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RAPID COMMUNICATION

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First isolation of an isoprene synthase gene from poplar and successful expression of the gene in *Escherichia coli*

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Abstract For the first time, the complete functional gene for isoprene synthase has been isolated from poplar (*Populus alba* × *Populus tremula*). The gene was quite similar to known limonene and other monoterpene synthases, but was found to specifically catalyze the formation of isoprene from the precursor dimethylallyl diphosphate with only a marginal activity for the formation of the monoterpene limonene from geranyl diphosphate as compared with limonene synthases. Omitting the part of the gene that putatively encoded the signal peptide necessary for transport into the chloroplast led to an enhanced rate of isoprene formation by the recombinant protein.

Keywords Dimethylallyl diphosphate · Isoprene synthase gene · Monoterpene synthase · *Populus* (isoprene synthase)

Abbreviations DMADP: dimethylallyl diphosphate · GDP: geranyl diphosphate · PCR: polymerase chain reaction

Introduction

Isoprene emission by plants was first described in 1957 (Sanadze 1957). Since this discovery, a series of trees and herbaceous plants that emit this volatile compound have been described (Kesselmeier and Staudt 1999). In the last decade, isoprene emission by trees has become of global interest as the compound contributes to the formation of photosmog in the lower atmosphere when NO_x is present (Paulson and Seinfeld 1992). Moreover, it reduces the concentration of atmospheric OH radicals required, for example, for the degradation of the greenhouse gas methane (Thompson 1992). Initially, it was suggested that isoprene is released by a chemical elimination of diphosphate from dimethylallyl diphosphate (DMADP), a central intermediate in the biosynthesis of isoprenoids, in the thylakoid lumen of the chloroplasts acidified during irradiation (Sanadze 1990). In the meantime, however, an enzyme that specifically catalyzes the formation of isoprene and diphosphate from DMADP, even at neutral pH, has been isolated from several tree species (Silver and Fall 1991; Kuzma and Fall 1993; Schnitzler et al. 1996; Wildermuth and Fall 1998). This so-called isoprene synthase was purified from aspen leaves, digested by cyanogen bromide (CNBr), and the initial 6–24 amino acids of three peptide fragments (25 kDa, 13 kDa and 6 kDa) were sequenced (Silver and Fall 1995). However, these partial sequences did not allow any identification of the enzyme in existing sequence libraries.

There are three main points of interest regarding the isolation of the gene. The first concerns its physiological role. In contrast to the higher volatile isoprenoids such as monoterpenes and sesquiterpenes, where in some cases their formation has a known physiological function (Bohlmann et al. 1998), the physiological function of isoprene release from trees is still unknown and controversially discussed (Sharkey and Singsaas 1995; Singsaas et al. 1997; Logan and Monson 1999; Logan et al. 2000). Mainly due to the lack of the isoprene synthase gene, the existing hypotheses could not be tested in transgenic plants by overexpression or repression. The second point of interest is to study the regulation of the gene to improve predictions of the isoprene formation rate by vegetation (Zimmer et al. 2000) for modeling atmospheric chemistry. The third point of interest is in the catalytic activity of the encoded protein. Isoprene is a basic molecule for the rubber and pharmaceutical industry, so that an isoprene synthase gene is of biotechnological interest, e.g. to produce isoprene in a bacterial fermentor. For these reasons the present work is aimed at the identification and isolation of the gene for isoprene synthase.

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Materials and methods

Strains and plasmids

Two-year-old poplar (*Populus alba* × *Populus tremula*) trees were obtained from the Institut für Forstbotanik und Baumphysiologie (Albert-Ludwigs-Universität, Freiburg, Germany) and cultivated in a greenhouse. The plasmids pQE30, pQE50 (Qiagen, Hilden, Germany) and their derivatives were amplified in *Escherichia coli* TG1 (Wain-Hobson et al. 1985).

Construction of a cDNA library

Total RNA was prepared from young leaves of *P. alba* × *P. tremula* (Barkan 1989). The mRNA fraction was isolated from total RNA by the use of the Oligotex mRNA mini kit (Qiagen). The cDNA was synthesized and the *Eco*RI linkers were ligated according to the Superscript Choice System (Gibco BRL). The cDNA segments were ligated into Lambda ZAP II (Stratagene), packaged into lambda particles (Gigapack III Packaging Extract; Stratagene) and used to transform *E. coli* XL1-Blue MRF′ (Stratagene). The resulting 1.2×10^6 colony-forming units were amplified in *E. coli* XL1-Blue MRF′. In-vitro excision of the identified phage was performed by the use of *E. coli* strain SOLR and the helper phage ExAssist (Stratagene).

Amplification of a cDNA segment of the isoprene synthase gene

Forward oligonucleotides (Roth) were designed from the published 26-, 13- and 6-kDa partial peptide sequences, and reverse oligonucleotides were designed for the 13- and 6-kDa partial peptide sequences (Silver and Fall 1995). In the case of the 13-kDa-forward oligonucleotide [5′ttc cta gct ctc tac aac ac(tcag) at(cta) aa(tc) g 3′] and the 6-kDa reverse oligonucleotide [5′gcg gtc tcg acg aa(agct) gg(gt) tt(agct) gc(ag) aa 3′], the polymerase chain reaction (PCR) resulted in a single product by the use of the following conditions: 36 cycles (1 min 94 °C, 1 min 56 °C, 1 min 72 °C) in an assay of 50 µl containing 2.5 U Taq-polymerase (Gibco BRL), 1×PCR buffer (Gibco BRL), 4.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide, 50 ng of cDNA from *P. alba* × *P. tremula* leaves. The amplified product was cloned into pCRII-Topo (Invitrogen BV) and was labeled by the incorporation of digoxigenin (DIG)-labeled nucleotides and used for hybridization according to the DIG Nucleic Acid Labeling and Detection kit (Boehringer).

Sequencing of DNA

Both strands of the 1.7-kb insert of pBS*iso* were sequenced using cycle sequencing dideoxy chain termination reactions with Big Dye Terminators (PE Applied Biosystems) and the universal forward and backward primer (Gibco BRL) or sequence-specific oligonucleotides (Roth). The sequence was analyzed on an ABI PRISM-System 310 (PE Applied Biosystems). The EMBL accession number of the complete 1,700-bp DNA sequence is AJ294819.

Construction of expression fusions

For the expression fusions with and without the plastidic leader, oligonucleotides (Roth) with restriction sites were designed (*Bam*HI at 5′end, *Kpn*I at 3′end): iso-forward: 5′gct gca gga tcc att act aga ggc 3′, Δiso-forward 5′gaa aca gga tcc gaa gcc aga cgg tc 3′ and iso-reverse 5′gca gac ggt acc atg gaa aac ctg 3′. PCR was performed in 36 cycles (1 min 94 °C, 1 min 58 °C, 3 min 72 °C) in a 50 µl assay containing 1 U Taq-polymerase (Gibco BRL), 1×PCR buffer (Gibco BRL), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each oligonucleotide, 1 ng of pBS*iso* DNA.

Purification of overexpressed isoprene synthase

To purify the gene products, 5 ml of an overnight pre-culture of *E. coli* strain TG1(pQE30*iso*) and strain TG1(pQE30Δ*iso*) were inoculated into 250 ml of LB medium and incubated for 1 h with vigorous shaking until an OD_{600} of 0.6 was reached. Expression was induced by adding 4 µM isopropyl β-D-thiogalactoside and the cultures were incubated for an additional 4–5 h. The his-tagged proteins were harvested and purified as described by Miller et al. (2000).

Assay and quantification of isoprene and monoterpene production

Isoprene synthase activity was assayed and isoprene formation quantified as described by Lehning et al. (1999) and Schnitzler et al. (1996). Monoterpene synthase activity was assayed according to the method of Fischbach (Fischbach et al. 2000). Quantification and identification of the monoterpenes was done by the use of external standards (α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene and limonene).

Results and discussion

To identify the gene for isoprene synthase, a series of oligonucleotides was synthesized on the basis of the published partial polypeptide sequences (Silver and Fall 1995). These oligonucleotides were used pairwise in PCR assays performed on a cDNA library made from young poplar leaves (*Populus alba* ×*P. tremula*). This led to the amplification of a 500-bp segment with an oligonucleotide combination (13 kDa forward, 6 kDa reverse) corresponding to the partial sequences of the published 13- and 6-kDa polypeptides of the aspen isoprene synthase. However, a PCR performed with poplar genomic DNA resulted in the amplification of an additional segment that was 100 bp bigger in size due to the presence of two introns. Hybridization of the PCR segment amplified from cDNA to a cDNA lambda library of *P. alba* × *P. tremula* leaves led to the identification of a full-length cDNA-carrying lambda phage clone. After isolation of the insert-carrying plasmid (pBS*iso*) via in vivo excision, the insert was sequenced (EMBL accession number AJ294819). The three partial peptide sequences known from the purified protein of *P. tremuloides* (Silver and Fall 1995) could be localized and were highly conserved in the deduced amino acid sequence of the complete gene from *P. alba* × *P. tremula* (Fig. 1). This was the first hint that the isolated gene might encode an isoprene synthase. Surprisingly, the deduced amino acid sequence showed the highest degree of identity (39%) and similarity (76%) to the limonene synthase sequence (Fig. 1) of *Mentha spicata* (Colby et al. 1993). The next highest similarity was found to other limonene synthases, followed by other monoterpene synthases (Table 1). In a distance matrix the isoprene synthase was as highly related to a limonene synthase as different monoterpene synthases were to each other (Table 1; myrcene synthase of *Arabidopsis thaliana* to the limonene synthase of *Mentha spicata* or to the sabinene synthase of *Salvia officinalis*). Especially

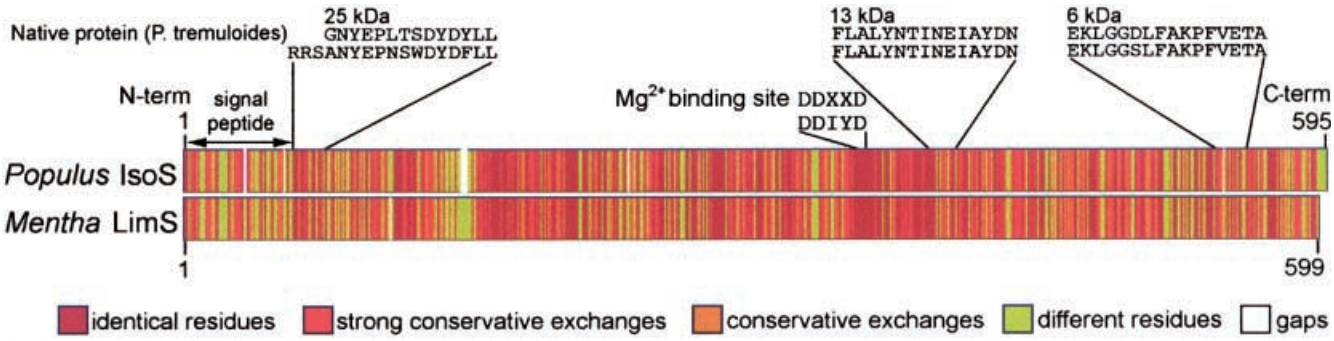

Fig. 1 Similarity of the deduced amino acid sequence of the isoprene synthase gene of poplar (*P. alba* × *P. tremula*) and the monoterpene synthase gene of *Mentha spicata*. The figure is constructed on the basis of an alignment of the two polypeptides by the program CLUSTALW version 1.8. Above the alignment are shown the published partial amino acid sequences of the isoprene synthase peptides obtained after CNBr treatment (Silver and Fall 1995) in comparison with the deduced amino acid sequence of the isoprene synthase gene of *P. alba* × *P. tremula*

noteworthy is the putative Mg^{2+}-binding motif "DDXXD" (Bohlmann et al. 1998) typical of monoterpene synthases in the deduced amino acid sequence of the isolated gene (Fig. 1). This corroborates the previous observation that the isoprene synthase activity of poplar is dependent on the presence of the divalent cations Mg^{2+} or Mn^{2+} (Silver and Fall 1995). As in monoterpene synthases, two typical arginine residues are present (Fig. 1), which are preceded by an N-terminal transit peptide sequence, indicating that the protein is transported into the plastids (Wise et al. 1998). Coincidently, the isoprene synthase is assumed to be a chloroplastic enzyme (Silver and Fall 1995) and thus will also need a transit peptide in the pre-mature form. All these features imply that the isolated gene encoded either a monoterpene synthase or an isoprene synthase.

However, to elucidate the function of the isolated gene, it was cloned into the expression vectors pQE50 and pQE30 (Qiagen), the latter of which encodes six histidines at the N-terminal end of the protein, allowing a one-step purification of the resulting protein. The resulting vectors were named pQE50*iso* and pQE30*iso*. As the peptide sequence preceding the pair of arginines was similar to a plastidial transit peptide (Fig. 1), a sequence lacking this 5′ part of the sequence was also cloned into the expression vectors which resulted in the vectors pQE50Δ*iso* and pQE30Δ*iso*. After transformation of the *E. coli* strain TG1 with the pQE50 derivates, the release of isoprene from these strains and several control strains was tested by gas chromatography (Table 2) using a method described previously (Schnitzler et al. 1996). Compared with the background release from a control strain without any plasmid, or with an insertless plasmid, the strain with the complete cloned putative isoprene synthase gene [TG1(pQE50*iso*)] released 100 times more isoprene, which strongly suggests that the isolated gene indeed encodes an isoprene synthase (Table 2). Isoprene release from the strain with the plasmid carrying the gene without the sequence encoding the plastidial leader [TG1(pQE50Δ*iso*)] was 1.9 times higher than that from strain TG1(pQE50*iso*), indicating that this sequence part is not necessary for the mature protein. Moreover, the release of isoprene could be further enhanced (3.6–4.1 times, Table 2) when the plasmids were expressed in the strain TG1(pGS*dxs*) in which the DMADP content was increased due to the overexpression of *dxs*, the initial gene of bacterial isoprenoid biosynthesis, which was constructed previously (Miller et al. 1999, 2000). In order to verify the function of the putative isoprene synthase gene, the protein was purified from the strains carrying pQE30*iso*

Table 1 Distance matrix of the isoprene synthase of poplar (*Populus alba* × *P. tremula*) and terpene synthases. The distance matrix was calculated by the Phylib program protdist vers. 3.5c using the Dayhoff PAM matrix. The deduced amino acid sequences are from: *Pa_iso* isoprene synthase of *P. alba* × *P. tremula* (this work), *Ms_lim* limonene synthase of *Mentha spicata* (Acc. No. L13459), *At_lim* limonene synthase of *Arabidopsis thaliana* (Acc. No. AB028607), *So_sab* sabinene synthase of *Salvia officinalis* (Acc. No. AF051901), *At_myr* myrcene/ocimene synthase of *A. thaliana* (Acc. No. AF178535), *Mp_far* farnesene synthase of *Mentha* × *piperita* (Acc. No. AF024615)

	Pa_iso	Ms_lim	At_lim	So_sab	At_myr	Mp_far
Pa_iso	0.00000					
Ms_lim	1.03544	0.00000				
At_lim	1.04469	0.98549	0.00000			
So_sab	1.11499	0.61671	1.02398	0.00000		
At_myr	1.11895	0.98677	0.44328	1.03568	0.00000	
Mp_far	1.69296	1.82588	1.62933	1.93262	1.74279	0.00000

Table 2 Isoprene formation of transconjugant *Escherichia coli* TG1 cells. Isoprene formation was detected by gas chromatography after 14 h of culture incubation in gas-tight vials, and is referred to mg of total cell protein. The experiments were performed at least with four independent repetitions

E. coli clones	Isoprene formation (nmol mg^{-1})
TG1	0.01 ± 0.00
TG1(pQE50)	0.01 ± 0.00
TG1(pQE50*iso*)	1.00 ± 0.08
TG1(pQE50Δ*iso*)	1.85 ± 0.22
TG1(pGS72dxs/pQE50)	0.04 ± 0.02
TG1(pGS72dxs/pQE50*iso*)	3.59 ± 0.53
TG1(pGS72dxs/pQE50Δ*iso*)	7.70 ± 0.67

Table 3 DMADP-dependent isoprene formation and GDP-dependent limonene formation catalyzed by recombinant isoprene synthase enzymes. Assays were incubated at 40 °C for 1 h in a volume of 100 µl. Limonene was the only monoterpene that was significantly increased after incubation with the recombinant enzyme. No appreciable formation of myrcene, sabinene, α-pinene, β-pinene, α-thujene and camphene was detected

Assay conditions	Isoprene formation (nmol s^{-1} mg^{-1})	Limonene formation (nmol s^{-1} mg^{-1})
Enzyme with leader without substrate	0.2 ± 0.2	0.0024 ± 0.0009
Enzyme with leader	0.9 ± 0.3	0.0021 ± 0.0002
Denatured enzyme with leader	0.0 ± 0.2	0.0022 ± 0.0002
Enzyme without leader without substrate	0.2 ± 0.2	0.0019 ± 0.0005
Enzyme without leader	2.6 ± 0.3	0.0141 ± 0.0035
Denatured enzyme without leader	0.2 ± 0.1	0.0000 ± 0.0005

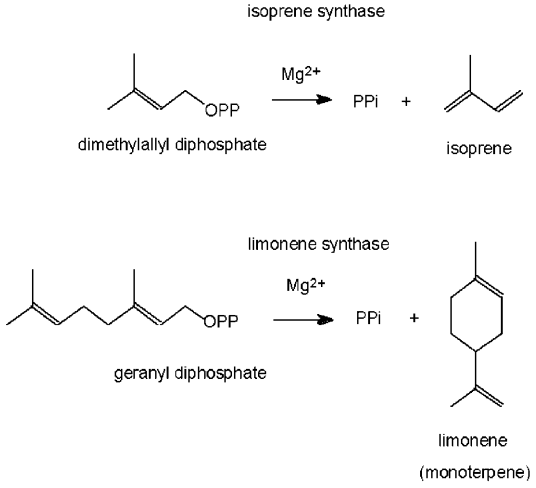

Fig. 2 Reaction scheme for the isoprene synthase and the limonene synthase

and pQE30Δ*iso* with the histidine-encoding region at the 5′ end of the sequence. The two proteins were incubated and the DMADP-dependent release of isoprene was quantified by gas chromatography (Table 3). Compared with the background level of the control assays, a 4-fold and a 13-fold increase in isoprene synthase activity was observed for the proteins isolated from TG1(pQE30*iso*) and TG1(pQE30Δ*iso*), respectively (Table 3). The specific activity of the recombinant leader-free isoprene synthase was 65% lower than the published activity of the highly purified native protein from aspen (Silver and Fall 1995). One explanation for the lower activity might be that the native protein has been isolated as a heterodimer with slightly different subunits (Silver and Fall 1995) whereas the recombinant protein consists only of one polypeptide. Moreover, keeping in mind that the recombinant protein has six additional histidines (his-tag) at the N-terminal site and was only purified in one step, a 65% lower activity is a satisfying result which indicates that the purified enzyme and the recombinant one are derived from the same gene.

As the sequence homology had identified strong similarity to monoterpene synthases (Fig. 2, Table 1) the incubation was also performed with geranyl diphosphate (GDP) instead of DMADP, as GDP is the precursor of monoterpene synthases (Table 3). Among the monoterpenes, which could be well separated and quantified in the chromatogram (see *Materials and methods*), the only detectable one after GDP incubation was limonene, but only in the assay with protein without leader. Compared to the isoprene-formation activity, the formation of limonene was about 200 times lower, indicating that the isolated enzyme indeed catalyzes the formation of isoprene in a highly specific manner. However, the high homology of the identified isoprene synthase to limonene synthases and the marginal activity of GDP-dependent limonene synthesis suggest the possibility that isoprene and limonene synthase genes have evolved from one ancestral gene after gene duplication.

This first successful cloning and expression of an isoprene synthase gene will now open new possibilities. The isolated gene will allow the production of transgenic plants with overexpressed or repressed isoprene synthase. Such plants will be helpful in answering the long unresolved question surrounding the physiological function of isoprene production in plants. The identification of the gene will also allow its regulation to be studied and support attempts to predict the rate of isoprene formation by vegetation (Zimmer et al. 2000) for modeling atmospheric chemistry. Moreover, by the use of *E. coli* strains, such as TG1(pGS72*dxs*/pQE50Δ*iso*), carrying the isoprene synthase gene it will be possible to produce isoprene for the rubber industry in a bacterial fermentor. As the compound isoprene is gaseous in an *E. coli* culture above 34 °C it can be condensed from the gas phase and no additional purification of the liquid product is necessary.

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References

Barkan A (1989) Tissue dependent plastid RNA splicing in maize: transcripts from four plastid genes are predominantly unspliced in leaf meristems and roots. Plant Cell 1:437–445

Bohlmann J, Meyer-Gauen G, Croteau R (1998) Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc Natl Acad Sci USA 95:4126–4133

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. J Biol Chem 268:23016–23024

Fischbach RJ, Zimmer I, Steinbrecher R, Pfichner A, Schnitzler J-P (2000) Monoterpene synthase activity in leaves of *Picea abies* (L.) Karst. and *Quercus ilex* L. Phytochemistry 54:257–265

Kesselmeier J, Staudt M (1999) Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. J Atmos Chem 33:23–88

Kuzma J, Fall R (1993) Leaf isoprene emission rate is dependent on leaf development and the level of isoprene synthase. Plant Physiol 101:435–440

Lehning A, Zimmer I, Steinbrecher R, Brüggemann N, Schnitzler J-P (1999) Isoprene synthase activity and its relation to isoprene emission in *Quercus robur* L. leaves. Plant Cell Environ 22:494–505

Logan BA, Monson RK (1999) Thermotolerance of leaf discs from four isoprene-emitting species is not enhanced by exposure to exogenous isoprene. Plant Physiol 120:821–825

Logan BA, Monson RK, Potosnak MJ (2000) Biochemistry and physiology of foliar isoprene production. Trends Plant Sci 5:477–481

Miller B, Heuser T, Zimmer W (1999) A *Synechococcus leopoliensis* SAUG 1402-1 operon harbouring the 1-deoxyxylulose 5-phosphate synthase gene and two additional open reading frames is functionally involved in the dimethylallyl diphosphate synthesis. FEBS Lett 460:485–490

Miller B, Heuser T, Zimmer W (2000) Functional involvement of a deoxy-D-xylulose 5-phosphate reductoisomerase gene harbouring locus of *Synechococcus leopoliensis* in isoprenoid biosynthesis. FEBS Lett 481:221–226

Paulson S, Seinfeld JH (1992) Development and evolution of a photooxidation mechanism for isoprene. J Geophys Res 97:20703–20715

Sanadze GA (1990) The principal scheme of photosynthetic carbon conversion in cells of isoprene releasing plants. In: Baltscheffsky M (ed) Current research in photosynthesis: Proc 8th Int Congr Photosynth. Kluwer, Dordrecht, pp 231–237

Sanadze JA (1957) Emission of organic matters by leaves of *Robinia pseudoacacia* L. Soobshch Acad Nauk Gruz SSR 19:83–83

Schnitzler J-P, Arenz R, Steinbrecher R, Lehning A (1996) Characterization of an isoprene synthase from leaves of *Quercus petraea* (Mattuschka) Liebl. Bot Acta 109:216–221

Sharkey TD, Singsaas EL (1995) Why plants emit isoprene. Nature 374:769–769

Silver GM, Fall RF (1991) Enzymatic synthesis of isoprene from dimethylallyl diphosphate in aspen leaf extracts. Plant Physiol 97:1588–1591

Silver GM, Fall RF (1995) Characterization of aspen isoprene synthase, an enzyme responsible for leaf isoprene emission to the atmosphere. J Biol Chem 270:13010–13016

Singsaas EL, Lerdau M, Winter K, Sharkey TD (1997) Isoprene increases thermotolerance of isoprene-emitting species. Plant Physiol 115:1413–1420

Thompson AM (1992) The oxidizing capacity of earth's atmosphere: probable past and future changes. Science 256:1157–1165

Wain-Hobson S, Sonigo P, Danos O, Cole S, Alison M (1985) Nucleotide sequence of the Aids virus, LAV. Cell 40:9–17

Wildermuth MC, Fall R (1998) Biochemical characterization of stromal and thylakoid-bound isoforms of isoprene synthase in willow leaves. Plant Physiol 116:1111–1123

Wise ML, Savage TJ, Katahira E, Croteau R (1998) Monoterpene synthases from common sage (*Salvia officinalis*). J Biol Chem 273:14891–14899

Zimmer W, Brüggemann N, Emeis S, Giersch C, Lehning A, Steinbrecher R, Schnitzler J-P (2000) Process-based modelling of isoprene emission by oak leaves. Plant Cell Environ 23:585–595