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

Rydén, Anna-Margareta

Chapter 4

The molecular cloning of dihydroartemisinic aldehyde reductase and its implication in artemisinin biosynthesis in *Artemisia annua*

Anna-Margareta Rydén[1,2,3], Carolien Ruyter-Spira[2], Wim Quax[1], Hiroyuki Osada[4], Toshiya Muranaka[3], Oliver Kayser[5] and Harro Bouwmeester[6]

[1] Pharmaceutical biology, University of Groningen, Groningen, The Netherlands
[2] Plant Research International, Wageningen, The Netherlands
[3] Kihara Institute for Biological Research, Yokohama City University, Yokohama, Japan
[4] Chemical Biology Department, Antibiotics Laboratory, Advanced Science Institute, RIKEN, Saitama, Japan
[5] Technical Biochemistry, Technical University of Dortmund, Dortmund, Germany
[6] Laboratory of Plant Physiology, Wageningen University, Wageningen, The Netherlands

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Abstract

A key point in the biosynthesis of the antimalarial drug artemisinin is the formation of dihydroartemisinic aldehyde which reprecents the key difference between chemotype specific pathways. This key intermediate is the substrate for several competing enzymes some of which increase the metabolic flux towards artemisinin and some of which – as we show in the precent study – may have a negative impact on artemisinin production. In an effort to understand the biosynthetic network of artemisinin biosynthesis, extracts of *Artemisia annua* (*A. annua*) flowers were investigated and found to contain an enzyme activity competing in a negative sense with artemisinin biosynthesis. The enzyme, Red1, is a broad substrate oxidoreductase belonging to the short chain dehydrogenase/reductase family with high affinity for dihydroartemisinic aldehyde and valuable monoterpenoids. Spatial and temporal analysis of cDNA revealed *red1* to be trichome specific. The relevance of Red1 to artemisinin biosynthesis is discussed.

Introduction

Since the discovery four decades ago that artemisinin from the plant *Artemisia annua* L. (*A. annua*) is the active compound against malaria there has been a keen interest in synthesizing it or derivatives thereof (Rydén et al., 2007). More recently biochemical studies revealed the probable intermediates in the biosynthetic pathway (Bertea et al., 2005; Wallaart et al., 1999; Wallaart et al., 1999). Given the leads from these biochemical studies, EST libraries were sequenced in the quest for genes involved in the pathway (Mercke et al., 2000; Ro et al., 2006; Teoh et al., 2006; Wallaart et al., 2001) complemented with a reverse genetics approach (Zhang et al., 2008). Currently, breeding programmes as well as microbial heterologous pathway expression studies are developed to satisfy the need for a stable supply of the drug. The focus on artemisinin derived from the plant has been on growth condition improvement, harvesting methods and marker assisted breeding. Microbial production systems on the other hand have focused on identification of the biosynthetic genes in the linear pathway. These efforts led to the identification of the terpene cyclase amorpha-4,11-diene synthase which catalyses the first committed step of the biosynthetic pathway of artemisinin (figure. 1) (Bouwmeester et al., 1999; Wallaart et al., 2001). The second step is catalysed by the cytochrome P450 Cyp71av1 that oxidizes amorpha-4,11-diene at C12 in three consecutive steps (figure. 1) (Ro et al., 2006; Teoh et al., 2006). Release of artemisinic aldehyde, which is the product after the second oxygenation step, is allowing Dbr2, a carbon double bond reductase, to reduce the Δ11(13) carbon double bond thereby producing dihydroartemisinic aldehyde (Zhang et al., 2008) (figure. 1). Thus a solid understanding of the production of the early and late intermediates in the pathway has been developed. However, given the global aim of increased production of artemisinin, it is important to study bottlenecks in the pathway both *in planta* and in heterologous systems as well as the impact of competing biosynthetic pathways on artemisinin production.

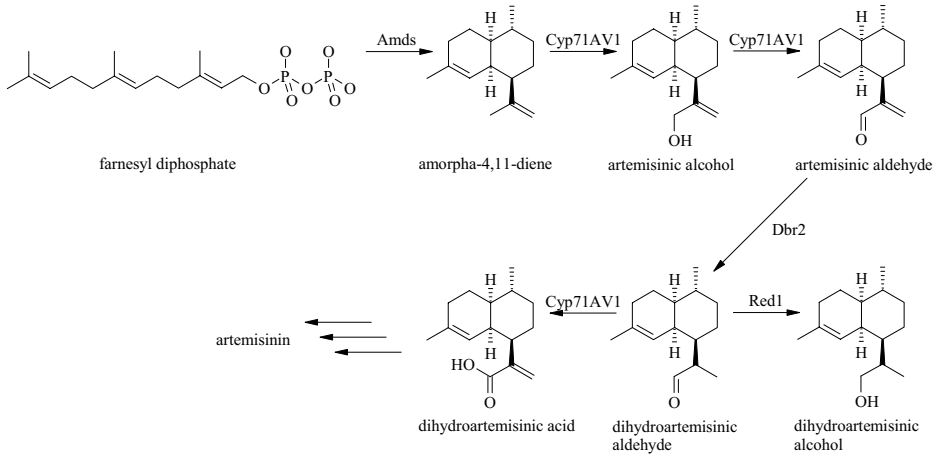

Figure 1. Biosynthetic pathway of artemisinin and the activity of Red1.

As part of an effort to understand the isoprenoid metabolism in *A. annua*, an EST library was employed to isolate genes involved in the biosynthetic pathway (Bertea et al., 2006). From the EST library, an oxidoreductase named *red1* was identified and cloned. Here we report on the kinetic characteristics of Red1 and its role in the biosynthetic pathway of artemisinin. The impact of Red1 on this biosynthetic network is discussed.

Results and Discussion

Several semi-quantitative PCR reactions were performed to compare the presence of transcripts in different tissues of *A. annua* (figure 2). The transcription patterns of other genes than *red1* relevant to the biosynthetic pathway of artemisinin were also investigated to be able to compare trends in transcription. Along with a spatial distribution of the transcripts, the temporal variable was investigated dividing tissues into two categories for leaves: young leaf and old leaf and

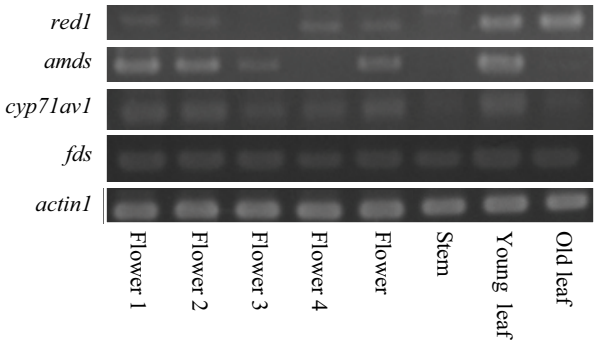

Figure 2. Semi-quantitative PCR on cDNA from *Artemisia annua*. *red1* – reductase 1, *amds* – amorpha-4,11-diene synthase, *cyp71av1* – cytochrome P450 71av1, *fds* – farnesyl diphosphate synthase.

four categories for flowers. In all tissues, *fds* was transcribed at similar levels (figure 2). This is an expected result as the product farnesyl diphosphate formed by the enzyme encoded by *fds*, is a common precursor of many ubiquitous molecules such as sterols and ubiquinones (Aharoni et al., 2005). The transcription of *amds*, which catalyses the first committed step of the artemisinin biosynthetic pathway by creating the amorphane skeleton from farnesyl diphosphate (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001), displayed a tissue and time dependent profile (Kim et al., 2008). The transcript levels of *amds* gradually decreased to undetectable levels in time in flowers and leaves while transcription in stems was not observed. The transcription of *cyp71av1* did not follow the same strict transcription profile as *amds* however it was confined in the same manner as *amds* to flowers and young leaves. In contrast to *amds*, *cyp71av1* was transcribed at a stable level throughout the life span of the flower. Transcription of *red1* occurred in flowers in a time dependent manner: it was high in buds, young flowers and old flowers with pollen but absent in completely open flowers without pollen (figure 2). Just as for transcription of *amds* and *cyp71av1*, there was no *red1* transcription in the stem. Just as for *amds, red1* transcription was high in young

leaves but in contrast to *amds,* it was also high in old leaves (figure 2). This is an expected result as it has been shown that amorpha-4,11-diene decease in time with maturing plant while dihydroartemisinic acid and artemisinin levels increase (Kim et al., 2008; Wallaart et al., 2000).

Analysis of *red1* with BlastX produced the enzymatically confirmed hits *Mentha x piperita* (*M. piperita*) (-)-isopiperitenone reductase, *M. piperita* (-)-menthone:(+)-neomenthol reductase (MNR) (Davis et al., 2005), *M. piperita* menthol dehydrogenase (MMR) (Davis, et al., 2005) and *Papaver bracteatum* salutaridine reductase (Geissler et al., 2007). These enzymes are categorized as belonging to the short chain dehydrogenase reductase (SDR) family (Kallberg et al., 2002; Oppermann et al., 2003; Persson et al., 2009). The common features of the SDR family are the N-terminal cofactor binding motif GxxxGxG (motif 1) and the catalytic domain YxxxK (motif 2) as well as the preference of NADP(H) over NAD(H) due to their basic lysine residue in motif 1 (Persson et al., 2003) which categorizes them as classical SDR enzymes. Consistent with the BlastX hits and biochemical studies, Red1 is categorized as belonging to the SDR family. Currently three cytosolic proteins involved in the biosynthetic pathway of artemisinin have been reported: Amds (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001), alcohol dehydrogenase 1 (Aldh1) (Teoh et al., 2007) and double bond reductase 2 (Dbr2) (Teoh et al., 2007; Zhang et al., 2008).

Biochemical studies of Red1 revealed it to be a broad substrate SDR family protein accepting among others menthone, menthol and neomenthol as substrates (Rydén et al., 2010) (table 1). Red1 accepts dihydroartemisninic aldehyde as a substrate converting it to dihydroartemisinic alcohol, but the closely similar substrate artemisinic aldehyde is not converted despite its close similarity with and greater chemical reactivity than dihydroartemisinic aldehyde (table 1, figure 3c-d).

Table 1. Apparent kinetic parameters for cytosolic reductases from *A. annua*, *Mentha x piperita* and *Carpoglyphus lactis*. Aldh1 – alcohol dehydrogenase 1 (*A. annua*) (Teoh et al., 2007), Dbr2 – Double bond reductase 2 (*A. annua*) (Zhang et al., 2008), GeDH – Geraniol dehydrogenase (*C. lactis*) (Noge, 2008), MNR – Menthone:neomenthol reductase (*Mentha x piperita*) (Davis et al., 2005), MMR – Menthone:menthol reductase (*Mentha x piperita*) (Davis et al., 2005), Red1 – Dihydroartemisinic aldehyde reductase (*A. annua*) (this study), n. r. – not reported.

Protein	v_{max} (pmol,s^{-1})	K_m (µM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1}, s^{-1})	Substrate	Product
Aldh1	n. r.	2.58	1.53	593000	AAA	AA
	n. r.	8.79	7.74	881000	DHAAA	DHAA
Dbr2	n. r.	19	2.6	0.14	AAA	DHAAA
	n. r.	790	1.8	0.0023	2-Cyclohexen-1-one	Cyclohexanone
	n. r.	650	0.86	0.0013	(+)-Carvone	(-)-Isodihydro-carvone, (-)-dihydrocarvone
GeDH	n. r.	51.0	2996	58800000	Geraniol	Geranial
MNR	n. r.	674 ± 78.0	0.06	89	(-)-Menthone	(+)-Neomenthol
	n. r.	> 1000	n. r.	n. r.	(+)-Isomenthone	(+)-Isomenthol
	n. r.	> 1000	n. r.	n. r.	(+)-Neomenthol	(-)-Menthone
MMR	n. r.	3.0 ± 0.6	0.6	200000	(-)-Menthone	(-)-Menthol
	n. r.	41 ± 5.0	n. r.	n. r.	(+)-Isomenthone	(+)-Neoisomenthol
Red1	24 ± 2.8	67 ± 15	0.28	4119	DHAAA	DHAAOH
	n. r.	> 1000	n. r.	n. r.	DHAAOH	DHAA
	n. r.	> 1000	n. r.	n. r.	AAA	AAOH
	52 ± 2.7	7.1 ± 1.4	0.6	83617	(-)-Menthone	(+)-Neomenthol
	79 ± 10	305 ± 101	0.9	2993	(+)-Neomenthol	(-)-Menthone

To further investigate the capability of Red1 to convert aldehyde substrates, perilla aldehyde was used as a substrate. Figure 3a and b

show that Red1 efficiently converts perilla aldehyde into its corresponding alcohol.

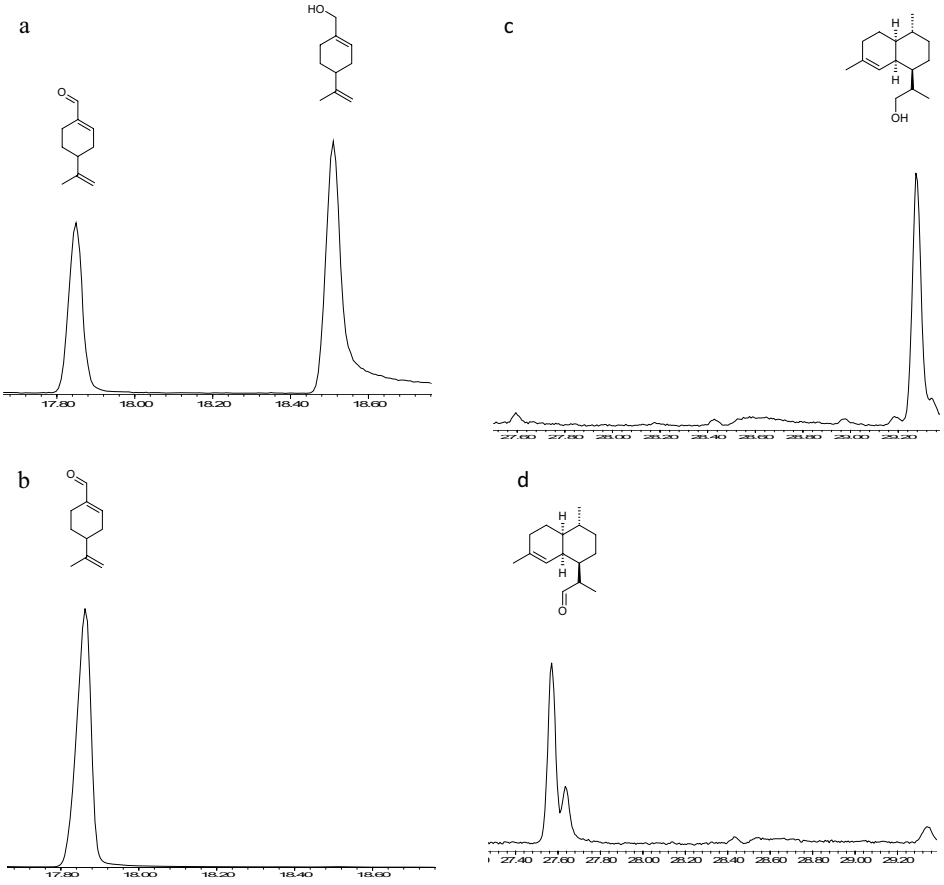

Figure 3. Substrate and product profile of Red1. a) Purified his-tagged Red1 with perilla aldehyde as substrate, b) Empty vector control with perilla aldehyde as substrate, c) Purified his-tagged Red1 with dihydroartemisinic aldehyde as substrate, d) Empty vector control with dihydroartemisinic aldehyde as substrate.

Also dihydroartemisinic alcohol, artemisinic aldehyde and artemisinic alcohol were investigated for their suitability as substrates for Red1 (data not shown), but no conversion activity could be detected using these substrates.

The apparent kinetic parameters of Red1 show that the monoterpenoid (-)-menthone is a preferred substrate (table 1) with K_m, k_{cat} and k_{cat}/K_m values of 7.1 µM, 0.6 s^{-1} and 83617 M^{-1}, s^{-1}, respectively. The sesquiterpenoid dihydroartemisinic aldehyde on the other hand is, albeit less efficiently than (-)-menthone, relatively efficiently converted into dihydroartemisinic alcohol. Interestingly the reverse reaction, oxidation of dihydroartemisinic alcohol to dihydroartemisinic aldehyde, could not be detected (table 1). The apparent kinetic parameters of Red1 converting dihydroartemisinic aldehyde were calculated to be K_m 67µM, k_{cat} 0.28 s^{-1} and k_{cat}/K_m 4119 M^{-1}, s^{-1}. These data show that Red1 is capable of efficient and specific reduction of ketone/aldehyde groups in dihydroartemisinic aldehyde as well as monoterpenoids.

Concluding remarks

The biosynthesis of artemisinin in *A. annua* is intensively studied due to its activity against the malaria parasite (Covello et al., 2007; Rydén et al., 2007; Zeng et al., 2008). The first committed step in the biosynthetic pathway, the cyclisation of farnesyl diphosphate to amorpha-4,11-diene (Bouwmeester et al., 1999; Mercke et al., 2000; Picaud et al., 2006; Wallaart et al., 2001) and the subsequent oxygenation to alcohol, aldehyde and acid are well characterized through biochemical studies on the plant (Bertea et al., 2005), cloning of responsible genes (Bouwmeester et al., 1999; Mercke et al., 2000; Teoh et al., 2006; Wallaart et al., 2001; Zhang et al., 2008) and heterologous characterisation of pathway intermediates (Ro et al., 2006; Zhang et al., 2008) (figure 1). There are two chemotypes of *A. annua* one the artemisinic acid the other a dihydroartemisinic acid accumulating chemotype, which have been shown to have low and

high artemisinin production, respectively (Wallaart et al., 2000). The cloning of *dbr2* and its efficient conversion of artemisinic aldehyde to dihydroartemisinic aldehyde (figure 1) suggests that the main chemotype shift occurs at the aldehyde level rather than at the alcohol or acid levels (Zhang et al., 2008) as has been postulated (Bertea et al., 2005). Expression of Dbr2 and other proteins necessary to reconstitute the artemisinin biosynthetic pathway in yeast produced a strain yielding 11.8 µg/mL artemisinic acid and 15.7 µg/mL dihydroartemisinic acid, as compared to the control strain lacking Dbr2 expression which produced 29.4 µg/mL artemisinic acid (Zhang et al., 2008). As the biosynthetic pathway has been removed from its context while reconstituted in the heterologous host, it is difficult to establish whether the comparatively high production of artemisinic acid even in strains expressing Dbr2 is due to imbalance in expression levels compared to the situation in the plant and/or due to an incomplete palette of expressed biosynthetic genes. Even so, the suitability of yeast as a factory for production of artemisinic acid has been previously established (Ro et al., 2008; Ro et al., 2006). The fate of the late intermediates artemisinic acid and dihydroartemisinic acid is less clear. There is evidence that points to a non-enzymatic conversion of dihydroartemisinic acid to artemisinin *in planta* (Brown et al., 2003; Brown et al., 2004).

The expression of Dbr2 in the yeast strain is interesting evidence for the chemotypic shift although it is to early to say that it is the only point at which the Δ11(13) carbon double bond reduction of amorpha-4,11-diene occurs. Precently there are no data for the kinetic parameters of Dbr2 catalysed conversion of dihydroartemisinic aldehyde to artemisinic aldehyde. It is possible that additional enzymes other than Cyp71av1 and to a minor extent Aldh1 exist, that convert dihydroartemisinic aldehyde to dihydroartemisinic acid. It is thus difficult to estimate the impact of Red1 in the biosynthetic network of artemisinin. Currently a system for stable transformation of *A. annua* is being developed to be able to

address this question. With the cloning of *red1*, there are a total of four known enzymes competing for the substrate dihydroartemisinic aldehyde, the other three being the alcohol dehydrogenase Aldh1 (Teoh et al., 2007), the cytochrome P450 Cyp71av1 (Rydén et al., 2009; Teoh et al., 2006) and Dbr2 (Zhang et al., 2008). Only for Aldh1 have the apparent kinetic data been reported (table 1). These data suggest that under those experimental conditions and employing $NADP^+$ as cofactor, Aldh1 is more efficiently converting dihydroartemisinic aldehyde into dihydroartemisinic acid than Red1 is converting dihydroartemisinic aldehyde into dihydroartemisinic alcohol. Earlier studies performed on cytosolic fractions from *A. annua* report that dihydroartemisinic aldehyde was reduced to dihydroartemisinic alcohol as well as oxidized to dihydroartemisinic acid (Bertea et al., 2005). Production of dihydroartemisinic alcohol can be seen as detrimental for the biosynthetic pathway of artemisinin as this metabolite is a less favored substrate of the major oxidizing enzyme Cyp71av1 than artemisinic aldehyde (Rydén et al., 2009). Also the chemical properties of dihydroartemisinic alcohol versus dihydroartemisinic aldehyde make the alcohol a less suitable substrate as it is less reactive than the aldehyde. The higher activation energy barrier of dihydroartemisinic alcohol compared to the dihydroartemisinic aldehyde is one explanation for the refusal of the metabolite as a substrate by Red1. Production of dihydroartemisinic alcohol will in a global sense lower the rate of production of dihydroartemisinic acid. Although there might be other enzymes involved in the reduction and oxidation of dihydroartemisinic aldehyde *in planta*, the overall scheme of a major flow from artemisinic aldehyde via dihydroartemisinic aldehyde to the chemically more stable dihydroartemisinic alcohol was established. The cloning and functional characterization of the broad substrate oxidoreductse *red1* is a reminder that to optimize a biosynthetic pathway in the original host, it is necessary to consider not only the forward biosynthetic route but also competing metabolic pathways. The ability of Red1 to convert dihydroartemisinic aldehyde to dihydroartemisinic alcohol makes it an interesting target

for knock down studies. Such investigation should clarify whether the function of dihydroartemisinic alcohol, given that it a less preferred substrate than artemisinic alcohol and dihydroartemisnic aldehyde for Cyp71av1, is as a storage molecule to prevent accumulation of the more reactive and potentially dangerous dihydroartemisinic aldehyde. The removal of Red1 from the biosynthetic network is also anticipated to assist in discovery of other genes that encode proteins acting on the biosynthetic intermediates of artemisinin. If Red1 has a role as a monitor protein keeping dihydroartemisininc aldehyde at suitable, non-toxic levels, it is likely that the removal of Red1 from the network will upregulate or downregulate other genes in a stress response against accumulated dihydroartemisinic aldehyde. However, in the long run it is anticipated that silencing of Red1 may lead to increased production of artemisinin in *A. annua*.

Material and Methods

Plant materials

Plants, originally identified by Wallaart et al. (1999), were grown for collection of flowers, leaf and root material using seeds obtained from University of Groningen. Seeds are deposited in the herbarium De Kruidhof in Buitenpost, the Netherlands, under registration number GR001. The *A. annua* line belongs to the high artemisinin chemotype and has the characteristic pattern of a high dihydroartemisinic acid to artemisinic acid ratio. Seeds were sown the 3rd of August 2006 and seedlings grown under green house conditions at 21/18°C (16/8 h) using potting compost. Daylight was supplemented with artificial light (SON-T AGRO) during the high-temperature period. Plants were watered as necessary without addition of fertilizer. When the plants started to flower, flowers were collected in four categories: bud (F1), young flower (F2), old flower (F3) and old flower with pollen (F4). Green young leaves and mature leaves (O leaf) were collected along with stem material. Plant

material was kept on ice during handling, then frozen in liquid nitrogen and stored at -80 °C.

Chemicals

Neat artemisinic alcohol, artemisinic aldehyde, dihydroartemisinic alcohol and dihydroartemisinic aldehyde were synthesized as described by Bertea et al. (2006). Neat perilla aldehyde and perilla alcohol were commissioned to the Department of Pharmaceutical Biology, Groningen, the Netherlands. Primers for semi-quantitative PCR were custom synthesized by BEX, Japan (table 2).

Table 2. Primers used for semi-quantitative PCR in this study.

Gene	Primer pair	Product size	Cycle number
red1	5' tac gga act aaa gga gta ac 3' 5' tga ggt tat ttg cat tcg ac 3'	477 bp	35
amds	5' cac aag gaa gag ctc agc cat gtg tgc a 3' 5' tca ttt agg cgt cga cca agt at acct g 3'	620 bp	30
cyp71av1	5' ttg gag cag gca cag aca ctt cct 3' 5' acg agt aac aac tca gtc ttt c 3'	583 bp	35
fds	5' tat tca ccg ccg aat tgt tc 3' 5' aag gat ttc aac acc gct tg 3'	456 bp	25
actin1	5' tcc aac tct ggt tct act atc 3' 5' ctt cga tct tca tgc tgc tc 3'	298 bp	30

GC/MS analysis

GC/MS analyses were performed on an Agilent 6890 series gas chromatograph system coupled to a JEOL JMS-SUN 200 mass selective detector in electron impact mode (70 eV) equipped with a guard column (1 m x 0.25 mm i.d., GL-Sciences) and a HP-5MS analytical column (30 m x 0.25 mm i.d., 0.25 µm film). The

temperature program was set to an initial temperature of 60°C followed by a first temperature gradient of 3°C min^{-1} to 220°C and then a second gradient of 30°C min^{-1} to 300°C with an endpoint hold of 4 min. Helium flow was set to 0.7 mL min^{-1}, detector temperature and injector temperature to 250°C using 500 scans min^{-1} with a scan range of 40-350 m/z.

Cloning of red1 and delineation of expression pattern in plant tissues

A glandular trichome specific cDNA library previously constructed was further sequenced (Bertea et al., 2006). Obtained sequences were compared with the literature using batch BlastX with standard settings. The EST fragments were further analyzed by alignment with EST libraries from tomato, potato, rice, *Arabidopsis thaliana*, lettuce and sunflower. It was argued that fragments having high similarity with EST sequences from lettuce and sunflower which both belong to the family Asteraceae but comparatively low similarity with the non-Asteracea family members potato, tomato and rice would be specific for Asteraceae species and likely to be involved in artemisinin biosynthesis. Based on the combined information from the two alignment approaches, one gene fragment named *red1* was selected for further analysis and 5' extension. Approximately 100 mg frozen flowers (stage F4) and mature leaves (O leaf) were ground in liquid nitrogen. Total RNA was isolated using TriPure (Roche Applied Science) and 1 ug of the isolated F4 and O leaf total RNA were used for 5' RACE cDNA amplification using the Smart RACE cDNA amplification kit from Clontech. PCR was performed on an aliquot of the obtained F4 cDNA using the internal primer Red1-RACE1 5' cgatttctctcattcgtgaggtgttcaatatcttggagc 3' and the universal primer mix from the Smart RACE cDNA amplification kit. The PCR product was purified from an agarose gel and ligated into the sequencing vector pGEMT-Easy (Promega) and sequenced. The full length gene was isolated form O leaf cDNA with the primers FL5-

AseI-red1 5’ accttggattaatatgtcatatgcaaccgagaaagatg 3’ and FL3-red1-XhoI 5’ accttggctcgagtcaaaatgaggttatttgcattcgac 3’ using Phusion DNA polymerase (Finnzymes) under reaction conditions recommended by the manufacturer (Finnzymes) using 1 µL cDNA. The 953 bp PCR product was purified using the QIAquick PCR Purification Kit of Qiagen and cloned into pGEMT for propagation and sequencing. Confirmed inserts were excised using AseI and XhoI and ligated into the expression vectors pET26b+ and pET28a+ previously restricted with NdeI and XhoI forming the constructs pET26b+-red1 and pET28a+red1. Constructs were cloned into *Escherichia coli* (*E. coli*) DH5α for propagation where after plasmids were isolated and transformed into the expression host *E. coli* BL21 (DE3)-Rosetta2 following manufacturer’s instructions (Novagen). The nucleotide sequence for *red1*has been deposited in the GenBank database under GenBank Accession Number GU167953. Semi-quantitative PCR was performed on obtained cDNA using primers listed in table 1 following the recommended reaction conditions of the Taq DNA polymerase manufacturer (Invitrogen). To ensure equal reprecentation of cDNA, actin was employed as a control.

Reductase assays

Initial conversion assays to determine substrate specificity were performed in 2 mL aliquots containing 20 µL purified protein or crude protein extract, 40 mM potassium phosphate buffer (pH 7.0), 1 mM DTT, 500 µM NADPH for reductase assays or $NADP^+$ for dehydrogenase assays and 10 or 100 µM substrate. Mixtures were incubated at 30°C with gentle shaking over night. All reactions were performed in duplicates. Protein extracts from *E. coli* harboring the empty expression vectors pET26b+ and pET28a+ served as negative controls.

Characterization of recombinant dihydroartemisinic aldehyde reductase

Seed cultures with 5 mL LB medium supplemented with 30 µg/mL kanamycine and 34 µg/mL chloramphenicol were initiated using one colony from fresh streak outs and incubated at 37°C over night with shaking. Main cultures of 200 mL 2xTY (2.4% tryptone, 1.5% yeast extract, 0.75% NaCl) supplemented with antibiotics as described above were initiated in 1000 mL Erlenmayer-flasks at a calculated OD_{600} of 0.02 using aliquots from the seed cultures. Cultures were incubated at 37°C with shaking for 4 hours and thereafter induced with 200 µL 1M IPTG followed by incubation at 16°C with shaking for 14 hours. Cytosolic proteins were extracted using Bugbuster (Novagen) following manufacturer's instructions. Proteins emerging from the pET28a-red1 construct were purified on a nickel column using Ni-NTA resin (Qiagen) and dialyzed at 4°C against buffer A (20 mM KH_2PO_4, 150 mM KCl and 20% glycerol) using a 14 kDa filter (Viskase Companies, Inc.). Proteins were stored at -20°C. Purity was verified using SDS-PAGE. The optimum pH of Red1 was determined to be 7.0 in an assay that included 20 mM phosphate and 60 mM Tris buffers with 150 mM KCl adjusted to pH 6.0 – 9.0 with 0.5 units increments, 1.5 µg purified protein and 60 µM substrate. For characterization of Red1, 300 µL reaction mixtures containing 1.5 µg protein, 500 µM NADPH or NADP+, 20 mM KH_2PO_4, 150 mM KCl adjusted to pH 7.0 were routinely used. Reductase assays were performed by adding dihydroartemisinic aldehyde as substrate covering the range 10 µM-200 µM or 60 µM-1mM dihydroartemisinic alcohol. Assays were pre-incubated at 30°C for 5 min and reactions initiated by adding Red1 followed by gentle mixing and incubation for 5 min. Reactions were stopped by adding 400 µL ethyl acetate followed by vigorous mixing and storage on ice for one hour before proceeding with two consecutive extractions using 400 µL ethyl acetate per extraction event. Extractions were pooled and evaporated to dryness on ice under a gentle flow of nitrogen gas. Dried samples were resuspended in 60-240 µL ethyl

acetate spiked with 10 µM cis-nerolidol as internal standard. Quantification of reaction products was carried out by GC-MS analysis. Control assays were performed with boiled protein. Reactions were performed in triplicates and the response of the internal standard was used to quantify and control reproducibility of the experiments. Reaction products were quantified by comparing peak areas with external standard curves of dihydroartemisinic aldehyde and dihydroartemisinic alcohol. The reaction conditions were chosen to limit conversion of the substrate to less than 10%. Apparent kinetic parameters were calculated with the software GraphPad Prism 5 using non-linear regression analysis in Michaelis-Menten modus (GraphPad Software Inc. San Diego, CA).

Sequence analysis

The amino acid sequences of Dbr2, Aldh1 and Red1 were compared using ClustalW2 (Larkin et al., 2007). BlastX analysis was performed using standard settings (Altschul et al., 1990).

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