10 mM)	1
HF-Buffer (5x)	10
NEB Phusion-Polymerase	0.5
Betaine (5M)	5
DMSO	2
ddH_2O	26.5
Total Volume	**50**

PCR Program:

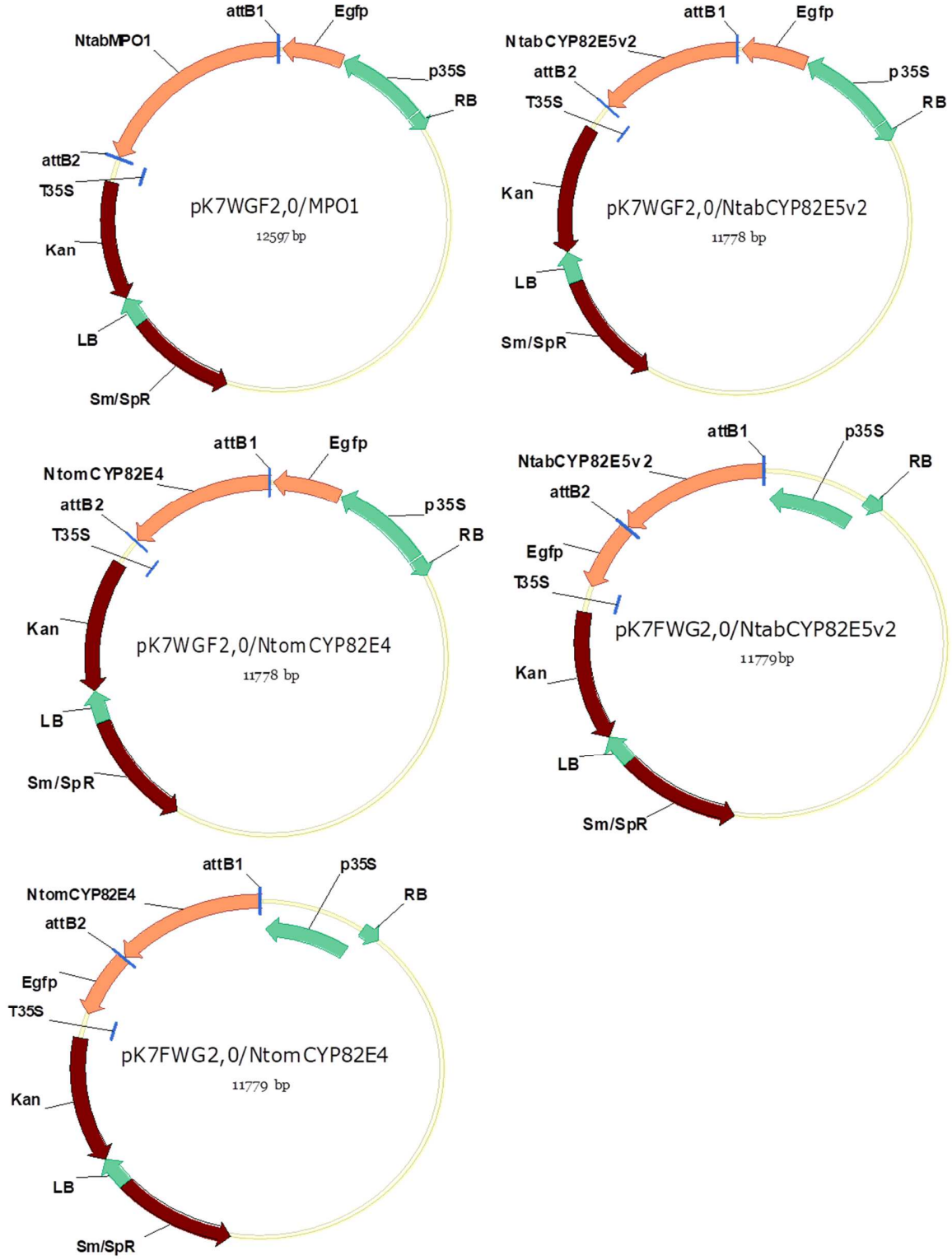
Pre-Heating		98 °C, 30 sec
38 cycles	Denaturation	98 °C, 10 sec
	Annealing	56 °C, 35 sec
	Extension	72 °C, 45 sec
Final Extension		72 °C, 7 min
End		4 °C, Hold

5.3 Primers used for Gateway® cloning

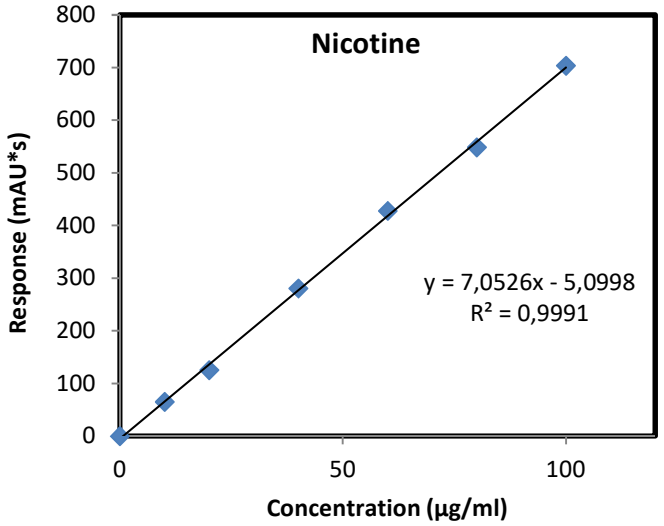
Primers	Sequences (5'- 3')
***Ntab*MPO1 Fw**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCACT ACTAAACAGAAAGTG
***Ntab*MPO1 Re N-terminal**	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAAGCTTG GCCAGCAAGC
***Ntab*CYP82E5*v2* Fw**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTTTCT CCCATAGAAGC
***Ntab*CYP82E5*v2* Re N-terminal**	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATAAAGC TCAGGTGCCAG
***Ntab*CYP82E5*v2* Re C-terminal**	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAAAGCTCA GGTGCCAG
***Ntom*CYP82E4 Fw**	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTTTCT CCCATAGAAGC
***Ntom*CYP82E4 Re N-terminal**	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATAAAGC TCAGGTGCCAG
***Ntom*CYP82E4 Re C-terminal**	GGGGACCACTTTGTACAAGAAAGCTGGGTCATAAAGCTCA GGTGCCAG

5.4 Gateway® destination vectors constructed

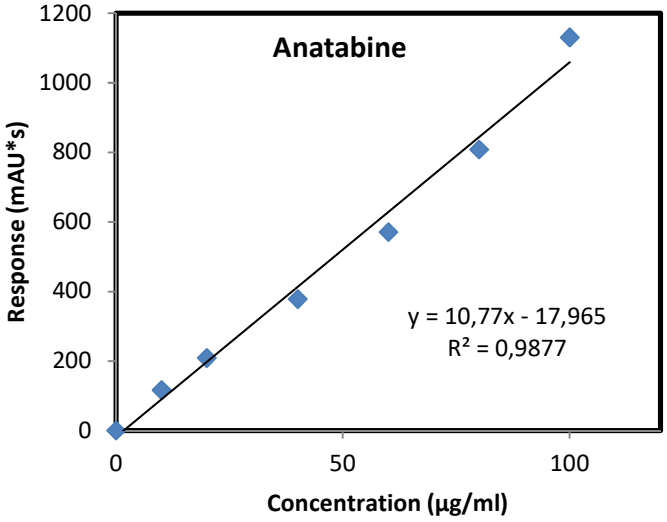
Overview of the constructs generated from this study:


attB1
Egfp
NtabMPO1
p35S
RB
attB2
T35S
pK7WGF2,0/MPO1
12597 bp
Kan
LB
Sm/SpR
attB1
Egfp
NtabCYP82E5v2
attB2
p35S
T35S
RB
Kan
pK7WGF2,0/NtabCYP82E5v2
11778 bp
LB
Sm/SpR
attB1
Egfp
NtomCYP82E4
attB2
p35S
T35S
RB
Kan
pK7WGF2,0/NtomCYP82E4
11778 bp
LB
Sm/SpR
attB1
p35S
NtabCYP82E5v2
RB
attB2
Egfp
T35S
pK7FWG2,0/NtabCYP82E5v2
11779bp
Kan
LB
Sm/SpR
attB1
p35S
NtomCYP82E4
RB
attB2
Egfp
T35S
pK7FWG2,0/NtomCYP82E4
11779 bp
Kan
LB
Sm/SpR

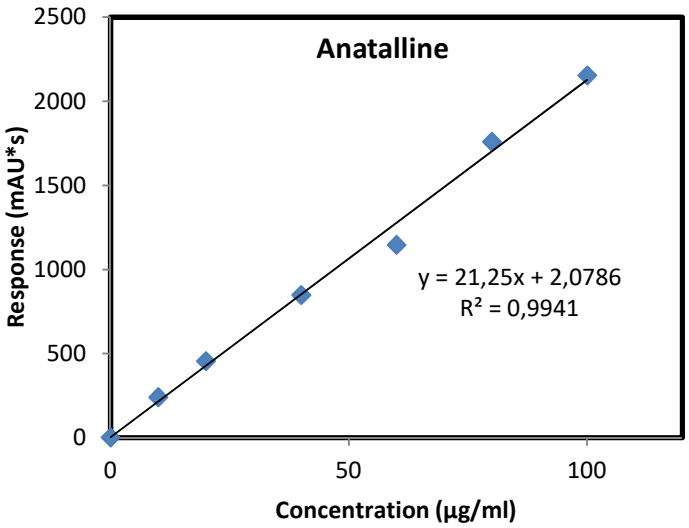
5.5 Calibration curves for quantitative analysis in HPLC



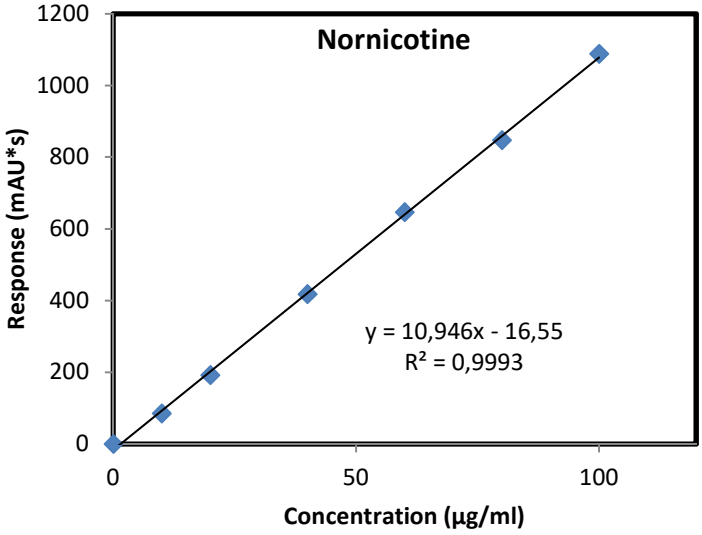
Nicotine (µg ml^{-1})	Peak area (mAU*s)
0	0
10	64.8994
20	125.398
40	280.4696
60	427.6054
80	548.6946
100	703.5349



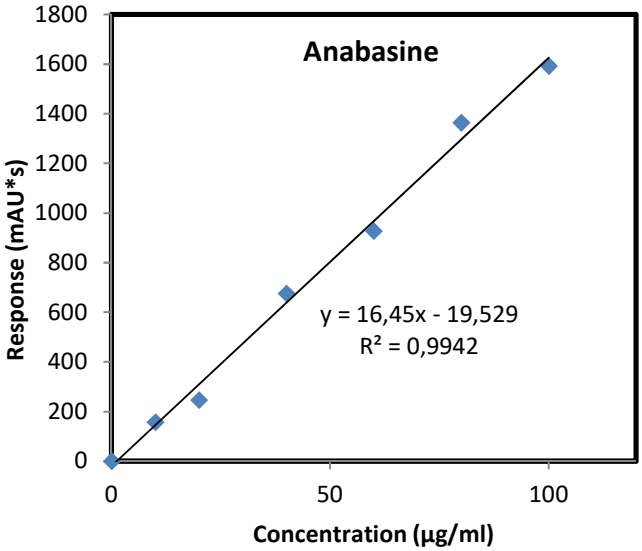
Anatabine (µg ml^{-1})	Peak area (mAU*s)
0	0
10	116.5218
20	208.2882
40	378.4631
60	570.6232
80	808.4061
100	1130.788



Anatalline (µg ml^{-1})	Peak area (mAU*s)
0	0
10	240.3472
20	455.2994
40	847.6521
60	1146.454
80	1758.227
100	2154.119



Nornicotine (µg ml^{-1})	Peak area (mAU*s)
0	0
10	85.44881
20	192.4632
40	418.1119
60	646.2044
80	847.0818
100	1088.069



Anabasine (µg ml^{-1})	Peak area (mAU*s)
0	0
10	157.6406
20	246.5203
40	675.7823
60	926.4616
80	1363.808
100	1592.659