Identification, characterisation and expression of early biosynthetic genes from Artemisia annua

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Chapter 7

Summary, general discussion and future perspectives

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Artemisia annua L. (*A. Annua*) is an annual herb growing in temperate and tropical regions of the world. It has the unique ability to produce an antimalarial compound, artemisinin, which has proved to be remarkably efficient in killing the malaria parasite while at the same time showing very minor side effects. The uniqueness of this compound and plant can be understood in the fact that *A. annua* is the only species capable of producing the artemisinin. From the moment when artemisinin was isolated and identified as the active ingredient against malaria in the 1970's, the natural compound has been the focus for intense research in organic chemistry and biochemistry. However, the complete synthesis of artemisinin proved to be a daunting task and was deemed uneconomical.

The natural yield of artemisinin is very low; levels ranging around 0.2-0.8 % artemisinin (dry weight) are routinely reported. There are several breeding programs currently in progress which aim to increase the production of artemisinin *in planta* without the use of genetic engineering. This is a plausible way forward but this strategy is hampered by the same disadvantages as the original plant: unstable supply due to natural weather variations, poor final yield and devotion of fertile soil to non-food production. An alternative solution is the use of microorganisms as production platforms for artemisinin. This is achieved by identification of the genes in the biosynthetic pathway of artemisinin in *A. annua* and their implementation in a safe microorganism such as *Saccharomyces cerevisiae* (*S. cerevisiae*). This allows for a stable, high, economical and controlled production of the drug. Furthermore, the knowledge of the genes involved in the pathway opens up for potential genetic improvement of the plant in terms of artemisinin yield should such an aim ever be accepted and desirable.

In this thesis, the biosynthetic genes of artemisinin in *A. annua* are identified and their potential in the heterologous expression platform

S. cerevisiae is investigated. **Chapter 1 and 2** provides background information of the history and chemistry of artemisinin. The suitability of *S. cerevisiae* as a production platform for artemisinin is discussed as well and examples are given for the implementation of synthetic biology.

Isolation of a broad substrate reductase from *A. annua* with implications in the artemisinin biosynthetic pathway

In **chapter 3** and **chapter 4**, data are presented relating to a new reductase isolated from the plant *A. annua.* In an effort to isolate genes involved in the biosynthetic pathway of artemisinin an EST library was sequenced and analyzed against other EST libraries and the published database collected at blastX (NCBI). A first analysis using blastX revealed 19 candidates out of 2180 EST sequences which possessed characteristic amino acid combinations of the reductase family. However, the enzymatic identities of the reductases remained largely obscure as the experimentally validated knowledge accumulated in the public database is very limited for plant reductases. To create a ranking list with the most promising candidates at the top, comparisons with EST libraries from tomato, potato, rice, *Arabidopsis thaliana* (*A. thaliana*), lettuce and sunflower EST libraries were pursued. To distinguish between ubiquitous reductases and reductases likely to be relevant in terpenoid biosynthesis, the following strategy was adopted: Reductases with a high similarity with lettuce and sunflower but significantly lower similarity with the non-Asteraceae outgroups potato (*Solanaceae*), tomato (*Solanaceae*), rice (*Poaceae*) and *A. thaliana* (*Brassicaceae*) were considered to be promising candidates in the terpenoid biosynthesis. Based on the bioinformatics analysis a single candidate, *red1*, was picked for further experimental studies. Several substrates, all members of the chemical class terpenoids, were tested for conversion using the cofactor NADP(H). The results presented in **chapter 3** clearly show that Red1 is a broad substrate oxidoreductase belonging to the short chain dehydrogenase/reductase

(SDR) protein family. It is particularly efficiently reducing (-)-menthone to (+)-neomenthol and can oxidize (+)-neomenthol to (-)-menthol via the intermediate (-)-menthone. This at efficiency rates rivalling those of the industrially important plant *Mentha x piperitea*. The menthone:neomenthol reductase of *Mentha x piperitea* attain a k_{cat}/K_m of 89 (M^{-1}, s^{-1}) which is the previously highest value recorded for a plant reductase whereas Red1 display a k_{cat}/K_m of 83620 (M^{-1}, s^{-1}), a 940 fold difference in efficiency. Other substrates such as dihydrocarvone and perilla aldehyde are converted by Red1 as well. All these compounds belong to the terpenoid subgroup monoterpenoids (C_5). However, the terpenoids in the artemisinin biosynthetic pathway belong to the subgroup sesquiterpenoids (C_{15}). A wider range of sesquiterpenoids were tested for conversion by Red1 but were consistently refused. As perilla aldehyde has a chemical similarity to artemisinic aldehyde and dihydroartemisinic aldehyde, these substrates were tested for conversion by Red1 (**chapter 4**). Red1 is a potent reducer of dihydroartemisinic aldehyde and will produce dihydroartemisinic alcohol while refusing the alcohol as a substrate. Red1 produce a k_{cat}/K_m value of 4119 (M^{-1}, s^{-1}) to be compared with another devoted reductase in the pathway, the carbon double bond reductase Dbr2 which show a k_{cat}/K_m value of 0.14 (M^{-1}, s^{-1}).

The impact of Red1 in the biosynthetic pathway of artemisinin can be better understood if one thinks in networks rather than simple straight down stream pathways. The effect of Red1 is to remove a valuable intermediate in the pathway, the dihydroartemisinic aldehyde. Currently there is only one reported enzyme which is able, albeit at low levels, to convert the alcohol back into the aldehyde so that it in its turn can be used for the production of artemisinin (**chapter 5**). We can speculate about the role of Red1 in the pathway; it may serve as a regulator of the flux or it might simply be one of many tasks performed *in planta* and the reduction of the aldehyde may simply be a, for our purpose, misfortunate side effect. It is, however, likely that the expression of Red1 on the artemisinin

production in *A. annua* is detrimental to the artemisinin yield. Currently a project aiming to develop a transformation system for *A. annua* is in progress and it is estimated that in the spring 2011 this system can be used to transform *A. annua* with a siRNA construct which will downregulate the expression of red1. The effect thereof is expected to lead to increased levels of artemisinin in the plant.

Investigating *Saccharomyces cerevisiae* as a drug production platform

Plants aside, the knowledge of Red1 and its function reminds us that it is important to investigate potential host platforms for their metabolic reaction on heterologous compounds. It may well be that the host already contains native genes that will perform functions in the heterologous pathway. If such genes can be identified, fewer foreign genes need to be introduced into the host and hence the stress on the organism is limited. To maximize the yield for the desired product it is important to reduce the stress on the organism. *S. cerevisiae* is a convenient host which is well characterized and which also produces the necessary precursors for artemisinin production. One way to adapt it to produce artemisinin is simply to transfer all the biosynthetic genes of the pathway from *A. annua* to the yeast. This, however, is putting a considerable stress on the organism which aims by various means to limit the production of drug intermediates either by excluding the genes or by over expressing stress response genes which results in an efflux of drug intermediates from the host. One strategy to lessen the stress response caused by expression of foreign genes is to employ the function of endogenous genes and to use them instead of foreign genes for production of the heterologous intermediates. In **chapter 5** is described how oxygen limitation and the choice of carbon source can change the genetic expression pattern of *S. cerevisiae* in such a way that it can convert an early intermediate into an interesting reduced intermediate which is dedicated to the production of artemisinin.

Future perspectives

A project aimed at adapting a protein fishing method has been developed in collaboration with RIKEN and Yokohama City University (**chapter 6**). The goal is to speed up the protein discovery procedure by using a reverse genetics approach. By coating beads with the substrate of the unknown protein and applying a crude extract of plant proteins it is possible to purify only the proteins that react with the substrate coated beads. Artemisinic acid is the last proven biosynthetic step in the artemisinin pathway. A pilot project using artemisinic acid coated on the beads and crude protein extract from *A. annua* produced two distinct protein bands on an SDS-PAGE gel. *De novo s*equencing of these proteins is currently in progress and the resulting amino acid sequence will be used to pick up candidates from the EST library. In parallel, biochemical work was performed to establish the next biosynthetic product after artemisinic acid and a biochemical test system was designed for later confirmation of enzymatic activity of the expressed cloned candidate genes.

Furthermore, the potential of different organelles in *S. cerevisiae* was delineated. Currently, synthetic biology efforts are focusing on manipulation in the cytoplasm of the host organism due to the easiness of this procedure. This ignores the potential of the mitochondria and peroxisomes as production places of the artemisinin intermediates. Mitochondria have a well documented large pool of the immediate precursor to the first artemisinin intermediate amorpha-4,11-diene. The prevalence of the necessary precursor in peroxisomes is uncertain although in human cells this precursor does exist. Constructs expressing amorphadiene synthase were designed in such a way as to target the synthase to either the cytoplasm, the mitochondria or the peroxisome. It would be interesting to see if both organelles can significantly contribute to the artemisinin intermediate production. It would be a benefit if both the mitochondria and the peroxisome could be used as they complement

each other in terms of carbon utility and hence allow for a maximized utilization of input material as the yeast would be able to produce the artemisinin intermediate during the entire batch cycle.

The suitability of *S. cerevisiae* was confirmed as a first choice for pilot studies. Initially, studies on *Xanthophyllomyces dendrorhous* and its ability to express amorphadiene synthase and produce amorpha-4,11-diene, the first committed compound in the artemisinin biosynthetic pathway, were performed. As the genetic tools are very limited for this organism, the gene was incorporated into redundant rRNA. Several copies of the genes were shown to be incorporated into the host but the expression of the enzyme proved irregular. It is possible that this is due to silencing of the redundant rRNA and possible the host can sense the foreign DNA and class it as a threat and deliberately silence that area. Currently there is no way of controlling which DNA stretch in rRNA will be active or inactive. At this moment there is a project in progress aiming to develop new genetic tools for *X. dendrorhous* and it is our hope that this organism will prove a better host than *S. cerevisiae* in terms of artemisinin intermediate yields.