Scale-Up of *Artemisia annua* L. Hairy Root Cultures Produces Complex Patterns of Terpenoid Gene Expression

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Abstract: Hairy roots grow quickly, reach high densities, and can produce significant amounts of secondary metabolites, yet their scale-up to bioreactors remains challenging. *Artemisia annua* produces a rich array of terpenoids, including the sesquiterpene, artemisinin, and transformed roots of this species provide a good model for studying terpenoid production. These cultures were examined in shake flasks and compared with cultures grown in two types of bioreactors, a mist reactor and a bubble column reactor, which provide very different environments for the growing roots. Mist reactors have been shown previously to result in cultures that produce significantly more artemisinin per gram fresh weight of culture, while bubble column reactors have produced greater biomass. We have compared expression levels of four key terpenoid biosynthetic genes: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and farnesyl diphosphate synthase (FPS) in the three culture conditions. In shake flasks we found that although all four genes showed temporal regulation, only FPS expression correlated with artemisinin production. Light also affected the transcription of all four genes. Although expression in reactors was equivalent to or greater than that of roots grown in shake flasks, no correlation was found between expression level within six different zones of each reactor and their respective oxygen levels, light, and root-packing density. Surprisingly, transcriptional regulation of HMGR, DXS, DXR, and FPS was greatly affected by the position of the roots in each reactor. Thus, relying on a single reactor sample to characterize the gene activity in a whole reactor can be misleading, especially if the goal is to examine the difference between reactor types or operating parameters, steps essential in scaling up cultures for production. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **83:** 653–667, 2003.
Keywords: immobilization; transformed roots; bioreactors

INTRODUCTION

Transformed "hairy" root cultures are a biochemically and genetically stable model for assessing biosynthesis and scale-up of pharmacologically important natural products (Flores et al., 1999). The ability of rapidly growing hairy roots to steadily produce a wide range of organic molecules offers additional advantages compared to conventional field production and unorganized cell suspension cultures (Shanks and Morgan, 1999). The latter, in particular, are generally unstable and unproductive over time. The ability of hairy roots to grow to high density and to produce significant amounts of secondary metabolites also makes them a suitable system for large-scale culture in reactors (Wilson, 1997).

Many factors are severely affected during scale-up of any kind of cell culture. These include oxygen transfer rate, heat transfer, mixing and the associated shear stress, superficial air velocity associated with impeller-mixed cultures, and culture age and stability (Humphrey, 1998; Kim et al., 2002a). For example, inadequate mixing is believed to create gradients of nutrients including oxygen and carbon sources throughout the bioreactor and can rapidly lead to nonoptimal large-scale performance (Bylund et al., 1998; Humphrey, 1998). Indeed, Schweder et al. (1999) reported that within 60 seconds *E. coli* cells in specific zones of a bioreactor showed differential transcription of genes in response to changes in glucose and oxygen levels.

Scale-up of hairy root cultures remains very challenging, and, despite recent advances, the objectives of optimal growth and production in bioreactors are far from being

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reached (Giri and Narasu, 2000; Kim et al., 2002a). The major drawback is often inadequate oxygen supply to the roots growing in liquid (Shiao, Doran, 2000; Weathers et al., 1999; Yu and Doran, 1994; Yu et al., 1997). Several types of reactors still being optimized for hairy root cultures include: liquid-phase reactors, such as stirred tank, bubble column, and air-lift reactors; gas-phase reactors, such as trickle bed, droplet phase and mist reactors; and finally, hybrid reactors that are a combination of both (Kim et al., 2002a; Shanks and Morgan, 1999; Weathers et al., 1997b; Wilson, 1997).

The terpenoids are a huge family of plant secondary products that are both crucial to normal plant function and a major source of scientifically and commercially important fine chemicals, including pharmaceuticals, such as paclitaxel and artemisinin, fragrances, flavors, food colors, and pesticides (Croteau et al., 2000). Despite their great diversity, all terpenoids found in nature share a unique biosynthetic precursor, isopentenyl diphosphate (IPP) (Chappell, 1995). It has now been established that the biosynthesis of IPP from the cytosolic mevalonate pathway originates from acetyl-CoA while, in plastids, the IPP pool derives from the mevalonate-independent pathway via the condensation of pyruvate and D-glyceraldehyde-3-phosphate (Fig. 1; Estevez et al., 2001; Croteau et al., 2000). Recent evidence suggests crosstalk between these two pathways and a possible exchange of IPP between cytosol and plastids (Fig. 1; Adam and Zapp, 1998; Adam et al., 1999; Lichtenthaler, 1999; Maier et al., 1998). The key enzymatic reaction in the mevalonate pathway is the reduction of 3-hydroxy-3-methylglutaryl CoA to form mevalonic acid, catalyzed by the highly regulated 3-hydroxy-3-methyl-CoA reductase (HMGR). In plastids, the condensation of pyruvate and D-glyceraldehyde-3-phosphate, catalyzed by deoxy-D-xylulose-5-phosphate synthase (DXS), represents the first enzymatic step of the mevalonate-independent pathway leading to the formation of 1-deoxy-D-xylulose-5-phosphate. However, the first committed step leading to IPP is the formation of 2-C-methyl-D-erythritol-4-phosphate (MEP) from 1-deoxy-D-xylulose-5-phosphate via the action of 1-deoxy-D-xylulose-5- phosphate reductoisomerase (DXR). As expected DXS and DXR are both regulated enzymes with growing evidence of isoform involvement (Carretero-Paulet et al., 2002; Estevez et al., 2001; Lois et al., 2000; Mahmoud and Croteau, 2001; Walter et al., 2002).

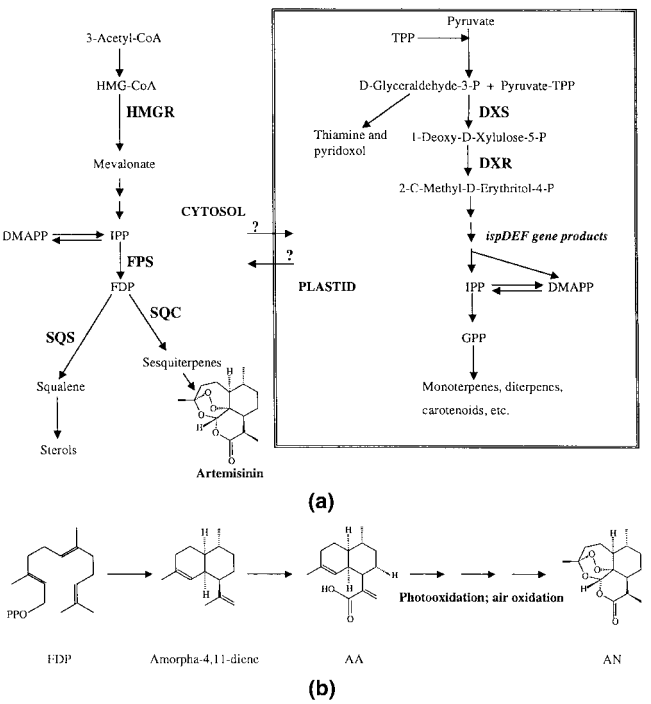

Figure 1. Simplified terpenoid biosynthetic scheme. (a) The two arms of terpenoid biosynthesis and relevant regulatory enzymes used in this study: HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; DXS, 1-deoxy-D-xylulose-5- phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; FPS, farnesyl diphosphate synthase; SQC, sesquiterpene cyclase; SQS, squalene synthase. (b) Shows a simplified biosynthetic scheme for the sesquiterpene, AN, artemisinin. FDP, farnesyl diphosphate; AA, artemisinic acid; TPP, thiamin diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate.

Hairy roots of *Artemisia annua* L. are a biochemically stable model system for studying production of terpenoids, including the sesquiterpene lactone, artemisinin (Jaziri et al., 1995; Weathers et al., 1994, 1996). Artemisinin is synthesized from farnesyl diphosphate via the action of the prenyltransferase farnesyl diphosphate synthase (FPS) (Fig. 1). Although a number of studies have focused on altering growth conditions to optimize the production of artemisinin in hairy roots of *A. annua* (Paniego and Giulietti, 1996; Smith et al., 1997; Weathers et al., 1996; 1997a; Wang, Ta, 2002), the regulation of terpenoid metabolism in this plant model has not been widely evaluated, mainly because the committed steps leading directly to artemisinin biosynthesis have not been fully identified. Only recently has the sesquiterpene cyclase, amorpha-4,11-diene synthase, leading to artemisinic acid been cloned (Bouwmeester et al., 1999; Mercke et al., 2000; Wallaart et al., 2001). However, the transcriptional regulation of the early enzymatic steps leading to farnesyl diphosphate biosynthesis can be assessed and, therefore, advance our fundamental understanding of terpenoid production. In hairy roots of *A. annua* L., in vitro alteration of growth and environmental conditions can lead to significant changes in both biomass production and artemisinin accumulation. For instance, artemisinin accumulation in transformed roots of *A. annua* L. varies during growth and aging of in vitro cultures (Weathers et al., 1996). Moreover, reports by Jaziri et al. (1995) and Wang et al. (2001) suggested a regulatory role for light in artemisinin biosynthesis. Strengthening this hypothesis were the findings by Wallaart et al. (1999; 2000) that the formation of dihydroartemisinic acid hydroperoxide, the direct precursor of artemisinin, was both light- and oxygen-dependent (Fig. 1B).

Since artemisinin is a highly oxygenated molecule, it was hypothesized that oxygen-rich cultures might have an increased yield of sesquiterpenes and, therefore, that the genes in the terpenoid biosynthetic pathway might also be affected. Indeed, Kim et al. (2001) reported a threefold in-

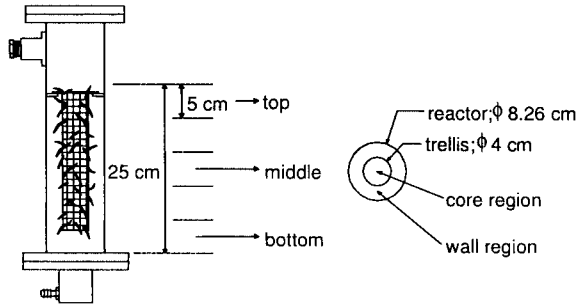

Figure 2. Diagram depicting the three levels inside the growth chamber, bottom, middle, and top, and the two different regions, wall and core, from where samples of transformed roots were harvested. (ϕ) diameter.

crease in artemisinin accumulation, on a dry weight basis, in hairy roots of *A. annua* grown in highly aerated mist reactors compared to those grown in oxygen-limited bubble column reactors. To our knowledge, however, no study has compared the changes occurring in small- (e.g., shake flask) and large-scale (e.g., bioreactors) culture of hairy roots in terms of terpenoid gene expression, and the effect of oxygen on those genes.

Here, we report the effects of light, oxygen, and culture age on the expression of four key regulatory enzymes involved in terpenoid metabolism in hairy roots of *A. annua.* Results were compared for cultures taken from shake flask and from different zones within bubble column reactors, and mist reactors (Fig. 2). The genes include three from the early steps of the terpenoid biosynthetic pathways leading to isopentenyl diphosphate: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). We also included farnesyl diphosphate synthase (FPS) from the first committed reaction leading to both sesquiterpenes and sterols.

MATERIALS AND METHODS

Plant Material and Shake-Flask Cultures

Transformed roots of *A. annua* were grown in 125 mL shake flasks containing 50 mL of autoclaved Gamborg's B5 medium as previously described (Weathers et al., 1994). They were subcultured every 2 weeks. For experiments, shake flasks containing the same medium were inoculated with 0.3 g FW of 2-week old, healthy root tips, and grown for 4

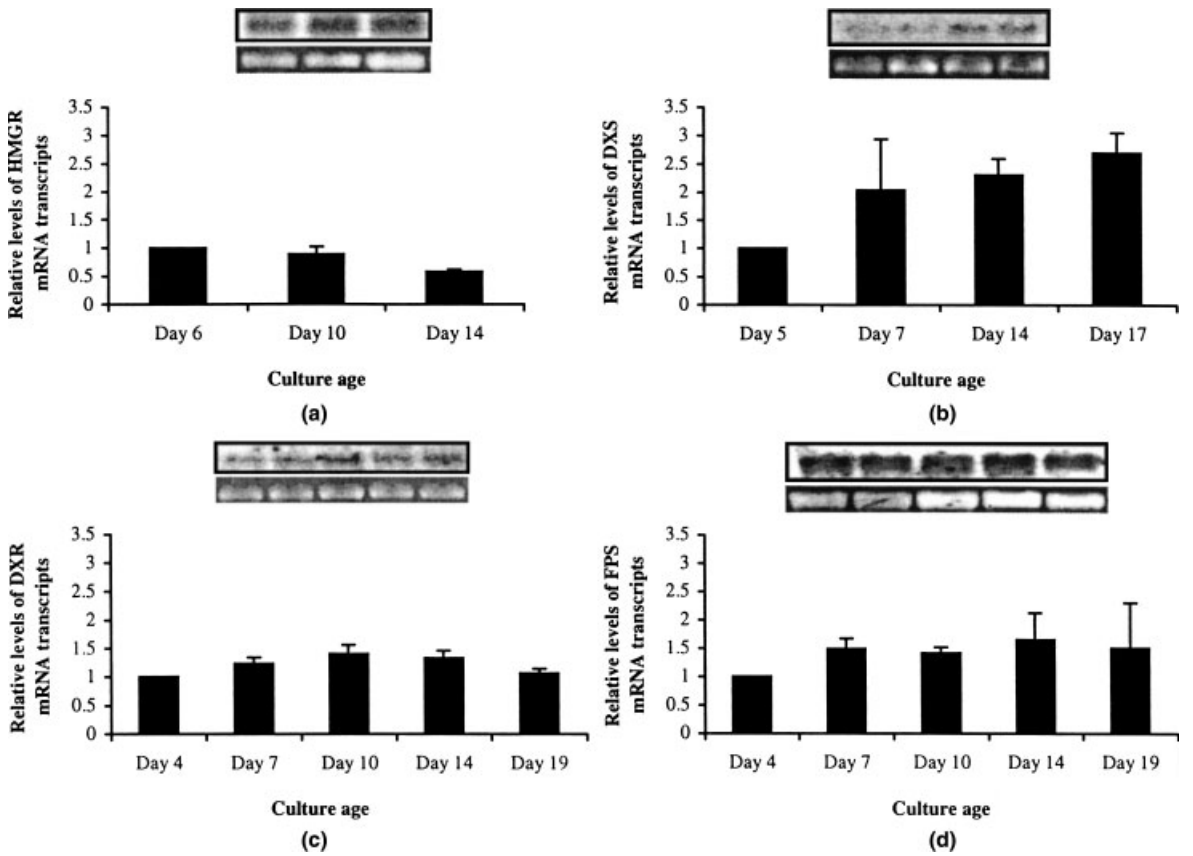

Figure 3. Temporal changes in steady-state mRNA levels for IPP and FDP biosynthetic enzymes in transformed roots of *A. annua* as a function of shake flask culture age. A representative time course experiment is presented with the RNA blot, the ribosomal bands were visualized by ethidium bromide or methylene blue dye, and the plot of the relative amounts of mRNA was determined by Northern blot. The genes examined and shown as averaged units of relative mRNA level (dimensionless) are normalized to the first sample. (a) 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), (b) 1-deoxy-D-xylulose-5- phosphate synthase (DXS), (c) 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and (d) farnesyl diphosphate synthase (FPS).

to 19 d at 23 ± 2°C under continuous cool white fluorescent light (60–100 μmol m^{-2} s^{-1}) or in the dark on an orbital shaker at 100 rpm. Each experiment was run 3 times and each condition had three shake-flask replicates. *Artemisia annua* YU strain seeds (kindly provided by Dr. Nancy Acton, Walter Reed Army Institute of Research), were also grown for 10 weeks in a greenhouse.

Fresh Weight Determination and RNA Extraction

At specific times, transformed roots were harvested, blotted to remove any excess media, and fresh weight determined. Roots were then immediately frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Roots, stems, and leaves from greenhouse-grown *A. annua* (strain YU) plants were harvested after 10 weeks and frozen. Total RNA was extracted from 1 to 2 g of tissue according to Downing et al. (1992) using a phenol-chloroform method.

Cloning Strategies for Conserved Regions of *Artemisia annua* HMGR, FPS, DXS, and DXR

Forward (5′-GAA GGG TGT TTG GTT GCT AG-3′) and reverse (5′-AAG ACC AGA AGC AAT AGC GG-3′) primers specific for HMGR, and forward (5′-TAT TCA CCG CCG AAT TGT TC-3′) and reverse (5′-AAG GAT TTC AAC ACC GCT TG-3′) primers specific for FPS cDNAs were synthesized based on the *A. annua* nucleotide sequences published in GenBank (GenBank Accession Number U14625 and AAU36376, respectively).

Single-strand cDNA was generated using 5 μg of total RNA extracted from 14 day-old transformed roots, the corresponding reverse primer, and Superscript II RT (Life Technologies, Grand Island, NY) following the manufacturer's instructions. Then, double-stranded cDNAs were obtained by PCR. The resulting RT-PCR products were analyzed on an agarose electrophoresis gel. Fragments of expected size were purified, then cloned into pGEM-T Easy vector (Promega, Madison, WI), and used to transform DH5α competent cells.

Sequencing of the cloned fragments was performed automatically to confirm the identity of the amplified partial cDNAs. Amplification and cloning of full-length cDNAs encoding *A. annua* DXS and DXR have been reported previously (Souret et al., 2002).

Northern Blot Analysis

Five micrograms of total RNA were fractionated on a 1% agarose formaldehyde denaturing gel and transferred to a nylon membrane (MSI brand, Fisher) using standard procedures (Ausubel et al., 1996). The RNA blot was then prehybridized for 2 h at 62–65°C in freshly prepared Church Buffer containing 1% BSA, 0.5M $Na_2HPO_4 \cdot 7H_2O$, and 7% SDS (Church and Gilbert, 1984). The hybridization was carried out at 58°C overnight. ^{32}P-labeled probes prepared by random priming (Stratagene, La Jolla, CA) of the cDNAs encoding HMGR, DXS, DXR, and FPS were used to detect the corresponding mRNAs. Unincorporated radiolabeled nucleotides were removed using NucAway™ spin columns (Ambion, Austin, TX). The blots were washed twice in 2× SSC, 0.5% SDS at room temperature for 5 min each, twice in 0.2× SSC, 0.2% SDS at 62°C for 20 min each, followed by a final wash at room temperature for 15–30 min in 0.2× SSC, 0.1% SDS. For each experiment, at least three RNA blots from independent experiments were prepared and analyzed, and a representative time-course profile presented. The membranes were then exposed to autoradiography film (Kodak BioMax) for 2–5 d with an intensifying screen. Densitometry was carried out using Kodak Digital Science 1D Image Analysis Software, Version 3.0 (Eastman Kodak, Rochester, NY). Equal loading was verified by comparing the ribosomal bands visualized on the ethidium bromide stained gel or methylene blue stained nitrocellulose membrane. The membrane was soaked in a solution of methylene blue (0.03% methylene blue, 0.3*M* sodium acetate pH 5.2) for 3 min, and then rinsed 3 times with autoclaved water, each time gently shaking the membrane for 15–30 s.

ADH northern analysis used a probe prepared from the ADH clone kAt3011 as a 1.6-kb *Sal*I/*Hind*III fragment and labeled with [^{32}P] dCTP with a random primer labeling kit (Chang and Meyerowitz, 1986; Stratagene, La Jolla, CA). Full details are provided in Weathers et al. (1999).

Artemisinin Assay

Artemisinin was assayed using the Q260 HPLC method described in Smith et al. (1997). Any putative artemisinin peak was validated by re-analysis of a sample that was co-injected with authentic artemisinin (Sigma Chemical, St. Louis, MO). Artemisinin content was then quantified by measuring the area of the original injection sample after comparison with known amount of standard.

Bubble Column and Nutrient Mist Reactors

Design, construction, and operation of the bubble column and nutrient mist bioreactors have already been described in detail in Weathers et al. (1999). Engineering analyses of the reactors used in this study can be found in Kim et al. (2002b; 2003).

To compare the performance of each reactor type, mist and bubble column reactors were usually run in pairs (#47, 48, and #49, 50), side-by-side with similar inoculum levels, and to minimize differences in culture room conditions including temperature and light (Table I; Kim et al., 2002b). An additional unpaired mist and a bubble column reactor (#55, 54) were also analyzed. Reactors were run and subsequently harvested. Harvested root bed dimensions were 8.3 cm diameter, and 25 cm in height; trellis diameter was 4 cm. For each reactor run, transformed roots were taken from six different sectors of the harvested root bed: three vertical sections, top 5 cm, middle 5 cm and bottom 5 cm, and then from inside (core) and outside (wall) the trellis for

Table I. Summary of reactor-culture conditions. Initial medium volume (1.5-L) was identical in each reactor type and for each run. In each case, the roots were grown for 28 days. In the mist reactor, roots were first grown in bubble-column mode for 6 days before medium was drained and mist mode began. Reactors 47 and 48, and then 49 and 50 were run as concurrent pairs; reactors 54, and 55 were not concurrent runs. Culture medium was autoclaved in the three sets of bioreactors used for the gene expression analysis (Kim et al. 2002b; 2003).

Culture conditions	Mist reactors			Bubble column reactors		
	47	49	55	48	50	54
Inoculum (g/L)						
Fresh weight	1.87	4.53	3.35	1.91	4.28	4.37
Dry weight	0.14	0.34	0.25	0.15	0.33	0.33
Harvest (g/L)						
Fresh weight	40.74	105.54	51.57	89.01	133.95	151.38
Dry weight	3.44	7.39	3.81	5.98	8.83	9.23
Remaining media (mL)	365	345	607	1,000	910	890
Sugar concentration at harvest (g glucose equivalent/L)[a]	53.13 ± 0.20	35.73 ± 0.28	34.94 ± 0.19	20.64 ± 0.60	13.76 ± 0.25	13.18 ± 0.26
Total carbon remaining at harvest (%)	39.5	23.7	40.0	42.3	24.4	22.3
Fructose:Glucose ratio at harvest	1.3	2.0	1.3	2.2	7.1	11.7
Nitrate concentration at harvest (m*M*)	32.4 ± 3.02	4.83 ± 0.13	17.97 ± 1.35	8.79 ± 0.71	0.02 ± 0.00	0.03 ± 0.00
Nitrate remaining at harvest (%)	28.4	4.4	28.0	22.4	0.05	0.07
Nitrate:Ammonium ratio at harvest	170	115	367	238	0.6	1.6
Artemisinin (μg/g FW)	0.29 ± 0.13	0.15 ± 0.09	0.07 ± 0.02	0.14 ± 0.07	0.01 ± 0.02	0.08 ± 0.06
Aeration direction	Top to bottom of root bed			Bottom to top of root bed		

[a]Sugar concentration at harvest especially in mist reactors is greater than at inoculation due to evaporation of media: about 33% loss in bubble column; about 66% loss in mist reactor.

each vertical section (Fig. 2). Total RNA was extracted and fractionated as described above. For each cDNA probe, at least three RNA blots from each of two independent reactor runs were analyzed by densitometry as already described. Three reactors of each type gave similar relative expression profiles and the responses were normalized for each gene within each reactor type to the sector with the lowest expression for that gene. The normalized response of each gene from each sector was averaged for three reactor runs of each reactor type.

Data Analyses

Analyses of chemical samples taken from reactors were done at least in triplicate and standard deviations calculated. The mRNA blots for each gene from each condition were assigned dimensionless numerical values based on densitometry after normalizing to that gene's lowest value on the blot. Multiple blots were then averaged and displayed ± SD.

RESULTS

Temporal Regulation of IPP and FDP Biosynthetic Enzymes in Shake Flasks

Typically, artemisinin accumulation on a per gram basis in hairy roots of *A. annua* grown in shake flasks is temporally restricted (Weathers et al., 1996). The highest specific content of artemisinin is generally found at the beginning of the exponential growth phase (day 6), and is followed by a rapid decrease until day 14 when the level begins to rise again continuing on into stationary phase (day 19). Based on these findings, the temporal regulation for a subset of key regulatory enzymes involved in early steps of terpenoid biosynthesis was studied to identify potential correlations between artemisinin accumulation and the expression of these enzymes in shake flask cultures.

The steady state levels of HMGR mRNA, encoding the key regulatory enzyme of the cytosolic mevalonate pathway, were monitored during a two-week culturing period. HMGR mRNA levels stayed relatively constant from the time the root cultures entered exponential phase (day 6) to the onset of the stationary phase (Fig. 3A), although 14-day old hairy roots accumulated slightly less HMGR mRNA than younger cultures. These results suggest that the rapid decrease in artemisinin accumulation between day 6 and day 14 cannot be explained solely by a decrease in HMGR gene expression. Although developmental regulation of HMGR has been widely studied in numerous plant systems, including suspension cultures (Enjuto et al., 1994; Korth et al., 1997; Lange et al., 1998b), to our knowledge, temporal changes in HMGR mRNA levels have not been reported for any other hairy root system.

Next, temporal changes in DXS and DXR transcript levels were investigated (Fig. 3B and C; Souret et al., 2002). There were clear differences in the expression pattern of these genes. While a steady increase in DXS mRNA levels was observed as the culture aged, the relative levels of DXR transcripts peaked midway through the culturing period, and then slowly decreased throughout the stationary growth phase (Fig. 3B and C). These results suggested that DXS and DXR were temporally regulated in transformed roots of *A. annua*. Accumulation of artemisinin, however, did not correlate with DXS and DXR transcript levels, as we would have expected a significant increase by day 6–7 and then a gradual decrease until day 14 when the artemisinin content

is low (Weathers et al, 1996). Together, these results suggested that regulation of artemisinin accumulation in transformed root cultures of *A. annua* does not appear to reside at the level of HMGR, DXS, or DXR gene regulation. There does not appear to be any coordinate regulation of these key terpenoid genes, as the patterns of expression were clearly distinct.

Farnesyl diphosphate, a branch point to both sesquiterpenes and sterols, is likely a key regulatory step in both pathways (Adiwilaga and Kush, 1996; Facchini and Chappell, 1992). In our hairy root cultures, the levels of FPS transcripts increased (Fig. 3D) as the hairy roots entered exponential growth (day 7) and then remained constant. Interestingly, this time point also corresponded to the highest artemisinin content found in the transformed roots per unit of fresh weight (Weathers et al., 1996).

Light and Tissue-Specific Regulation of IPP and FDP Biosynthetic Enzymes in Shake Flasks and Whole Plants

Light can significantly stimulate the biosynthesis of numerous secondary metabolites including alkaloids, flavonoids, betalains, carotenoids, and anthocyanins (Kurata et al., 1994; Stafford, 1994). In transformed roots, light activation of plant secondary metabolite accumulation has been investigated in numerous plant systems, including *H. albus*, *T. patula*, *L. erythrorhizon*, and *C. roseus* (Bhadra et al., 1998; Lange et al., 1998b; Mukundan and Hjortso, 1991; Sauerwein et al., 1992). Earlier reports by Jaziri et al. (1995) and Wang et al. (2001) suggested that light enhanced artemisinin production in *A. annua* transformed roots. In addition, light has been shown to play a role in the later steps of artemisinin biosynthesis (Fig. 1B; Wallaart et al., 1999; 2000). Since some key regulatory enzymes involved in plant secondary metabolite biosynthetic pathways are known to be light regulated (De Luca and St-Pierre, 2000; Kurata et al., 1994; Learned, 1996), we investigated the effects of light on key regulatory enzymes involved in the early steps of terpene biosynthesis. In addition, since the key biosynthetic enzymes involved in terpenoid metabolism are also generally differentially regulated in specific tissues (McGarvey and Croteau, 1995), mRNA levels of HMGR, DXS, DXR, and FPS in greenhouse-grown *A. annua* YU plants were also investigated. This analysis allowed us to measure the differences in gene expression between photosynthetic (stems and leaves) and nonphotosynthetic tissues (roots) from untransformed *A. annua* YU plants. We were also able to compare expression levels in untransformed roots with levels in transformed roots from the YU strain of *A. annua*.

A comparison of the level of expression of the four biosynthetic genes in roots, stems, and leaves of whole plants again reveals very different patterns of expression (Fig. 4). While DXS mRNA is most abundant in stems, HMGR accumulates preferentially in roots and stems. On the other hand, FPS and DXR mRNAs showed similar levels of transcripts in all tissues analyzed. Interestingly, although artemisinin concentrations in whole plants are highest in leaves and inflorescences (Ferreira et al., 1997), none of the genes analyzed in this study showed peak levels in leaves.

A comparison of normal roots, grown in soil and therefore in the dark, to dark-grown hairy roots showed only small differences in the observed levels of steady state mRNA of the four genes, with the exception of HMGR. Indeed, HMGR showed substantially less expression in dark-grown transformed roots than in normal roots (Fig. 4). Interestingly, light appeared to affect the expression of the four target genes in hairy root cultures (Fig. 4), while biomass production and artemisinin levels were not significantly affected (data not shown). The largest effect observed was the level of DXS mRNA, which was at least twice as high in light-grown roots than in roots grown for an equivalent time in the dark. This resulted in levels of DXS mRNA comparable to that seen in leaves of normal plants. Smaller effects were seen with DXR and FPS, and only on day 17 for HMGR. Roots that were dark-grown for 2 weeks followed by a 3-day exposure to light consistently yielded equal or higher levels of all mRNAs analyzed than were seen in cultures grown for 17 d in either continuous light or continuous dark.

Terpenoid Biosynthetic Gene Expression in Shake Flasks Versus Reactors

While investigating the regulation of terpenoid biosynthetic genes in hairy roots of *A. annua* grown in bubble column and mist reactors, we identified an unusual gene expression profile suggesting that transcriptional regulation of all our genes of interest, HMGR, DXS, DXR, and FPS, was greatly affected by the position of the roots in reactors. Thus, relying on a single reactor sample to characterize the gene activity in a whole reactor can be misleading, especially if the goal is to examine the difference between reactor types or operating parameters. We, therefore, investigated this phenomenon further to better understand the response of *A. annua* hairy roots to growth in different zones of bubble column and mist reactors by analyzing the spatial variation in gene expression of HMGR, DXS, DXR, and FPS.

Shake-flask culture, the most common method for studying the growth of transformed roots, is often used as a baseline for comparisons with reactors (McKelvey et al., 1993; Toivonen et al., 1990). We, therefore, compared terpenoid gene expression of hairy root cultures of *A. annua* grown in shake flasks with roots grown in the bubble column and mist reactors. Multiple, independent northern blots for each type of reactor and shake flasks were prepared and analyzed for each gene, and an average of multiple blots is presented (Fig. 5). Although the relative gene response profile from each type of reactor was similar, the magnitude of the changes was sometimes variable. Data in Figure 5 are, thus, normalized for each gene to the lowest responding sector in each reactor prior to averaging multiple reactor blots.

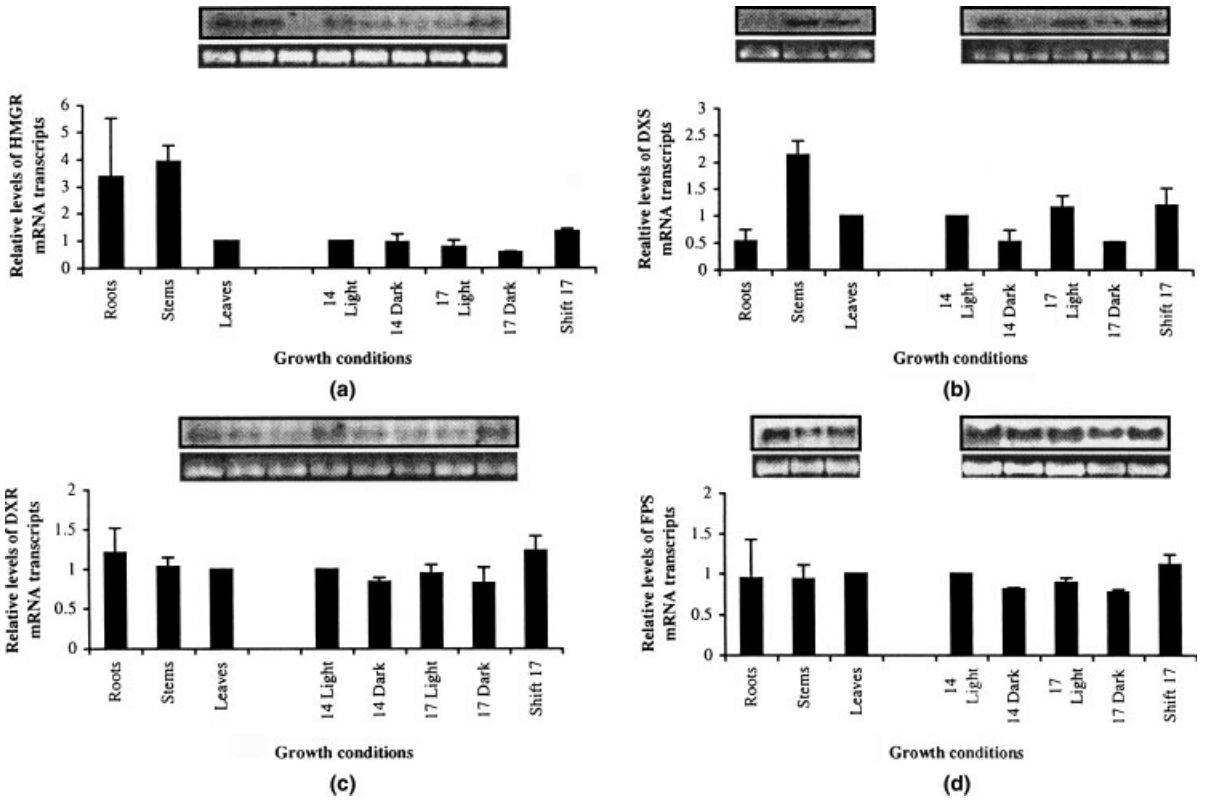

Figure 4. Tissue-specific and light effects on steady-state mRNA levels on IPP and FDP biosynthetic enzymes in normal plants and transformed roots of *A. annua* grown in shake flasks. A representative RNA blot is included, the ribosomal bands were visualized by ethidium bromide or methylene blue dye, and the plot of the relative amounts of mRNA was determined by Northern blot. The genes examined and shown as averaged units of relative mRNA levels (dimensionless) are normalized to the leaves for the tissue-specific expression analysis and to the 14 Light cultures for the light experiments. (a) 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), (b) 1-deoxy-D-xylulose-5-phosphate synthase (DXS), (c) 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), and (d) farnesyl diphosphate synthase (FPS). Total RNA was extracted from roots, stems, and leaves of 10-week-old greenhouse-grown *A. annua* plants, and from 14-day-old light-grown transformed roots (14 Light), 14 day-old dark-grown transformed roots (14 Dark), 17 day-old light-grown transformed roots (17 Light), 17 day-old dark-grown transformed roots (17 Dark), and 17 day-old transformed roots grown in the dark for 14 days, followed by 3 days continuous light (Shift 17).

As seen in Figure 5, there are several similarities in the patterns of expression of HMGR, DXR, and DXS. Roots grown in shake flasks typically yielded the minimum level of expression of these mRNAs, with expression in any given zone in either reactor being greater than or equal to their corresponding shake flask level (Fig. 5). Another general trend was that the mRNA levels of these three genes increased from the bottom to the top of the reactors. However, in the mist reactor the higher levels of expression at the top of the reactor were restricted to roots outside the trellis. Roots inside the trellis at each vertical level of the mist reactor generally had mRNA levels approximately equivalent to the levels from roots grown in shake flasks. The exception to these trends is FPS whose expression varied little from reactor to reactor and from sector to sector within the reactors, although the center of the bubble column reactor and the top of the mist reactor showed some variation.

DXR mRNA always appeared as a major and minor band in roots taken from both shake flasks and reactors. Although two DXR bands suggests the presence of two isoforms, both bands responded similarly regardless of changing experimental conditions (data not shown).

Terpenoid Gene Expression Under Oxygen-Limited and Nonlimited Conditions

Liquid-phase bioreactors such as the bubble column reactor suffer from inadequate liquid circulation, resulting in formation of chemical gradients (Kim et al., 2002a; Weathers et al., 1997b). Oxygen deficiency is an important growth-limiting factor in this type of bioreactor, mainly due to inadequate mass transport (Kim et al., 2002a; Yu and Doran, 1994; Yu et al., 1997). In contrast, gas-phase bioreactors, such as the mist reactor, provide high rates of oxygen transfer (Kim et al., 2002a; Williams and Doran, 2000). Using alcohol dehydrogenase (ADH) as a marker for hypoxia in roots grown in the mist and bubble column reactors, we found that the relative levels of ADH mRNA were undetectable in the mist reactor. In contrast ADH response in the bubble column reactor was significant and varied between the six regions in the growth chamber (Weathers et al., 1999). Indeed, roots harvested from the wall and core regions of the middle zone and from the core regions of the bottom zone of a bubble column accumulated nearly twice the level of ADH transcripts compared to other parts of the root bed (Weathers et al., 1999). In addition, yields of ar-

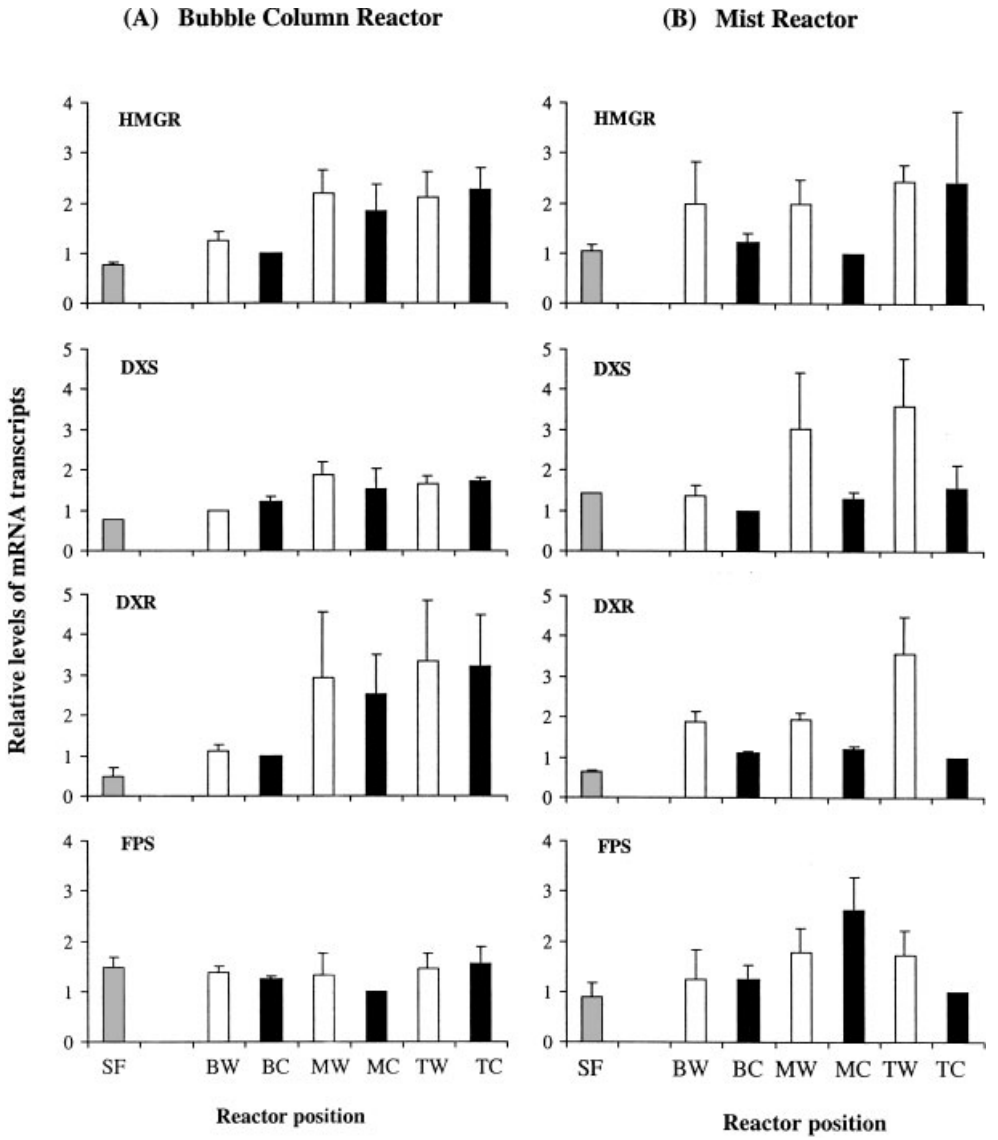

Figure 5. Quantitative comparison of terpene gene expression in different zones within reactors. (A) Bubble column; (B) mist reactor. Total root RNA was isolated, separated on a denaturing agarose gel, and hybridized to radiolabeled cDNA probes directed toward 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) 1-deoxy-D-xylulose-5- phosphate synthase (DXS), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), farnesyl diphosphate synthase (FPS). Gene expression is shown as averaged relative mRNA levels ± SD. B = bottom level; M = mid level; T = top level; W = wall region; C = core region in reactors. Wall regions are white; core regions are black; shake flasks are gray. The genes are shown as averaged units of relative mRNA levels (dimensionless) normalized to the lowest level of transcript for each specific gene in each reactor examined.

temisinin, a highly oxygenated molecule, were found to be significantly higher in transformed roots grown in the mist reactor compared to those in the bubble column bioreactor (Kim et al., 2001; Table I). Therefore, it was hypothesized that the different oxygen environments experienced by the roots in these reactors may promote the observed differential response in terpenoid gene expression.

As depicted in Figure 6, ADH response in transformed roots of *A. annua* did not uniformly correlate with changes in transcriptional induction of any of the terpenoid genes throughout the different zones of the growth chamber (bottom, middle, and top). Although the ADH mRNA levels were higher at the bottom of the bubble column reactor compared to the top, terpenoid biosynthetic gene mRNA accumulation was generally higher at the top compared to the bottom, suggesting an inverse relationship between ADH response and transcriptional induction of HMGR, DXS, DXR, and FPS (Fig. 6A). However, such a relationship is refuted by an analysis of expression levels in the mist reactor (Fig. 6B) where the ADH levels are undetectable. Likewise, artemisinin in roots harvested from wall and core regions of each reactor level do not correlate with ADH expression in those levels (data not shown).

It is important to note that although shake flasks and bubble column reactors have very similar ADH expression levels indicating similarity in oxygen tension (Weathers et al., 1999), terpenoid mRNA levels are different between the flask and reactor cultures. This is almost certainly caused by

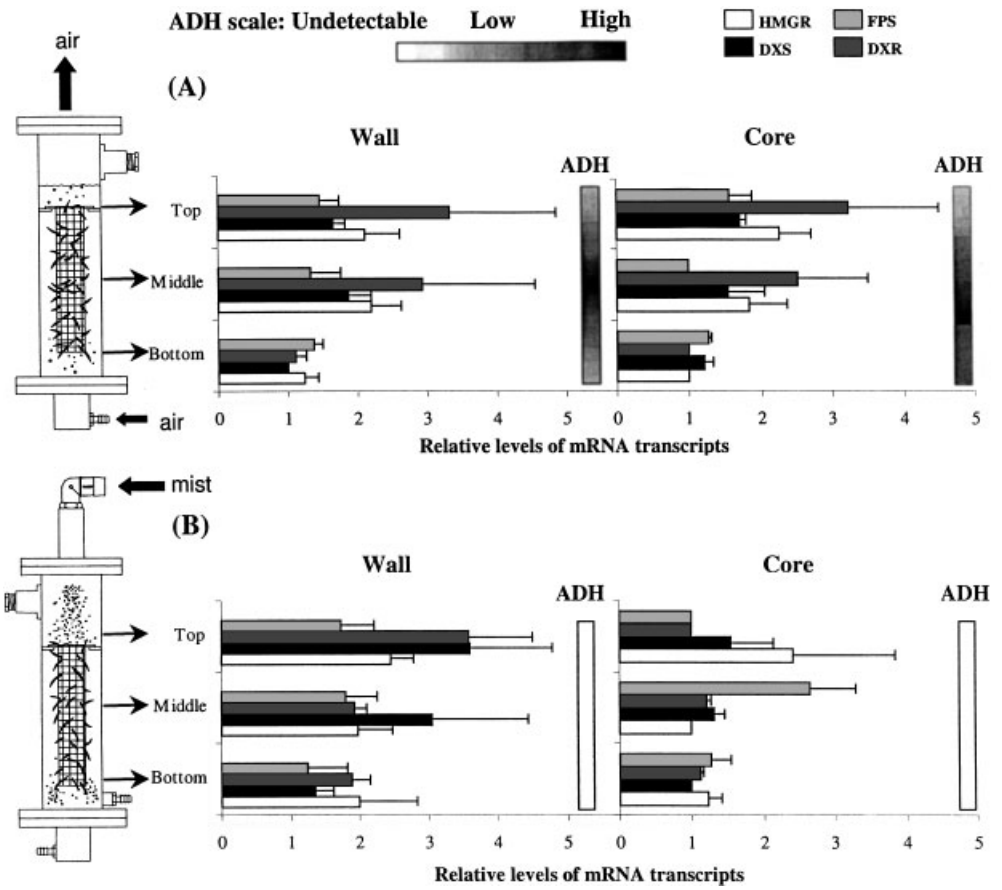

Figure 6. Comparison of ADH expression with relative mRNA levels of four terpenoid biosynthetic genes at three levels, bottom, middle, and top, and in two separate regions, wall and core, of (A) the bubble column reactor, and (B) the mist reactor used for *A. annua* transformed root cultures. Total root RNA was isolated, separated on a denaturing agarose gel, and hybridized to radiolabeled cDNA probes directed toward 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), **1-**deoxy-D-xylulose-5- phosphate synthase (DXS), **1-**deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), farnesyl diphosphate synthase (FPS), and alcohol dehydrogenase, (ADH) (Weathers et al., 1999). A gray-scale showing the relative mRNA level of ADH is presented at the top, and ADH levels are shown on the right of each figure for wall and core sections of each reactor. The genes are shown as averaged units of relative mRNA levels (dimensionless) normalized to the lowest level of transcript for each specific gene in each reactor examined.

multiple interacting factors, of which oxygen could be one. Certainly, a multifactor analysis would prove interesting.

DISCUSSION

Although extensive studies have been conducted to optimize in vitro production of secondary metabolites by altering growth conditions, very few studies have focused at the molecular level to study the pattern of gene expression under conditions affecting their biosynthesis in plants or in bioreactors. To gain a better fundamental understanding of both the branched pathway leading to isopentenyl diphosphate, the common precursor to all terpenoids, and the first regulatory step leading to sesquiterpene and sterol biosynthesis, we used artemisinin-producing hairy roots of *A. annua* as a model system to analyze the gene expression pattern of four key enzymes with results summarized in Figure 7.

Some of the challenges in studying the terpenoid biosynthetic enzymes reside in the fact that most of them, including HMGR and FPS, belong to small gene families whose isoforms exhibit complex developmental and environmental regulation (Chappell, 1995; Korth et al., 1997; Newman and Chappell, 1999). In *A. annua*, three putative HMGR cDNAs, and three putative FPS cDNAs have been isolated. Although it is unknown whether *A. annua* HMGR and FPS isoforms are differentially regulated, one might expect a different pattern of gene expression for each isozyme, including divergent temporal, spatial, and light-mediated regulation. For example, induction of transcript accumulation of specific HMGR isoforms in potato has already been shown to correlate with either sesquiterpene phytoalexin accumulation (HMGR2), or steroid glycoalkaloid production (HMGR1) (Korth et al., 1997), suggesting that the HMGR isoforms were active in separate pathways leading to the biosynthesis of distinct end-products (Chappell, 1995; Choi et al., 1994). Furthermore, by using transgenic *A. thaliana* plants containing the HMGR1:*uidA* reporter gene, Learned and Connolly (1997) determined that in root tissues, HMGR1 gene expression was restricted to the elongation zone and, in contrast to the results found in aerial

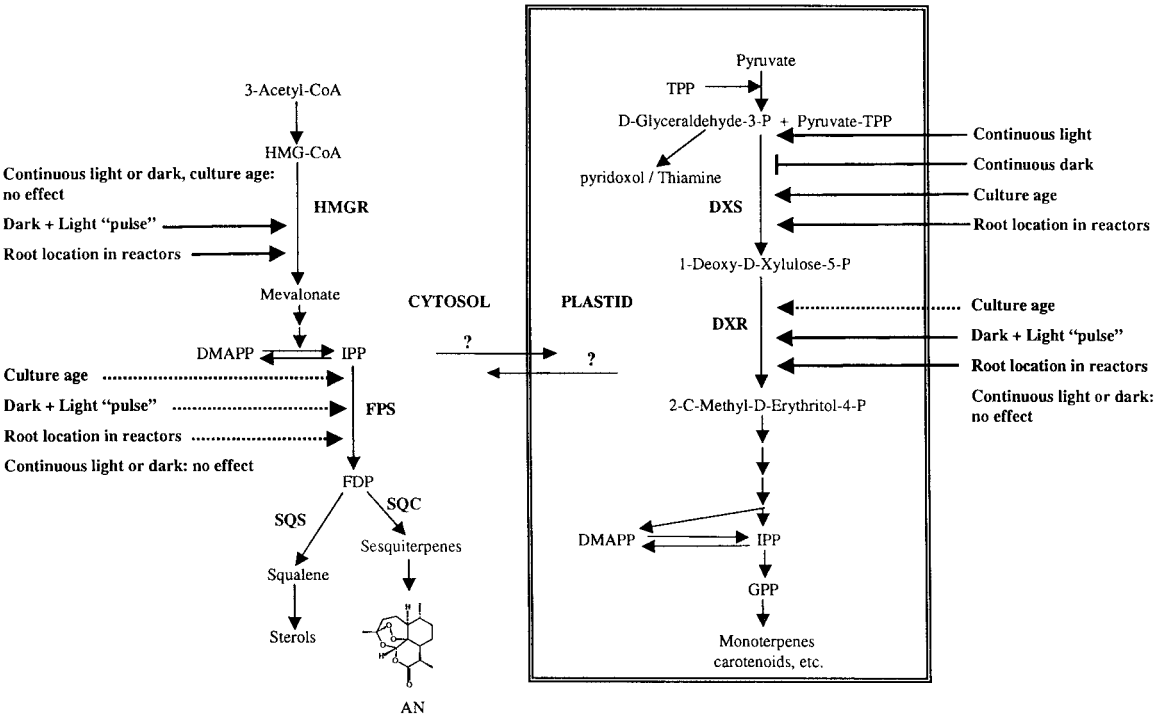

Figure 7. Summary depicting the global regulation of four terpenoid biosynthetic enzymes and the factors studied that affected their gene expression profile. The key abbreviations are indicated as follows: DMAPP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5- phosphate synthase; FDP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; GPP, geranyl diphosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IPP, isopentenyl diphosphate; SQC, sesquiterpene cyclase; SQS, squalene synthase; TPP, thiamine diphosphate. Arrows in and out of plastid indicate a potential, but limited exchange of IPP pools between the plastid and the cytoplasm. Arrow ending with vertical line indicates inhibition of step. Solid arrows indicate an important upregulation at the transcriptional level, while the dashed arrows indicate a slight increase in mRNA accumulation. Dark + Light "pulse" indicates that transformed roots were grown in shake flasks for 14 days in darkness and then shifted to continuous light for an additional 3 days.

parts, did not respond to light. Since our study used probes generated from highly conserved regions of genes, differential expression of isoforms would not be detected, and therefore the potential differences in expression of individual isoforms is potentially much greater than the differences seen in this study which measured total populations of each class of mRNA. However, this preliminary analysis also clearly demonstrates that there are significant differences in the level of expression of these genes under the various conditions used to grow the root cultures.

A second level of complication to studying regulation of gene expression is the possibility that there is posttranscriptional regulation, perhaps at the level of translation or of protein activity. A recent study by Korth et al. (2000) showed that developmental and light-regulated control of one specific HMGR isoform in potato plants occurred posttranscriptionally. Similarly, posttranscriptional regulation of DXS in *Arabidopsis* roots was also reported (Estevez et al., 2000). This level of control is not unexpected since it allows a level of fine-tuning that is crucial for the regulation of complex pathways. Future work should also focus on how expression of specific isoforms and posttranscriptional events function to regulate terpenoid biosynthesis in roots grown in reactors and shake flasks.

Whole Plant Expression Analysis

In plants, artemisinin has been shown to accumulate exclusively in aerial parts, specifically in highly specialized glandular tissues, and is undetectable in normal roots (Duke et al., 1994; Van Geldre et al., 1997). In addition, numerous other terpenoids isolated from *A. annua* have been isolated exclusively in glandular trichomes (Tellez et al., 1999). Yet in our work, similar or higher levels of HMGR, DXR, and FPS expression were often found in roots compared to aerial plant parts (Fig. 4A, C, and D). Moreover, the biochemical distribution of plant secondary metabolites is often rigidly dependent on the specific localization of the participating enzymes that are spatially and temporally expressed in the same way in which accumulation of the substrate occurs (Pichersky and Gang, 2000). Therefore, the differential tissue-specific accumulation of HMGR, DXS, DXR, and FPS mRNAs in greenhouse-grown *A. annua* YU strains suggests intercellular translocation of pathway intermediates throughout the plant. This is not surprising since the terpenoid pathway is the source of many compounds, e.g., carotenoids, sterols, phytohormones, essential for overall plant growth (Croteau et al., 2000).

Shake-Flask Culture Analysis

Initially, we studied the changes in expression of HMGR, DXS, DXR, and FPS during a normal culturing period (Fig. 3). Although steady-state levels of HMGR transcripts remained relatively constant, our results showed an increase in steady-state DXS mRNA levels as the culture aged. FPS and DXR mRNA accumulation also appeared to be temporally regulated, although to a lesser extent, with reproducible increases to day 7 (Fig. 3C and D). After day 10, however, transcripts of FPS remained constant, while those of DXR decreased. Together, these results suggest that these terpene genes of *A. annua* hairy roots are not coordinately regulated during culture growth.

Although recent evidence suggests that increases in DXS and DXR transcript accumulation are associated with increases in a variety of plastid-produced terpenoids, for example, carotenoids (Bouvier et al., 1998; Lois et al., 2000; Walter et al., 2000), and monoterpenes (Lange et al., 1998a), artemisinin levels in *A. annua* do not appear to respond similarly (Weathers et al., 1996; Fig. 3B and C). Artemisinin is a sesquiterpene, and likely produced in the cytoplasm. Thus, it is expected that IPP from the mevalonate pathway will be the predominant precursor, although Adam and Zapp (1998) showed that in chamomile, sesquiterpenes are also produced, in part, by the mevalonate-independent pathway. Carbon labeling and inhibition studies may further clarify responses in *A. annua*.

Increases in FPS transcripts early in exponential growth, however, do appear to coincide with increases in artemisinin levels (Weathers et al., 1996; Fig. 3D). This correlates with the observations of Chen et al. (1999; 2000) who showed transgenic lines expressing FPS under the control of the CaMV 35S promoter, had a significantly greater growth rate and accumulated 2 to 4 times more artemisinin than controls. This is not surprising since FPS is expected to be a key regulatory point in the biosynthesis of artemisinin.

Light has previously been shown to affect the expression of some terpenoid genes including HMGR (Learned, 1996), FPS (Sanmiya et al., 1997), SQC (Back et al., 1998), and DXS (Carretero-Paulet et al., 2002; Souret et al., 2002). Our analysis of light effects produced some interesting conclusions. First, the level of expression of the four biosynthetic genes studied was similar in normal roots and in dark-grown hairy root cultures with the notable exception of HMGR, which was substantially reduced in hairy roots. Second, light did appear to affect the mRNA accumulation of all the genes studied here. DXS transcript accumulation was substantially increased when the roots were grown in light as compared to the dark-grown roots (Fig. 4B; Souret et al., 2002). In contrast, the differences in HMGR, DXR, and FPS mRNA levels between light-adapted and dark-adapted cultures were much smaller and appeared to be more significant in younger root cultures (day 14 vs. day 17). This could be because of a decline in expression of these genes with time (Fig. 3). Interestingly, shifting the cultures from 14 days of growth in continuous dark to continuous light for an additional 3 days seemed to significantly increase the accumulation of HMGR and DXR mRNA transcripts, and to a certain extent those of FPS compared to 17-day old dark-adapted root cultures (Fig. 4). Although there is no obvious explanation for this phenomenon, several plausible hypotheses can be posed to explain these results.

First, shifting the dark-adapted root cultures to continuous light might induce a short-term "light-stress" response. In this scenario, the addition of light induces a variety of gene responses along with metabolic changes that directly or indirectly trigger a "later" increase in isoprenoid biosynthetic enzyme transcript levels. Consistent with this hypothesis is the fact that plants have a number of photoreceptors that can regulate gene expression via a variety of signal molecules, thereby enabling plants to rapidly respond to changes in light conditions in their environment (Fankhauser and Chory, 1997). In addition, it is known that the developmental changes induced by light at nearly every stage of the plant cycle can also directly or indirectly modulate the expression of numerous genes, and the activity of many enzymes (Kuno and Furuya, 2000).

Second, we could hypothesize that the sudden change in light condition (from darkness to continuous light) might more directly induce the rapid biosynthesis of light-absorbing and photoprotective pigments such as carotenoids, thereby leading to a more long term "light stress" response. Carotenoids are tetraterpenes derived from the mevalonate-independent IPP pool (Croteau et al., 2000). Interestingly, expression of both DXS and DXR was significantly induced when dark-adapted transformed root cultures were shifted from dark to continuous light. Yet, when grown in continuous light or dark, our roots do not accumulate detectable levels of chlorophyll or carotenoids (unpublished results). Furthermore, the light-mediated gene regulation of DXS and DXR might be distinct as these two enzymes not only responded differently to light in hairy roots of *A. annua* (Fig. 4B, C), but also in *Medicago truncatula* (Walter et al., 2002) and in *A. thaliana* (Carretero-Paulet et al., 2002).

Finally, a monoterpene synthase for β-pinene was recently shown to be transcriptionally regulated in a circadian pattern in *A. annua* plants (Lu et al., 2002). The dark-to-light switch then, could be resetting the circadian clock resulting in increased transcriptional activity of these genes.

It is becoming apparent that light quality, rather than simply the presence or absence of light, is inducing specific changes in terpenoid genes. For example, although Cunillera et al. (1996) found no significant difference in FPS expression between light- and dark-grown *A. thaliana* seedlings, Sanmiya et al. (1997) found a stimulatory response in blue light. Furthermore, in *A. annua* hairy roots, Wang et al. (2001) recently showed that red light treatment improved biomass and artemisinin accumulation. Considering the differential responsiveness of *A. thaliana* HMGR1 and *O. sativa* FPS to red- and blue-light (Hata et al., 1997; Learned, 1996; Sanmiya et al., 1997), investigating red- and blue-light induced expression of HMGR, DXS, DXR, and FPS in

transformed roots of *A. annua* might further our understanding of the role of light in terpenoid metabolism. Although there is evidence that light plays a role in artemisinin biosynthesis downstream of the genes studied here (Jaziri et al., 1995; Wallaart et al. 1999, 2000; Wang et al., 2001), the light-mediated responses at the mRNA level of the genes analyzed here appear to be complex and do not correlate closely with artemisinin production (Souret et al., 2002).

Comparison of Shake-Flask Culture to Reactor-Scale Cultures

This study also investigated the expression of these four terpenoid biosynthetic genes in transformed roots of *A. annua* when scaled up from a 50-mL shake flask to a 1.5-L bioreactor. Analysis of RNA isolated from roots grown in different zones within the growth chamber demonstrated that gene expression significantly differs from region to region within both types of reactors (Figs. 5 and 6). Thus, a single reactor sample is not representative of the entire culture.

In the bubble column reactor, higher DXR, DXS, and HMGR mRNA accumulation was observed in the middle and top zones compared to the bottom one (Fig. 5). However, FPS mRNA transcripts did not follow this trend, having slightly lower accumulation in the middle zone than in the others. In addition, changes were also noticed between the wall and core regions making the spatial regulation response of terpenoid biosynthetic genes very complex in the bubble column reactor.

In the mist reactor, mRNA transcripts for all four of the investigated genes accumulated at a higher level in the middle and the top, but mainly in the wall region (Figs. 5 and 6). In the core region, while the levels of HMGR were greatly increased in the top region that of FPS was highest in the middle zone. Furthermore, DXS and DXR mRNA levels remained constant throughout all levels of the core region. Taken together, these results show that terpenoid gene expression in transformed roots of *A. annua* is different in a bubble column reactor compared to a mist reactor. Moreover, expression of terpenoid biosynthetic genes in bioreactors was greatly affected by the location of the roots in the growth chamber of both bubble column and mist bioreactors.

Many factors affect the growth of cells and tissues during the scale-up of cultures, including oxygen, mass and heat transfer, and inadequate mixing that can create confined gradients of nutrient concentration (Humphrey, 1998). For example, Enfors et al. (2001) observed a rapid but transient transcriptional induction of stress genes when *E. coli* passed through a compartment containing high glucose and low oxygen content. Thus, we searched for any correlations that might exist between terpenoid gene expression and different reactor conditions including oxygen availability, light, and packing density.

Changes in oxygen level, as determined by measuring ADH response in transformed roots of *A. annua* in different reactor levels, did not uniformly correlate with artemisinin levels or changes in terpenoid gene mRNA levels in the two bioreactors (data not shown and Fig. 6). Although no ADH mRNA was detected in transformed roots grown in the mist reactor, the changes in HMGR, DXS, DXR, and FPS expression were as significant as those observed under the oxygen-limiting conditions experienced by transformed roots grown in the bubble column reactor suggesting that oxygen does not have a direct impact on the terpenoid genes studied.

Since we showed that light is a strong regulatory factor in DXS gene expression (Souret et al., 2002; Fig. 4B), we hypothesized that the light intensity inside the core area of the reactor is considerably decreased as root packing density increases. Consequently, roots harvested from the core will not have received as much light as roots grown in the wall region. Thus, if the differential light intensity between wall and core regions was important, we might expect upregulation of DXS in the wall compared to the core in both reactors. However, DXS gene expression did not show the expected changes in response to this illumination gradient indicating that light intensity does not appear to play a role in any of the observed changes in gene expression between reactors or zones within each reactor.

Packing density is another factor that can affect homogeneous distribution of nutrients as well as light within the growth chamber of a reactor. Dense root beds hinder the efficient circulation of nutrients especially in liquid-phase reactors (Kim et al., 2002a; McKelvey et al., 1993). Consequently, increased packing density leads to changes in local nutrient concentrations thereby affecting biosynthetic reactions. In both of the reactors used in this study, however, we saw no significant correlation between packing density and terpenoid gene expression or artemisinin levels (data not shown).

The generally lower gene expression that is observed in roots taken from the bottom region of both reactors suggests that those roots may be responding to the weight of the stacked root bed. We could not discern, however, any apparent physical reason for the response. Thus, the generally lower level of gene expression in the bottom sectors of both reactors is rather perplexing.

The pattern of spatial heterogeneity in terpenoid biosynthetic gene expression throughout these reactors remains, so far, a challenging phenomenon to explain. With respect to this, though, there have been numerous reports demonstrating heterogeneity within reactors in terms of nutrient concentration. Bylund et al. (1998) reported that glucose concentration gradients occurred in large-scale *E. coli* cultures with declining concentrations away from the glucose input. This heterogeneous microenvironment could lead to the formation of starvation zones, and consequently to physiological changes inside the cells (Schweder et al., 1999). Williams and Doran (2000) recently reported an interesting observation of *A. belladonna* hairy roots grown in a liquid-dispersed (spray) reactor. They noticed higher biomass density, lower sugar concentration, lower volume of inter-

stitial liquid, and higher root alkaloid content present in the top zone compared to the bottom. They suggested there was a greater level of metabolic activity toward the top of the bioreactor. They further commented on the problem of characterizing transformed root growth in bioreactors using averaged culture properties knowing that great variation in the measured parameters existed within different regions of the bioreactor. Our results concur with their findings, but add a new dimension, with an analysis at the gene level.

In addition to the differences in gene expression seen in different sectors of these reactors, we see differences in other parameters of the reactors. As shown in Table I, the total sugar and nitrate concentrations at harvest found in the bulk medium were not only different between the two types of reactors, but also between the three runs of each type of reactor. Although not measured, it is likely that the nutrient delivery system in the mist reactor and the mixing conditions in the bubble column reactor create zones of uneven concentrations of nutrients, and that nutrient concentration gradients likely exist within the reactors.

This work demonstrated the great heterogeneity in terpenoid gene expression in hairy roots of *A. annua* grown in shake flasks and in two types of bioreactors, a bubble column and a mist reactor. While these systems differ significantly in terms of growth conditions, in particular oxygen levels, the changes in terpenoid gene expression could not be directly assigned to a single factor. It appears that a combination of multiple factors that could affect gene expression locally and/or at a distance might be involved. Similarly, it appears that none of the conditions tested coordinately regulates this subset of terpenoid genes. This raises not only the question of which factors are playing a significant role, but also the notion of "matrix effects" in which gene response is dependent on multiple conditions, including environment, development, and metabolic situations (Coruzzi and Zhou, 2001). These studies, therefore, emphasize the need for the use of genomic and proteomic tools to provide a more rapid and global survey of genes relevant to the scale-up of in vitro cultures to reactors.

This initial study also demonstrates the value of fundamental studies at the gene expression level for making commercial production of secondary metabolites viable from in vitro culture systems. With a more complete understanding of the regulatory mechanisms of the pathway of interest, culture conditions can be designed for optimum production of the product of interest. In vitro cultures allow a level of flexibility not possible with whole plants, the ability to systematically alter conditions dramatically at various times during the culture period, or use conditions that would not support whole plant growth (dark). This ability would certainly facilitate our ability to harvest larger quantities of the product of interest.

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