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SUMMARY

Plants interact with their environment with a great variety of volatile organic compounds (VOCs), isoprenoids (≡ terpenes), i.e. isoprene, mono-, homo-, di- and sesquiterpenes, being the most prominent group. Isoprene, a hemiterpene, is the simplest isoprenoid compound whose main source, woody plant species, comprises 75% of the 500 Tg C isoprene emitted to the atmosphere per year. Due to the significant influence of isoprene in atmospheric chemistry, growing research interests have focused to investigate this C5 compound. However, physiological function(s) of isoprene emission *in planta* is not elucidated to date. Actual studies indicate that isoprene can enhance thermotolerance or quench oxidative stress, but the underlying mechanisms are widely unknown. The work presented here significantly contributes to the understanding of physiological function of isoprene and regulation of isoprene biosynthesis by exploiting transgenic *Arabidopsis thaliana* and *Populus* x *canescens* as model systems.

The first part of the work aimed to elucidate whether isoprene biosynthesis in plants is triggered by endogenous regulatory mechanisms like the circadian clock. Isoprene emission varies diurnally in several species, also in the natural isoprene emitter Grey poplar (*P.* x *canescens*). Moreover it was recently proved that the poplar isoprene synthase gene (*PcISPS*) displays diurnal variation in its expression. Working on shoot cultures of Grey poplar, placed under different light regime in climate chambers, it was possible to show that under continuous light *PcISPS* expression, measured by quantitative reverse transcriptase PCR, oscillated with amplitude of approximately 24 hours testifying for endogenous clock regulation. Furthermore, circadian rhythms were not only limited to the level of gene expression. Isoprene emission rates also displayed circadian changes. In contrast, on the protein level circadian changes could not be detected. It, however, appeared that PcISPS activity and protein content became reduced under constant darkness, while under constant light activity and protein content were higher than under day/night regime. Measurement of additional selected isoprenoid genes revealed that phytoene synthase (*PcPSY*; carotenoids pathway) also displays circadian fluctuations of gene expression whereas 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*PcDXR*), the first committed enzyme of the methylerythritol phosphate (MEP)-pathway only shows a light regulation of its expression.

In the second part of the work *Arabidopsis thaliana* (ecotype Columbia-0), a natural non-emitter of isoprene, has been constitutively transformed with *PcISPS* from Grey poplar. Over-expression of poplar *ISPS* in Arabidopsis resulted in isoprene emitting rosettes that showed enhanced growth rates compared to wild type under moderate thermal stress. The fact that highest growth rates, higher DMADP levels and ISPS enzyme activities were detected in young developing plants indicates that enhanced growth of the transgenic plants under thermal stress is due to the

introduced *PcISPS* gene. However, the emission rates did not reach the level of natural isoprene emitters, like poplars, suggesting possible different regulation of the isoprene biosynthesis and/or lack of substrate for that. To study the physiology of these plants a dynamic gas exchange system was developed allowing miming the natural rapid fluctuations of leaf temperature and light intensity ('sun- or lightflecks') in order to study isoprene emission. The results showed that wild type Arabidopsis is already well enough thermotolerant against transient and moderate light and/or heat stress. In contrast, when the same conditions, combining light and heat flecks, were applied to wild type and transgenic Grey poplar lines in which gene-expression of *PcISPS* was knock-down by RNA interference technology, assimilation was impaired in the non-isoprene emitting lines compared to wild type. Thus, for poplar the ability to emit isoprene can be detrimental.

Transgenic Arabidopsis lines were further applied in ecophysiological studies to investigate the role of isoprene in plant-insect interactions. Feeding of herbivores on plants makes plants release volatile compounds that attract herbivore enemies. In all the performed studies the parasitic wasp *Diadegma semiclausum* searching for its host *Plutella xylostella* (Diamondback moth) preferred the volatiles emitted by wild type Arabidopsis to those from transgenic, isoprene emitting Arabidopsis plants. Furthermore low external isoprene concentration in the volatile blend of either Arabidopsis or herbivore-infested *Brassica oleracea*, the natural host of the Diamondback moth, repelled the parasitic wasps. The behaviors of the two examined herbivores (*Pl. xylostella* and *Pieris rapae* (Small White Cabbage butterfly)) were not affected by isoprene emission, and GC-MS detection showed despite of isoprene no other differences in the VOC blends of wild type and transgenic plants. These findings suggest that isoprene emission of plants plays a complex ecophysiological role, influencing biotic interactions between plants and insects.

Molekularbiologische und (öko)physiologische Untersuchungen zur Isoprenemission bei Arabidopis und Graupappel

ZUSAMMENFASSUNG

Pflanzen stehen mit ihrer Umgebung in ständiger Wechselwirkung durch die Abgabe verschiedener flüchtiger organischer Verbindungen. Die größte Stoffklasse dieser Verbindungen stellen die Terpene dar; zu ihnen gehören u. a. Isopren, Mono-, Sesqui-, Homo- und Diterpene. Isopren, ein Hemiterpen, ist das am einfachsten gebaute Terpen und wird vor allem von holzigen Pflanzen emittiert. Aufgrund seines bedeutenden Einflusses auf die Chemie der Atmosphäre steht Isopren verstärkt im Fokus der Forschung. Trotz dieser Bedeutung ist die physiologische Funktion der Isoprenemission in Pflanzen größtenteils ungeklärt. Aktuelle Studien weisen darauf hin, dass die Produktion von Isopren die Resistenz der Pflanzen sowohl gegenüber thermalem als auch oxidativem Stress erhöhen kann. Die vorliegende Arbeit liefert einen wichtigen Beitrag zum Verständnis der physiologischen Funktion von Isopren und der Regulierung der Isoprenbiosynthese. Als Modellpflanzen für die Untersuchungen wurden transgene *Arabidopsis thaliana* und *Populus* x *canescens* herangezogen, in denen die Isoprenbiosynthese verändert war.

Im ersten Teil der vorliegenden Arbeit sollte untersucht werden, inwieweit die Isoprenbiosynthese von Pflanzen durch endogene Regulationsmechanismen wie die „Innere Uhr“ gesteuert wird. Gewöhnlich weist die Isoprenemission einen ausgeprägten, vom Licht abhängigen, Tageslauf auf. Diese Diurnalität ließ sich auch auf der molekularen Ebene für die Genexpression der Isoprensynthase (PcISPS) nachweisen. Durch kontrollierte Klimakammerversuche mit verschiedenen Lichtregimes konnte an Sprosskulturen der Pappel nachgewiesen werden, dass dieser charakteristische Tagesverlauf nicht nur durch Licht, sondern auch endogen durch circadiane Faktoren gesteuert wird. Quantitative Messungen der Transkriptmengen unter Dauerlicht zeigten, dass die Genexpression der *PcISPS* mit einer Amplitude von ca. 24 Stunden oszilliert. Diese endogene Rhythmik konnte auch für die Isoprenemission selbst nachgewiesen werden. Der Proteingehalt und die Enzymaktivität der ISPS wurden hingegen durch die „Innere Uhr“ nicht beeinflusst. Allerdings wurden bei anhaltender Helligkeit ein höherer Proteingehalt und eine stärkere Enzymaktivität festgestellt als bei dauerhafter Dunkelheit. Die Messung weiterer Gene des Isoprenoidstoffwechsels zeigte, dass auch die Phytoensynthase (*PcPSY;* Gen aus dem Carotinoid-Stoffwechsel) circadian reguliert wird, während die 1-Deoxy-D-xylulose 5-reduktoisomerase (*PcDXR*), das Eingangsenzym des Methylerythritol-Stoffwechsels, lediglich einer Lichtregulation unterliegt.

Im zweiten Teil der Arbeit wurde *Arabidopsis thaliana*, (Ökotyp Columbia-0), eine natürlicherweise nicht Isopren emittierende Pflanze, mit dem Gen der Isoprensynthase (*ISPS*) aus der Pappel transformiert. Die Expression der *PcISPS* in Arabidopsis führte zu einer Emission von Isopren, die jedoch verglichen zu der Emission aus Pappeln sehr viel geringer war. Wachstumsanalysen zeigten, dass die transformierten Isopren emittierenden Pflanzen unter moderatem Temperaturstress ein besseres Wachstum als der nicht emittierende Wildtyp aufwiesen. Die Beobachtung, dass die stärksten Wachstumsunterschiede, die höchsten Konzentrationen an Dimethylallyldiphosphat (DMADP), dem Substrat der ISPS, und die höchsten Enzymaktivitäten in jungen, sich entwickelnden Blättern auftraten, deutet auf einen funktionellen Zusammenhang zwischen dem Einbringen des *PcISPS*-Gens und der physiologischen Reaktion hin. Für die physiologischen Studien an diesen Pflanzen musste ein neues dynamisches Gasaustauschsystem entwickelt werden, das Photosynthese- und Emissionsmessungen an Arabidopsis-Rosetten und Pappelblättern bei schnellem Wechsel von Lichtintensitäten und Blatttemperaturen ermöglicht. Es zeigte sich, dass Arabidopsis-Blätter generell eine hohe Thermotoleranz aufweisen. Dagegen wiesen transgene, nicht Isopren emittierende Pappellinien, bei denen die Genexpression der *ISPS* über RNA-Interferenz (RNAi) unterdrückt wurde, unter identischen Bedingungen eine starke Beeinträchtigung der Photosyntheseleistung und des photosynthetischen Elektronentransports bei kurzfristigem Licht- und Temperaturstress im Vergleich zu entsprechenden Wildtypen auf. Die Ergebnisse liefern einen klaren Beweis dafür, dass die Fähigkeit Isopren zu emittieren eine wichtige Rolle bei der Stabilisierung von photosynthetischen Prozessen in der Pappel einnimmt.

Transgene Linien von Arabidopsis wurden des Weiteren für ökophysiologische Studien herangezogen, um die Rolle von Isopren bei Pflanzen-Insekten-Interaktionen zu untersuchen. Fressen herbivore Insekten an Pflanzen, löst dies eine Abwehrreaktion aus, die zur Abgabe von flüchtigen organischen Verbindungen führen kann, die als Lockstoffe für Feinde der Herbivoren fungieren. In den durchgeführten Studien konnte bewiesen werden, dass die parasitische Schlupfwespe *Diadegma semiclausum* auf der Suche nach ihrem Wirt *Plutella xylostella* (Kohlmotte) jeweils den Wildtyp gegenüber den Isopren emittierenden Pflanzen beworzugte. Wurden der Schlupfwespe zwei befallene Kohlpflanzen (*Brassica oleracea*), der natürliche Wirt der Kohlmotte, oder Wildtyp Arabidopsis-Pflanzen angeboten, eine jedoch mit Isopren begast, bevorzugte die Schlupfwespe auch hier die Isopren-freien Varianten. Das Verhalten der beiden untersuchten Herbivoren (*Pl. xylostella* und *Pieris rapae* (Kleiner Kohlweissling)) wurde dagegen nicht durch Isopren beeinflusst. Bei diesen Untersuchungen gab es keine Unterschiede bei der Emission von anderen Terpenen zwischen den transgenen und nicht transgenen Arabidopsis-Pflanzen. Diese Erkenntnisse weisen darauf hin, dass die Isopren Emission eine komplexe Rolle bei biotischen Interaktionen zwischen Pflanzen und Insekten spielt.

CONTENTS

ABBREVIATIONS

A	absorption
ABA	abscisic acid
ANOVA	analysis of variance
At	*Arabidopsis thaliana*
ATP	adenosine-5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHT	butylated hydroxytoluene
bp	base pair
BSA	bovine serum albumin
BVOC	biogenic volatile organic compounds
C	carbon
cDNA	complementary DNA
CHES	2-(cyclohexylamino)ethanesulfonic acid
CMK	4-diphosphocytidyl-2C-methyl-D-erythritol kinase
CMS	4-diphosphocytidyl-2C-methyl-D-erythritol 4-phosphate synthase
CO_2	carbon dioxide
CTAB	cetyltrimethylammonium bromide
DD	continuous darkness
ddH_2O	didistilled water
DEPC	diethyl pyrocarbonate
DHA	dehydroascorbate
DIG	digoxigenin
DMADP	dimethylallyl diphosphate
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxyribonucleoside 5'-triphosphate
DOX	1-deoxy-D-xylulose
DOXP	deoxy-D-xylulose-5-phosphate
DTT	dithiothreitol
DXR	1-deoxy-D-xylulose-5-reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMBL	European Molecular Biology Laboratory
ETR	electron transport rate
FC	flow controller
FDP	farnesyl diphosphate
FIS	fast isoprene sensor
FSM	fosmidomycin
FW	fresh weight
g	gram
GC	gas chromatograph
gDNA	genomic DNA
GDP	geranyl diphosphate
GGDP	geranylgeranyl diphosphate
GHG	greenhouse gas
gH_2O	stomatal conductance
GLV	green leaf volatile
GSH	reduced glutathione
GSSG	oxidized glutathione
h	hours
HDR	1-hydroxy-2-methyl-butenyl 4-diphosphate reductase
HDS	2C-methyl-D-erythritol 2,4-cyclodiphosphate reductase
HPLC	high performance liquid chromatography
IAA	isoamylalcohol
IDI	isopentenyl diphosphate isomerase
IDP	isopentenyl diphosphate
ISB	isoprene synthase buffer

ISPS	isoprene synthase
K	Kelvin
kat	katal = mol s^{-1}
kb	kilobases
kDa	kilo-Dalton
LB	Luria-Bertani
LL	continuous light
LD	light/dark rhythm within a 24h cycle
M	molar
m	meter
m	milli (10^{-3})
MBB	monobromobimane
MDA	malondialdehyde
MDHA	monodehydroascorbate
2-ME	2-mercaptoethanol
MEP	2-C-methylerythritol-4-phosphate
MeSA	methylsalicylate
min	minutes
µ	mikro (10^{-6})
mRNA	messenger ribonucleic acid
MS	mass spectrometer
MS	Murashige and Skoog (medium)
MT	monoterpene
MVA	cytosolic mevalonate
N	nitrogen
n	nano (10^{-9})
n	number of parallel experiments
NADPH	nicotinamide adenine dinucleotide phosphate
NBT	nitroblue tetrazolium chloride
NO_x	nitrogen oxide
n.s.	no significant difference
O	oxygen
O_2^-	singlet oxygen
O_3	ozone
OD	optical density
OH^-	hydrogen radical
P	propability in statistical tests
PAGE	polyacrylamid gel electrophoresis
PBS	phosphate-buffered saline
Pc	*Populus x canescens*
PCR	Polymerase chain reaction
PEB	protein extraction buffer
PEP	phospho*enol*pyruvate
pH	a value to measure the acidity or alkalinity of a solution (potential Hydrogen)
ppbv	parts per billion by volume
PPFD	photosynthetic photon flux density
ppmv	parts per million by volume
pptv	parts per trillion by volume
PTR-MS	proton transfer reaction - mass spectrometer
RH	relative humidity
RNase	ribonuclease
RT	room temperature
s	seconds
SD	standard deviation
SE	standard error
SDS	sodium dodecylsulfate
SOA	secondary organic aerosol
SQT	sesquiterpene
SSC	sodium citrate buffer
TAE	tris-acetate-EDTA electrophoresis buffer

TBA	thiobarbituric acid
TCA	trichloroacetic acid
TE	tris-EDTA-Buffer
TMTT	(3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetradiene
TPS	terpene synthases
TRIS	tris(hydroxymethyl)aminomethane
V	volt
VOC	volatile organic compound
v/v	volume per volume
w/v	weight per volume
WT	wild type
y	year

I INTRODUCTION

It is not largely known that in addition to photosynthesis plants influence atmospheric chemistry in a multifaceted manner. After oxygen plant produced hydrocarbons are the most abundant reactive chemicals synthesized and emitted by plants. They are highly reactive in atmosphere with lifetimes ranging from minutes to hours and play an important role in the chemistry of the troposphere contributing to ozone production, methane oxidation and carbon monoxide budget (Guenther et al., 1995; Lerdau, 2007) Together these compounds are referred as biogenic volatile organic compounds (BVOC) including all other organic atmospheric trace gases except carbon dioxide or monoxide: isoprenoids (≡ terpenes), alkanes, alkenes, carbonyls, alcohols, esters, ethers and acids (Kesselmeier and Staudt, 1999). From all biogenic VOCs two dominate the global flux: methane and isoprene (2-methyl 1,3-butadiene) each comprising around one third of the overall BVOC flux. Methane is a relatively stable compound remaining unchanged in the troposphere up to years (Guenther et al., 2006) whereas isoprene has much shorter lifetime. It reacts from minutes to hours, e.g. with NO_x and OH-radicals (Guenther et al., 2006). This high reactivity in addition to vague knowledge about the regulation of isoprene emission leads to difficulties estimating global annual isoprene fluxes and its impacts.

Isoprene is contributing to atmospheric reactions in diverse manners. One of the most important reactions for life on earth is isoprene's ability to contribute to tropospheric O_3 formation. Isoprene oxidized through hydroxyl radicals can interfere in NO_x cycling and thus is involved in O_3 production (under high NO concentration) or decomposition (under low NO concentration) in the troposphere (Williams et al., 1997; Lerdau, 2007). Oxidized isoprene is able to convert NO back to a NO_2 molecule that is photolyzed into NO and O_3 molecules under high light intensities and presence of oxygen (Lerdau, 2007). Thus, isoprene can up-regulate O_3 concentration in anthropogenic polluted areas. Given that plants can emit isoprene, e.g., to protect against oxidative condition (Loreto and Velikova, 2001, will be discussed in detail in chapters below) and that anthropogenic pollution of, e.g., NO_x is continuously increasing, a self-feeding loop can be created (Lerdau, 2007).

Isoprene reacting with hydroxyl radicals also has other significant influences on atmospheric processes. Reaction of isoprene with OH-radicals reduces the atmospheric concentration of these radicals. As consequence the lifetime of other greenhouse gases (GHGs), e.g. methane, which also reacts with OH-radicals, can turn longer (Thompson, 1992). In addition, isoprene and other volatile isoprenoids [mono- (C10), sesqui- (C15) and homo- terpenes (C11, C16)] act themselves as GHGs by contributing to the formation of secondary organic aerosols (SOAs) and haze development that scatter and absorb solar radiation above forest canopies (Claeys et al., 2004;

Joutsensaari et al., 2005). Mono- and sesquiterpenes, which are generally lower volatilized than isoprene, are more important for SOA formation (Bonn and Moortgat, 2002). However, as isoprene alone contributes almost half of all non-methane BVOCs, as a single compound it has a considerable influence on SOA formation (Claeys et al., 2004).

According to model predictions by Guenther et al. (1995), the amount of global biogenic non-methane VOC emissions is estimated to be about 1150 Tg (C) Y^{-1} of which isoprene contributes ~ 44% and monoterpenes ~ 23%. Main isoprene sources are woods and shrubs, e.g. alone isoprene emitted from woody species composes over one third from total non-methane BVOCs. However, especially atmospheric concentrations of volatile isoprenoids are highly variable depending on environmental (biotic and abiotic) and plant developmental factors that are still largely under debate, resulting in imprecise modelling data. According to Guenther and co-authors (1995) a reliable atmospheric emission model must contain (1) accurate estimates of source type and density of the chemical species, (2) information to which extend these chemical species are produced, (3) which are the drivers of the chemical species and (4) how these drivers are regulated (Guenther et al., 1995). For all these points actual information is insufficient. Before reliable estimates are possible, more knowledge must be gathered about e.g. how genetic variations and predisposition, light, temperature, humidity, CO_2 concentration, stomatal conductance, leaf development, diurnal and seasonal variation and biotic and abiotic stresses influence plant emission levels (Guenther et al., 1995). At the moment enormous efforts are given in many laboratories to better understand the regulation of VOCs and especially that of isoprene.

The more the regulations of VOC biosynthesis and emission are studied, the more complex they seem to get. Plant isoprenoids are synthesized through condensations of the five-carbon precursors: isopentenyl diphosphate (IDP) and its allylic isomer dimethylallyl diphosphate (DMADP), which can be synthesized from two pathways localized in two separate cell compartments. The cytosolic mevalonate (MVA) or 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway has been known since many years (Agranoff et al., 1960) and is responsible for the synthesis of ubiquinone (Disch et al., 1998), cytokinins, sesquiterpenes (C15), triterpenes (C30), sterols and brassinosteroids (Suzuki et al., 2004). The MVA pathway is found in animals, fungi and phototrophic organisms (Chappell et al., 1995).
DMADP in chloroplasts is synthesized through the 2-C-methylerythritol-4-phosphate (MEP)-pathway that has been discovered in 1993 by Rohmer et al. almost 40 years after isoprene emission was first described (Sanadze, 1957). This pathway is also found in Eubacteria, green algae and *Plasmodium sp*. (Eoh et al., 2007; Cassera et al., 2004; Grauvogel and Petersen, 2007; Okada and Hase, 2005; Massé et al., 2004). It leads to the synthesis of IDP and its isomer DMADP

which are the building units of isoprene (C5), monoterpenes (C10), diterpenes (C20), carotenoids (C40), phytol, tocopherols, phylloquinones, and some sesquiterpenes (C15) (Lichtenthaler, 1999). The precursors of the MEP-pathway are glyceraldehyde-3-phosphate (GA-3-P) and pyruvate. 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is the first acting enzyme in this pathway and is suggested by several studies to have a regulatory role as its expression positively correlates with the concentration of several isoprenoid end products (Lois et al., 2000; Estévez et al., 2001; Muñoz-Bertomeu et al., 2006). Estévez et al. (2001) showed that over-expression of *DXS* in *Arabidopsis thaliana* leads to up-regulation of carotenoid and ABA levels. Similar results were obtained by Lois et al. (2000) in tomato (*Lycopersicon esculentum*) and by Muñoz-Bertomeu et al. (2006) in lavender (*Lavandula latifolia*). DXS forms 1-deoxy-D-xylulose-5-phosphate (DOXP) from which the first truly MEP-pathway specific enzyme 1-deoxy-D-xylulose-5-reductoisomerase (DXR) catalyzes MEP (Fig. 1). DOXP is not only the first intermediate of the MEP-pathway since it is also involved in the thiamine/shiki mate pathway (Julliard and Douce, 1991; Belanger et al., 1995). Therefore, it was assumed that DXR rather than DXS plays a regulatory role in the MEP-pathway (Mayrhofer et al., 2005). Supporting this suggestion, transformation of peppermint (*Mentha* x *piperita*) with *DXR* increased the essential oil yield in the plants. Moreover, Carretero-Paulet et al. (2002; 2006) showed recently that over-expression of *DXR* in *Arabidopsis thaliana* increased the concentration of isoprenoid end products. However, as the *DXR* gene expression does not correlate with the rhythm of isoprene emission (Mayrhofer et al., 2005) its regulatory role remains contradictory. The complete sequence of genes involved in the following five steps of MEP-pathway until the synthesis of isoprenoid direct precursors isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) has already been identified (Eisenreich et al., 2001), however, to elucidate their regulation(s) more investigations are needed.

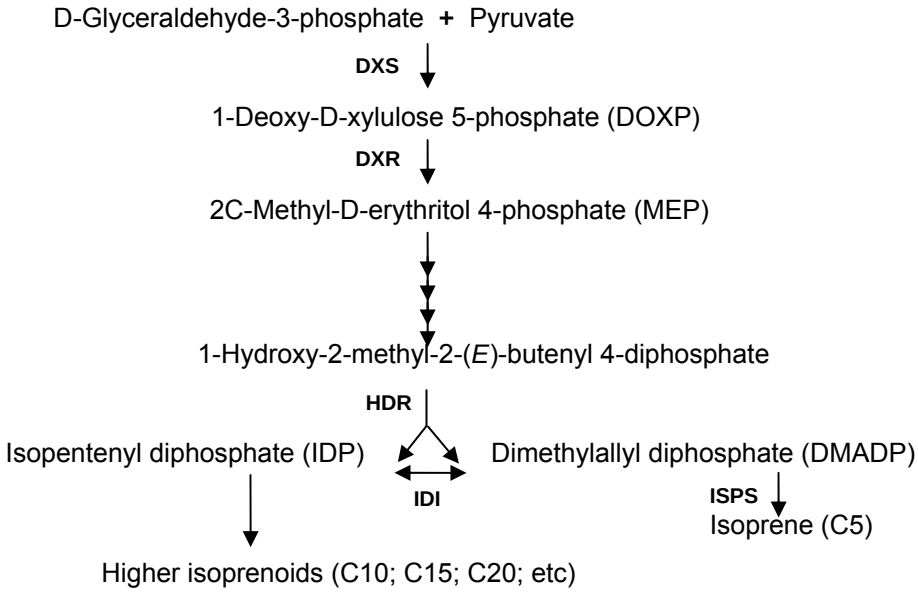

Fig. 1. Diagram of the MEP-pathway that takes place in the chloroplasts (modified after Guevara-Garcia et al., 2005). The enzymes catalyzing the reactions are shown in bold as follows: 1-deoxy-D-xylulose-5-phosphate synthase (DXS); 1-deoxy-D-xylulose-5-reductoisomerase (DXR); 1-hydroxy-2-methyl-butenyl 4-diphosphate reductase (HDR); isopentenyl diphosphate isomerase (IDI); isoprene synthase (ISPS).

The last step in the MEP-pathway is catalyzed by the enzyme 1-hydroxy-2-methylbutenyl 4-diphosphate reductase (HDR) which converts 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate to DMADP, the immediate precursor of isoprene and its isomeric form IDP, the precursor of higher isoprenoids. IDP can also be isomerized by isopentenyl diphosphate isomerase (IDI) to DMADP from which isoprene synthase (ISPS) synthesizes isoprene. In order to synthesize higher isoprenoids the two diphosphorylated C5-units, DMADP and IDP condense in a head-to-tail reaction to produce geranyl diphosphate (GDP; C10). The reaction can be repeated to further produce farnesyl diphosphate (FDP, C15) or geranylgeranyl diphosphate (GGDP; C20) (Ramos-Valdivia, 1997).

It is not only the regulation of the MEP-pathway that remains to be solved but also the interaction between plastidic MEP- and cytosolic MVA-pathway (Lichtenthaler, 1999; Laule et al., 2003; Rodríguez-Concepción et al., 2004). Some studies suggest a cross talk between the two pathways: MVA derived precursors can be used for biosynthesis of isoprenoids in plastids and other way round, in at least some species and plant tissues (Lichtenthaler, 1999). Laule et al. (2003) showed that blocking the MVA-pathway with lovastatin can cause change in plastidic carotenoid and chlorophyll levels (not only in end product levels, but also in transcript levels of the prominent enzymes), whereas cytosolic sterol levels remained constant after recovering from an

initial drop. On the other hand blocking the MEP-pathway with fosmidomycin (FSM), which inhibits the production of MEP from DOXP (Kuzyama et al., 1998) resulted in decreased concentrations of all the isoprenoid metabolites, also in cytosolic sterols. Thus, the results from Laule et al. (2003) suggest rather unidirectional transport from plastids to the cytosol. However, Rodríguez-Concepción et al. (2004) isolated Arabidopsis mutants that could survive in the presence of a blocked MEP-pathway. This was most likely due to enhanced uptake of MVA-derived isoprenoid precursors by plastids.

Even if the fundamental mechanism how isoprene emission is regulated through the MEP-pathway remains unclear, several studies are committed to investigate the initial drivers changing the isoprene emission pattern in the plants. Since 50 years now the question has been: “Why plants emit isoprene?”. The decoding of this question so far has led to three main hypotheses about the physiological role of isoprene for the plant itself:

Isoprene is a costly molecule needing 20 ATP and 14 NADPH for its biosynthesis (Sharkey and Yeh, 2001). Under certain conditions plants can emit up to 15% of photosynthetically fixed carbon back to the atmosphere as isoprene (Sharkey et al., 1996) and even under “normal conditions” a few percent of photosynthetically fixed carbon is directed to synthesize isoprene (Guenther et al., 1995). It is somewhat contradictory that even if isoprene means a significant loss of carbon, the strongest isoprene emitters are fast growing tree species, e.g. from the genera *Populus*, *Salix*, *Eucalyptus* and *Pueraria*. Also many, even not all, of *Quercus* species emit isoprene. Thus, it seems to be evident that isoprene should have a significant advantage for a plant. Such an advantage might be an ability to protect against thermal stress. Indeed, isoprene and DMADP levels are controlled at least by 2 different factors: by the recent CO_2 fixation rate (Schnitzler et al., 2004) and by the temperature dependence of the ISPS activity (Eisenreich et al., 2001; Brüggemann and Schnitzler, 2002a). Monson et al. (1992) showed that isoprene emission fluctuates in temperature dependent manner with its maximal emission above 40°C, and later on it was finally proved by Sharkey and Singsaas (1995) and by Sharkey et al. (2001) that isoprene can protect against thermal stress. Sharkey and co-workers used FSM combined with isoprene fumigation in their studies. Photosynthesis of FSM-fed Kudzu (*Pueraria montana*) leaves that were fumigated with exogenous isoprene was less affected by high temperature episodes than similar leaves that were not fumigated (Sharkey et al., 2001). A similar thermoprotective effect by isoprene has been shown later to be a more general phenomenon in several species (Velikova and Loreto, 2005; Velikova et al., 2005; Wiberley et al., 2005). The most recent study was done by Behnke et al. (2007) who used transgenic non-isoprene emitting poplars where *ISPS* gene expression was knock-down by RNA interference (RNAi) technology. In this study assimilation of the non-isoprene emitting poplars dropped remarkably when the plant had to face fast changing transient

temperature cycles. The result supports the suggestion that isoprene would rather protect against transient thermal stress than against a permanent high temperature (Sharkey et al., 2001). This would explain why isoprene emission capacity is not more common within tropical species and has not at all been found e.g. within desert plants.

The mechanism how isoprene protects against thermal stress is not clear. As well it is not clear how thermal stress inhibits photosynthesis. A reason for the latter can be increasing fluidity of thylakoid membranes under thermal stress. Increased leakiness reduces electron transport capacity resulting in inhibited photosynthesis (Pastenes and Horton, 1996). Here isoprene might play a role by stabilizing the hydrophobic interactions of the membrane lipid bilayer structures (Sharkey and Singsaas, 1995; Singsaas et al., 1997; Sharkey et al., 2001).
Not only high temperature but also oxidative stress increases fluidity of membrane structures in plant tissues. Active oxygen species $O_2^{-\bullet}$, H_2O_2 and $OH^{-\bullet}$, secondary ozone reaction products, are toxic for plant tissues causing peroxidation and denaturation of membrane lipid bilayer (Pell et al., 1997; Noctor and Foyer, 1998). Moreover, isoprene (as well as other alkenes) is able to react with singlet oxygen (O_2), ozone and other OH radicals (Sauer et al., 1999) and this could be another mechanism of protection against photo-oxidative stress (Loreto and Velikova, 2001; Affek and Yakir, 2002; Velikova et al., 2005). Several studies have shown that the ability to emit isoprene can indeed protect a green plant tissue against oxidative stress. Loreto et al. (2001) showed that isoprene can function as antioxidant in tobacco (*Nicotiana tabacum*) and birch (*Betula pendula*) leaves that were fumigated either with ozone or ozone combined with isoprene. In the same year Loreto and Velikova (2001) showed that FSM-fed reed (*Phragmites australis*) plants treated with 100 ppbv ozone for 8h had, e.g., lower assimilation rates and higher lipid peroxidation levels than the respective control plants.

In addition to protection against thermal or oxidative stress, isoprene was suggested to serve as overflow mechanism for excess of carbon intermediates or photosynthetic energy (Logan et al., 2000; Rosenstiel et al., 2004). Rosenstiel et al. (2004) suggested a so called "safety valve" hypothesis to explain why plants emit isoprene. Isoprene as a metabolic ‘safety valve’ could prevent the unnecessary sequestration of phosphates. The hypothesis is based on the observation that chloroplastic DMADP level can be regulated extrachloroplastically by foliar assimilation of NO_3^- that competes for the PEP in cytosol. Competition has a direct effect on PEP- and further DMADP-levels in chloroplast and could thus alter the isoprene emission rate.

Isoprene emission is dependent on the availability of substrate which is mainly derived from recently fixed CO_2. Thus it is evident that it also depends on photosynthetic photon flux densities (PPFD) and on intraday fluctuations. Mayrhofer et al. (2005) proved that diurnal rhythms influence

the PcISPS activity and *PcISPS* gene expression rate which have their maximum before midday in Grey poplar. Furthermore, several studies exist about the seasonal variations of isoprene emission (Monson et al., 1994; Kempf et al., 1996; Funk et al., 2005; Mayrhofer et al., 2005) showing an increase in basal isoprene emission capacity in spring and rapid decrease of it in autumn. Furthermore, the basal isoprene emission was shown to depend on other leaf physiological and environmental factors, i.e., on leaf developmental stage (Kuzma and Fall, 1993; Mayrhofer et al, 2005), previous growth conditions (Wiberley et al., 2005), nutrition (Rosenstiel et al., 2004), atmospheric CO_2 level (Rosenstiel et al., 2003; Scholefield et al., 2004; Calfapietra et al., 2007), atmospheric O_3 level (Calfapietra et al., 2007) and leaf position within the canopy (Sharkey et al., 1996). The latter factors, however, have rather minor influence on basal isoprene emission capacity whereas temperature and light can increase or decrease isoprene emission rapidly by several order of magnitudes (Behnke et al., 2007).

Despite of the fact that isoprene is a widely studied compound, so far nothing is known about its possible interactive role between plants and their environment. Plant released VOC are namely used as a kind of language to enable communication between plants and their environment. Activation of the plant signaling network by pathogen or insect attack induces a plant and herbivore species specific blend of VOC that can support plants self-defence by repelling herbivores and pathogens or by luring herbivore enemies to the plants (Pichersky and Gershenzon, 2002; Degenhardt et al., 2003). The latter, indirect defence provides plants a top-down control of herbivore populations (Baldwin and Preston, 1999) that was for the first time observed within spider mite-infested Lima beans calling carnivorous mites for help (Dicke and Sabelis, 1988) and was later shown to be a more general phenomenon between several plant and predator or parasitoid species (Degenhardt et al., 2003).

The commonly used model plant *Arabidopsis thaliana* is known not to emit isoprene. However, Arabidopsis, thought to have a relatively simple metabolism, has been shown by *in silico* analysis to have over 30 putative genes belonging to the multigene family of terpene synthases (*TPS*) (Aubourg et al., 2002; Chen et al., 2003). Most of these genes are almost exclusively expressed in flowers (Chen et al., 2003; Tholl et al., 2005; Aharoni et al., 2006), but low terpene emissions from leaves and siliques (Van Poecke et al., 2001; Chen et al., 2003) and even monoterpenes (namely 1,8-cineole) from roots (Steeghs et al., 2004; Chen et al., 2004) have been detected. The rate of terpene emission from Arabidopsis is comparatively low relative to insect-pollinated species (Chen et al., 2003), however, when induced the emitted compounds are far enough to attract herbivore enemies (Van Poecke et al., 2001; Van Poecke and Dicke, 2002). This relatively low constitutive isoprenoid emission from leaves (Van Poecke et al., 2001; Chen et al., 2003) makes Arabidopsis a suitable tool to study the role of individual compounds by means of transformation. For example

over-expression of linalool/nerolidol synthase (*Fa/NES1*) in chloroplasts (Aharoni et al., 2003) or in mitochondria (Kappers et al., 2005) of Arabidopsis plants, improved plant fitness by repelling aphids or attracting carnivorous predatory mites, respectively. Similarly, introducing maize TPS10 gene, responsible of several sesquiterpenes synthesis, into Arabidopsis, showed that activation of a single gene can be enough for a parasitoid, *Cotesia marginiventris*, to learn to locate its host (Schnee et al., 2006). Even if some of the attempts to elucidate the function of individual compounds in a VOC blend were less successful due to e.g. lack of substrate (Beekwilder et al., 2004) or remodification of the product (Lücker et al., 2001), these studies provided important information about possible problems and pave the way for more successful applications.

II AIM OF THE THESIS

The present thesis contributes to the efforts to study the regulation and function of isoprene emission. It aims to find answers to some of the open questions why plants release such an amount of carbon to the atmosphere. Aims of the present thesis were:

- To investigate the regulation of isoprene emission from a native isoprene emitter in order to find out whether the diurnal rhythm of isoprene emission is mainly light or rather circadian regulated. The aim was to use sterile Grey poplar cultures cultivated under controlled conditions in climate chambers in which un-predicted environmental factors would not disturb the study. Thus the obtained rhythm could be ascribed to a circadian clock control.
- To introduce the isoprene emission capacity in normally non-isoprene producing plants by genetic transformation. The previous isolation of the isoprene synthase gene (*PcISPS*) from Grey poplar (*Populus* x *canescens*) (Miller et al., 2001) provided the opportunity to use the isoprene synthase gene from poplar. Thus in the present work isoprene emitting *Arabidopsis thaliana* plants expressing constitutively the isoprene synthase (*PcISPS*) gene should be created.
- To create Arabidopsis mutants over-expressing the *PcDXR* gene from Grey poplar cDNA. The previous isolation of *PcDXR* from Grey poplar (Mayrhofer, 2006) made it possible to use this gene. Aim of this approach was to later co-transform Arabidopsis with both genes, *PcDXR* and *PcISPS*, thereby enhancing the metabolic flow through the MEP-pathway to provide more substrate for isoprene biosynthesis.
- To prove the proposed ‘thermotolerance hypothesis’ of isoprene by using positively selected and biochemically characterized transgenic lines of isoprene emitting Arabidopsis and isoprene non-emitting Grey poplar.
- The final aim of the thesis was to use the transgenic isoprene emitting Arabidopsis as tools to investigate the completely unknown role of isoprene in plant-insect interactions.

III MATERIALS AND METHODS

1 PLANT AND INSECT MATERIAL, CULTIVATION AND HARVESTING

1-1 Arabidopsis

Arabidopsis thaliana subsp. Columbia 0 (Col0) (Brassicaceae) were mostly cultivated in climate chambers with 22 ± 2°C (day:night) and a L16:D8h photoperiod (Photosynthetic Photon Flux Density (PPFD) 80 µmol photons m^{-2} s^{-1} during the light period) like described in Loivamäki et al. (2007b (**VII: 2**)). In the experiments investigating plant-insect interaction, the plants were cultivated in a climate chamber of the Laboratory of Entomology (Wageningen University) with 21°C ± 1°C and a L8:D16h photoperiod (PPFD 80-110 µmol photons m^{-2} s^{-1} during the light period) as described in Loivamäki et al. (2008 (**VII: 3**)). Plants were either selected on antibiotic containing agar or directly sown to the soil (Loivamäki et al., 2007b/2008 (**VII: 2,3**)).

Before plating on MS (Murashige and Skoog, 1962) agar (**C2**) the seeds were sterilized to inhibit the growth of unwanted microorganisms. Sterilization was performed by submerging the seeds for 5 minutes in a chloral mixture containing 30 % (v/v) Danklorix[11]and 0,2% (v/v) Triton X100[4]. After sterilization the seeds were washed 5 to 6 times with sterile ddH_2O and plated immediately on MS-agar (**C2**) containing the proper antibiotics. The plates were stored at +4°C for 3 to 5 days after which they were transferred to climate chamber conditions. One-week old plants that had developed 2 to 4 leaves were then transferred to soil (Loivamäki et al., 2007b (**VII: 2**)). Several small plants were placed in one 7x7 pot[1] to ensure the survival of one. In the age of 2 to 3 weeks the extra plants were removed. The plants were used in the vegetative stage. When a plant started to make the first stem, it was cut in order to either create bigger rosettes or more flowers and seeds.

Fig 2. A transgenic Arabidopsis rosette in the age of 5 weeks.

1-2 Grey poplar

1-2-1 Propagation of shoot cultures and design of circadian experiments

Six to seven wild type *Populus* x *canescens* (Aiton) Sm. (Salicaceae) shoots were grown in 1L glass containers on half-concentrated MS medium (**C1**). Plants grew under standard conditions at 27:24°C (day:night), using a L16:D8h photoperiod and 65 µmol m^{-2} s^{-1} PPFD during the light

period. Initially several (up to 25) small plant parts were transferred into medium (**C1**) and let to grow a new shoot. After approximately 4 weeks the obtained shoots were transferred onto new medium, one jar containing approximately 7 poplar shoots (Fig. 3). The plants were used in experiments in the age of 7 to 8 weeks.

Fig 3. Grey poplar shoots in sterile cultures.

For circadian studies three different photoperiods were necessary: L16:D8h; L24h; D24h. For this experimental design darkness was achieved by wrapping the jars in aluminium foil after which temperature in a jar stayed at 25 °C. Nine containers (3 containers per day over 3 days) were necessary per condition (long day, continuous darkness, continuous light). For all the experiments, plants were placed in test conditions at 22:00 the day before first sampling. For all experiments samplings were done at 5:00 (one hour before the end of the standard night), 7:00, 14:00, 22:00 (just before start of the standard night) and 24:00. For the darkness condition, containers were opened and samples were taken under red light. All samples, consisting of all leaves of one shoot, were immediately frozen in liquid nitrogen and stored at -80 °C until further use.

1-2-2 Cultivation of Grey poplar plants under greenhouse conditions for light- and temperature-fleck simulation experiment

Wild type and transgenic *Populus* x *canescens* lines were cultivated for the second summer in a greenhouse compartment in Garmisch-Partenkirchen, Germany. One and a half year before the experiment the plants had been transferred from sterile cultures into the soil as described by Behnke et al. (2007). Plant shoots were cut for the winter and at the time of the experiment (July, 2006) the resprouted new shoots reached again a size of approximately 1 ± 0.5 m. In addition to wild type, two transgenic non-isoprene emitting Grey poplar lines were investigated. According to emission rates given in Behnke et al. (2007) and by personal communication with Katja Behnke, the isoprene-free lines RA1 and RA22 were chosen for the present studies.

To create standard and controllable conditions for the light- and temperature-fleck simulation experiment, poplars were transferred into a climate chamber (28 ± 2°C, 300 µmol m^{-2} s^{-1} PPFD and photoperiod of L16:D8h) (Fig. 4) 5 days before the start of the experiment. The gas exchange

measurements were started with each leaf sample 2 hours after onset of light. To perform the light fleck simulation experiments with 3 samples per day the plants faced the L16:D8h photoperiod in different rhythms: light for 16h was turned on either at 4:00, 6:00 or 8:00 on the morning. The leaf no. 9 from the top was used as a sample leaf. The control leaf was chosen in its direct proximity.

Fig. 4. Wild type and transgenic isoprene non-emitting poplars in a climate room

1-3 Insects and Brassica sprouts

Brassica oleracea was used for herbivore rearing and moreover in Y-tube olfactometer experiments. The plants were cultivated in commercially available soil in a greenhouse compartment with a temperature of 23 ± 2°C and relative humidity of 60 ± 10 %. Bioassays were performed with plants having 6 to 8 leaves per plant.

Pieris rapae (Lepidoptera, Pieridae) and *Plutella xylostella* (Lepidoptera, Plutellidae) were continuously reared on *Brassica oleracea* var. *gemmifera* cultivar Cyrus (Brassicaceae) plants in separate climate rooms at 21 ± 1°C, 60 ± 10% relative humidity (RH), and a L16:D8h photoperiod. Caterpillar-infested plants were obtained by placing 20 first instar larvae (either *P. rapae* or *Pl. xylostella*) distributed over several leaves of each plant. The larvae were allowed to feed for 24h on the plants before the plants were used for the experiments.

The parasitic wasp *Cotesia rubecula* (Hymoneptera, Braconidae) was reared on *P. rapae* larvae feeding on *B. oleracea* as described in Loivamäki et al. (2008, (**VII: 3**)), and the parasitic wasp *Diadegma semiclausum* (Hymoneptera, Ichneumonidae) (Fig. 5) was reared on *Pl. xylostella* larvae feeding on *B. oleracea.* Detailed descriptions of rearing procedures can be found in Loivamäki et al., 2008 (**VIII: 3**).

Fig. 5. A female of the parasitic wasp *Diadegma semiclausum* next to a larva of *Plutella xylostella*. Photo: Tibor Bukovinszky / www.bugsinthepicture.com.

For bioassays the parasitic wasps were collected and transferred to separate cages in which they were provided with honey and water. Male and female wasps were kept together until the experiment started. Both wasp species were originally collected from a Brussels sprouts field in the vicinity of Wageningen, the Netherlands. Adult wasps that were used in the experiments did not have oviposition experience before and are therefore referred to as naïve wasps.

2 MOLECULAR BIOLOGICAL METHODS

2-1 Description of basic methods

2-1-1 Plasmid isolation

2-1-1-1 Plasmid isolation according to Birnboim and Doly

The plasmid isolation was performed according to the method described by Birnboim and Doly (1979) when screening transformed bacterial cells (**III: 2-3-1**). For isolation 2 ml of overnight bacterial culture (**C3**) was centrifuged (20,000 x *g*) in plastic tubes[16] for 2 minutes (Sigma, 3K15, Sigma Laborzentrifugen GmbH, Osterode, Germany) and the bacterial pellet was used in further steps. The bacteria were first resuspended in BD1 (**E2**) and the suspension was incubated at room temperature (RT) for 5 minutes, centrifuged and the supernatant was discarded. The cells were opened by resuspending in BD2 buffer (200 µl) (**E3**). The samples were mixed by inverting the tubes and incubated on ice for 5 minutes. For precipitation of the salts buffer BD3 (**E4**) was added to the samples and the twice inverted tubes were incubated for 5 minutes at RT. The tubes were centrifuged for 10 minutes (20,000 x *g*) and the liquid phase was transferred to a new tube. At the next step a one time phenol[4]:chloroform[4] (1:1 (v/v)) (latter with isoamylalcohol[4] 24:1 (v/v)) extraction was performed (1:1 (v/v)) (**III: 2-1-5-2**). The aquatic phase was transferred into 100% ethanol[4] (1 ml) and DNA precipitated for 30 minutes on ice. After centrifugation (16 min at 4°C) the supernatant was removed and the pelleted DNA was washed with 70% ethanol. The DNA was dried (Speedvac, Eppendorf, Hamburg, Germany), and the pellet was resuspended in ddH_2O. The obtained plasmid was diluted in TE-buffer (**G5**).

2-1-1-2 Plasmid isolation by Qiagen Spin Minikit

Alternatively to the above described plasmid isolation plasmids were also isolated and purified with the Qiagen Spin Minikit[5] according to manufacturer's protocol from 2 ml of overnight bacteria culture (**C3**). The method was used mostly, only not when transformed bacterial cells were to be screened due to the large quantity of samples.

2-1-2 Plant DNA and mRNA isolation and cDNA synthesis

2-1-2-1 DNA isolation by 2x CTAB

For genomic DNA (gDNA) isolation 100 mg or 1 g leaf material was homogenized under liquid nitrogen with mortar and pestle. 200 µl (or in the case of gDNA 5 ml) pre-warmed (65°C) 2x CTAB-buffer (with 2% (v/v) 2-mercaptoethanol[8]) (**E1**) was added onto the frozen leaf material and mixed with a vortex (Heidolph, Reax 2000, Schwabach, Germany). Samples were incubated at 65°C in a water bath (6A, U3, Julabo, Seelbach, Germany) for 20 min. The extraction was enhanced by mixing with a vortex a few times during the incubation. DNA isolation was continued by phenol:chloroform:isoamylalcohol-extraction like described in **III: 2-1-5-2** and by precipitation like described in **III: 2-1-5-3**. Finally the RNA was digested from the DNA samples by adding RNase (20 µg ml^{-1}) and incubated at 37°C for 30 minutes or in the case of gDNA over night. In order to avoid breakage of DNA bonds the gDNA was treated very gently during the extraction process.

2-1-2-2 mRNA isolation and cDNA synthesis

Total RNA from leaves, roots and inflorescences (similarly from poplar and Arabidopsis leaves) was isolated with the Qiagen RNeasy Minikit™[5] following the Qiagen standard protocol. Amount and purity of isolated RNA was determined spectrophotometrically as described in **III: 2-1-4**.

For first strand cDNA synthesis 3 µg of total RNA were reverse transcribed immediately after the RNA extraction. The cDNA synthesis was performed with 11 µl RNA in DEPC-H_2O (with 3 µg RNA) and 1 µl oligo (dT) primers[3] in 0.2 ml plastic tubes[16]. The synthesis was started by linearising the RNA at 70°C for 10 min (in a Trio 48 Thermocycler, Biometra, Göttingen, Germany) after which primers were let to anneal onto the RNA by cooling the reaction mixture on ice for 3 min. Simultaneously a master mix containing 4 µl 5xBuffer[3], 1 µl dNTPs[3], 2 µl DTT[3], 0.5 µl RNase inhibitor[3] and 1 µl Superscript II reverse transcriptase™ [3] per sample was prepared. This master mix was then added to each RNA sample. Reverse transcription was performed at 42°C for 50 min. The transcriptase was inactivated within 15 min at 70°C. Obtained cDNA was stored at -20°C prior to analysis.

2-1-3 Gel electrophoresis

Gel electrophoresis was performed to separate DNA, RNA or protein on an agarose gel. This was done by applying an electric current (120V). The negative charged nucleic acids move through the

agarose matrix towards the cathode according to their size (bigger size of nucleic acids or higher agarose concentration make the movement slower). A standard agarose gel was prepared by adding 3 µl of ethidiumbromide (**G3**) in 1x TAE buffer (**G1**) with 1% (w/v) agarose[3]. The sample was prepared by adding 20% (v/v) bromphenolblue loading buffer (**G2**) to the total volume (maximum 20 µl) of a sample. The size of the fragment was determined by 1kb standard ladder[3] that was loaded into one well of each prepared gel.

2-1-4 DNA and RNA concentration and purity determination

Amount and purity of isolated DNA and RNA were determined spectrophotometrically. Optical density (OD) of 1 at the wavelength 260 nm means that the sample involves 33, 40 or 50 µg ml^{-1} of single-stranded DNA, RNA or double-stranded DNA, respectively. When the OD was determined by a Perkin Elmer UV/VIS Spectrophotometer (Perkin Elmer, Waltham, MSC, USA) the nucleic acids were diluted normally 1:50. When the OD was determined by a NanoDrop® ND-1000 (Peqlab Biotechnologie GmBH, Erlangen, Germany) 2 µl of the pure nucleic acid solution was used. An absorbance ratio 260/280 nm of around 1.8 to 2 testified for pure RNA. If the value was under 1.6 the sample was considered to be contaminated by proteins or phenols.

2-1-5 Restriction, purification, precipitation, ligation

2-1-5-1 Restriction

Restriction enzymes cleave double-stranded DNA by producing either complementary or blunt ends. Vectors contain a so called “multiple cloning site” (MCS) in which known different restriction enzymes can cleave. The MCS site makes it easy to clone a sequence of interest into a vector and in addition makes it possible to cleave it out again. A restriction was prepared as follows:

2 µl 10x Buffer (specific for each enzyme)[3]
2 µl DNA from plasmid mini preparation
15.8 µl ddH2O
0.2 µl restriction enzyme[3]

The restriction mixture was carefully mixed and centrifuged shortly after which the restriction was performed in an incubation oven (Heraeus ST5042, Heraeus, Hanau, Germany) at 37°C for approximately 2 h.

2-1-5-2 Phenol/chloroform/isoamylalcohol extraction

Phenol:chloroform:isoamylalcohol extraction was performed in order to separate DNA from protein. Proteins partition into the organic phase whereas DNA remains water soluble in the aquatic phase. Equal volumes of phenol:chloroform (latter with isoamylalcohol 24:1 (v/v)) (1:1) and the DNA

sample were mixed. The mixture was incubated at RT for 10 min and centrifuged for 10 min at 22,000 x *g* to separate phases. The liquid phase was carefully transferred to a new centrifuge tube without touching the organic phase. The extraction was repeated similarly once and followed by precipitation with ethanol (**III: 2-1-5-3**).

2-1-5-3 Precipitation

Ethanol precipitation is a method used to concentrate DNA. DNA is insoluble in the relatively nonpolar ethanol and therefore precipitates. The precipitation was performed with 5% (v/v) Na-acetate and 2.5 volume ethanol for at least 20 min or overnight on ice or at 4°C. The DNA was collected by centrifugation for 20 min at 22,000 x *g* at 4°C after which the supernatant was removed. The DNA pellet was washed with 70% ethanol and centrifuged again. DNA was dried either in a rotating exsiccator (Speedvac, Eppendorf, Hamburg, Germany) (approx. 5 min) or under a hood (overnight).

2-1-5-4 Ligation

DNA ligase is an enzyme that links together cutted DNA strands. Ligation of a gene (e.g. *PcISPS*) and a binary vector (e.g. pBinAR), that were both cleaved with the same restriction enzymes, (e.g. *Kpn*I and *BamH*I) was prepared as follows:

3 µl gene and vector mixture
1 µl ligase buffer[3]
1 µl ligase[3]

The ligation was performed at 4°C overnight. On the next morning 2 µl of the ligation-solution were carefully mixed with competent Top 10 cells[3] on ice and the clone transformed into *Escherichia coli* as described in **III: 2-3-1**.

2-1-6 Polymerase Chain Reaction (PCR)

A PCR is performed in order to amplify a DNA fragment and generate many copies of it. This is done by help of the heat stable Taq-polymerase enzyme that, elongating specific primers, amplifies the DNA fragment of interest. A standard reaction was prepared in 0.2 ml plastic tubes as follows:

2 µl 10xBuffer[3]
0.8 µl 50 mM MgCl[3]
0.8 µl dNTPs[3]
1 µl 50 mM Forward primer[4]
1 µl 50 mM Reverse primer[4]
1 µl Taq[3]
12.4 µl ddH_20
2 µl sample e.g. plasmid, diluted 1:10

The PCR reactions were run with a Thermal cycler (Trio 48 Thermoblock or Personal Cycler, Biometra, Göttingen, Germany). The reaction started with 3 minutes initializing (denaturizing) step at 94°C. After the hot start 30 cycles of denaturizing and re-annealing were performed:

45s at 94°C	→ denaturizing
30s at adequate T	→ annealing
120s at 72°C	→ elongation

The annealing temperature was chosen according to the melting temperature of the specific oligonucleotide primers. The list of primers that were used in the different applications of the thesis is summarized in Table 1. The elongation temperature was at 72°C optimal for Taq-polymerase activity. The PCR ended with 10 min at 72°C. The obtained products were stored at -20°C until use.

Table 1. Sequences of the primers used in polymerase chain reaction (PCR) and/or in quantitative Reverse Transcript (RT)-PCR.

gene	Name of primer	Sequence in 5`-3` order
PcISPS	Isoprensyn.For.spez.	TTT GCC TAC TTT GCC GTG GTT CAA AAC
	Isoprensyn.Rev.spez	TCC TCA GAA ATG CCT TTT GTA CGC ATG
	IS.sequ.1 (for)	GAC TCT GCT TGA ACT GAT AGA TAA TGT CC
	IS.sequ.2 (rev)	TAG AGA GCT AGG AAG CAG AGC TTC
	IS.sequ.3 (for)	GAT CTT CGC GAG ACA TCA AGG TG
PcDXR	DXR-4f	GGA ATA GTA GGT TGC GCA GGC
	Dxr-2r	GCA TAT GTC TTT TCC AGC TTC TAT TGC
	Dxr 3f	GCT CTT CTT GCA GAC CAG GTG
PcPSY	5104-Psy-RT155F rev	CTG CTC TAT CAA ACA CTG TCT CCA A
	5105-Psy-RT241R	TTT TCA AGT CCA TTC TCA TTC CTT C
PcTUB	tub_150_for	GAT TTG TCC CTC GCG CTG T
	tub_150_rev	TCG GTA TAA TGA CCC TTG GCC
AtAct	Act for 17	CGA TGC CTG AGA ACA TAG TGG TT
	Act rev 134	GCT GCT GGA ATC CAC GAG AC
NPTII	nptII-for	ATT CGG CTA TGA CTG GGC AC
	nptII-rev	ACA AGA CCG GCT TCC ATC C

2-1-7 Sequencing of the fragments obtained from PCR

To verify the amplification of the correct genes from poplar, the amplified cDNA fragments were isolated from a 0.8% agarose gel (**III: 2-1-3**). The fragment purification from gel was performed with NucleoSpin® Extract 2 in 1-kit[15] as described in the manufacturer's protocol. The concentration and purity of the DNA was determined with NanoDrop® ND-1000 and the samples were externally sequenced by MWG-Biotech AG, Ebersberg, Germany.

2-2 Construction of binary vector cassettes containing the gene of interest

2-2-1 *PcISPS* into pBinAR through ligation reaction

To clone *Populus* x *canescens* isoprene synthase *(PcISPS)* (EMBL AJ294819) a partial sequence from position 39 to 1868 from the original sequence was ligated into the bacterial expression vector pQE50 (Qiagen, Hilden, Germany) by introducing a *BamH*I restriction site at the 5'-end and a *Kpn*I restriction site at the 3'-end (Bachl, 2005). This subclone, harboring the complete coding sequence of *PcISPS*, was shown to produce isoprene when heterologously expressed in *E. coli* (described in the PhD thesis of Bachl, 2005) and was therefore used for the further cloning steps.

In the next step the *BamH*I–*Kpn*I - fragment from pQE50 was ligated into the MCS of the binary vector pBinAR (Höfgen and Willmitzer, 1990). The gene was cut out from the vector with a *BamH*I-

*Kpn*I restriction and after electrophoretic separation purified from the 0.8% (w/v) Agarose gel (**III: 2-1-3**) with NucleoSpin® Extract 2 in 1-kit[15] as described in the manufacturer's protocol. Simultaneously the pBinAR vector was digested by the same enzymes and purified with phenol:chloroform:isoamylalcohol (IAA) extraction (**III: 2-1-5-2**, until the first phenol:chloroform:IAA extraction). The *PcISPS* gene and the linearized vector were pooled together and a phenol:chloroform:IAA extraction was repeated. The obtained DNA was precipitated as described in **III: 2-1-5-3**. The received pellet was diluted in 3 µl ddH_20 in which 1 µl 10x ligase buffer[3] and 1 µl ligase[3] was added (**III: 2-1-5-4**). The ligation reaction was incubated overnight (16h) and the obtained clone harboring the *PcISPS* gene was then transformed into *E. coli* (**III: 2-3-1**). The full length *PcISPS* in pBinAR was now under the control of the constitutive 35S-promotor and an OCS-terminator (see construct in Fig. 1A, in Loivamäki et al., 2007b (**VII: 2**)) that where provided by the binary vector (Höfgen and Willmitzer, 1990).

2-2-2 *PcDXR* into pH2GW7 through Gateway™ recombination

To clone the *Populus* x *canescens* 1-deoxy-D-xylulose-5-reductoisomerase gene (*PcDXR,* EMBL AJ574852) into the Gateway destination vector pH2GW7 (University of Gent, Belgium), the Gateway™-system was used. The Gateway™ technology is based on bacteriophage λ mediated site specific recombination reactions. A DNA-fragment of interest is cloned between specific *Att*-recombination sites after which the DNA-fragment can be transferred into other vectors that contain compatible *att*-recombination sites. Such compatible sites are *attB*x*attP* and *attL*x*attR* between which the recombination reaction is catalyzed by BP-clonase™ or LR-clonase™.

The *PcDXR*-gene used in the present work was previously sequenced and cloned between *attR* sites in the Gateway™ destination vector pDEST17™ by Mayrhofer (2006). For its use in the present work the gene was cloned back between the *attP*-sites of the donor vector pDONR221™ that serves as a general donor vector. The reaction was mediated by the Gateway™ BP-clonase™ enzyme mix as described in the Gateway™ manual. The obtained entry clone was transformed into *E. coli* strain DH5α[3] (**III: 2-3-1**) and positive clones were selected on LB-agar (**C4**) with kanamycin[14] (25 µg ml^{-1}), an antibiotic to which pDONR221™ has resistance.

In the next step the *PcDXR*-gene was transferred into the destination vector pH2GW7 containing the *attR*-sites. This binary vector for plant transformation contains 35S promoter, NOS terminator and genes for hygromycin (for plant selection), spectinomycin and streptomycin (for bacterial selection) resistances. This vector was selected to insert *PcDXR* into transgenic Arabidopsis already containing the *PcISPS* gene and kanamycin as selection marker. The recombination of the *PcDXR* fragment from pDONR221 into the destination vector was mediated by Gateway™ LR-clonase™ enzyme mix. The reaction was performed as described in the Gateway™ manual. The obtained expression vector was further transformed into *E. coli* as described in the next chapter

(**III: 2-3-1**). The positive clones were selected with streptomycin[10] (20 µg ml^{-1}) containing LB-agar (**C4**).

2-3 Transformation and selection

2-3-1 Transformation and selection of *E. coli* cells

Products obtained from ligation as described above were transformed into *Escherichia coli* (Top10[3] (in the case of *PcISPS*) and DH5α®[3] (in the case of *PcDXR*), both chemically competent cells) by using the TOPO-TA Cloning® Kit[3]. The TOPO cloning reaction was performed according to manufacturers' protocol using 2 µl of the PCR product, 1 µl of salt solution[3], 2 µl of ddH_2O and 1 µl of TOPO® vector[3] per reaction. The reaction tubes were gently mixed and incubated at RT for 30 minutes after which they were placed on ice.

The transformation was further performed by thawing the *E. coli* competent cells on ice. TOPO® cloning reaction[3] (2 µl) was added into the vial containing the competent cells, mixed and the reaction was further incubated on ice for 30 minutes. For transformation the cells were heat-shocked without shaking in a water bath at 42°C for 30 seconds. 250 µl of SOC-medium[3] was added onto cells directly after the heat shock. The tubes were incubated at 37°C for 1h after which 10 to 50 µl of the cells were transferred on LB-plates (**C4**) containing the appropriate antibiotica (kanamycin[14] (125 µg ml^{-1}) in the case of *PcISPS* transformation and streptomycin[10] (20 µg ml^{-1}) in the case of *PcDXR* transformation).

Clones were cultivated (37°C) overnight. 24 individual colonies were then further cultivated in 5 ml LB medium (**C4**) containing the antibiotics of interest. The plasmid was purified according to Birnboim and Doly (**III: 2-1-1-1**) and the transformation verified by digesting (**III: 2-1-5-1**) the *PcISPS* fragment (approximately 1800 bp) from the plasmid with *BamH*I[3] and *Kpn*I[3] or *PcDXR* with *Apa*I[3] and *Spe*I[3]. The fragments were visualized by gel electrophoresis (**III: 2-1-3**). Additionally, the presence of *PcISPS* in DNA level (plasmid isolation see **III: 2-1-1-2**) was verified by PCR (**III: 2-1-6**) that was performed with the primers "IS.sequ.1" and "IS.sequ.2" (Table 1). The presence of the expected fragment was proved by gel electrophoresis (**III: 2-1-3**).

2-3 Transformation and selection of *A. tumefaciens* cells

The purified plasmid was further transformed into *Agrobacterium tumefaciens* strain C58C1 pMP90. The transformation started by cultivating *A. tumefaciens* cells overnight at 28°C in LB-medium (**C3**) containing gentamicin[2] (25 µg ml^{-1}) and rifampicin[2] (100 µg ml^{-1}). On the next morning two large *A. tumefaciens*-cultures (à 150 ml) were started from the overnight culture and let to grow until an OD_{550nm} of 0.5 to 0.6 was reached. The cells were centrifuged for 20 min at 2,000 x *g* at 4°C and the supernatant was discarded. The cells were washed on ice twice by resuspending them into 150 ml cold ddH_2O, centrifuged and the supernatant again discarded. The

same was repeated with 50 ml ddH_2O. The obtained cells were resuspended in 1 ml ddH_2O, washed two-times more with 1 ml ddH_2O and resuspended until a viscous solution was obtained.
The *A. tumefaciens* cells were mixed with DNA in a cold cuvette. The cells were transformed electrochemically using a Gene Pulser II (Bio-Rad, Hercules, CA, USA) with the settings 25 µF, 400Ω, 1.8kV. After a pulse (9.6 to 9.8 time constant) the cells were transferred into SOC-medium[3] and incubated at 28°C for 2h. Clones were transferred and selected on LB-Agar (**C4**) containing gentamicin[2] (25 µg ml^{-1}), rifampicin[2] (100 µg ml^{-1}) and kanamycin[14] (25 µg ml^{-1}) (*PcISPS*) or streptomycin[10] (20 µg ml^{-1}) (*PcDXR*).

2-3-3 Transformation of *Arabidopsis thaliana*

For transformation healthy *A. thaliana* plants were cultivated until they were flowering. The inflorescences were about to open at the time the transformation was performed. Arabidopsis plants were transformed using floral-dip technology (for detail see Clough and Bent, 1998). A small (approx. 5 ml) overnight culture of *Agrobacterium* carrying the gene of interest was grown (in LB with the proper antibiotics). On the next morning a bigger (150 ml) culture from the overnight culture was started and grown for approximately 6 hours until an OD_{550nm} of 0.9 ± 2 was reached. While the *Agrobacterium* were growing, the infiltration medium in which the bacteria were to be resuspended was prepared (100-200 ml per transformation of 5% sucrose[8], 0.05 – 0.1 % Silwet L-77 [13]).
The *Agrobacterium* cells were harvested by centrifugation and resuspended in infiltration medium until an OD_{550nm} = 0.9 ± 2 was reached again. The inflorescense of *A. thaliana* was dipped for 2 to 3 seconds into *Agrobacterium*-infiltration-solution. After that the *A. thaliana* inflorescence was placed on a plate with watered paper towels in order to have higher relative humidity that helps *Agrobacterium* to carry the gene of interest into the plants. Plants were kept in darkness overnight after what they were let to make seeds in the climate chamber conditions (**III: 1-1**). When the seeds had dried (approximately 4 to 5 weeks after the transformation) they were harvested in order to generate F1 generation.

2-3-4 Selection of transformed plants

Positive transformants of the F1 generation were selected on MS-plates (**III: 1-1**) containing the selective antibiotica (50 µg ml^{-1} kanamycin[14] for *PcISPS*-transformed plants and 40 µg ml^{-1} hygromycin[4] for *PcDXR*-transformed plants). The transgenic plants showed a normal green phenotype, whereas non-transgenic plants were white or pale green (Fig. 6).

Fig. 6. Selection of transformed Arabidopsis plants on selective agar. The transgenic plants showed a normal green phenotype, whereas non-transgenic plants were white or pale green.

To select *PcISPS* transformed Arabidopsis lines with the desired chemotype, 56 plants showing a dark green phenotype on selective agar were transferred to soil (**III: 1-1**). From these plants isoprene emission was measured as described in **III: 4-1**. Eleven lines with significant isoprene emission rates were chosen in order to generate the homozygotic F2 generation. According to the functional emission screening of F2 generation plants the lines 3, 5 (low isoprene emitters), 8, 9 and 10 (high isoprene emitters) were chosen for further experiments. The presence of *PcISPS* and *NPTII* (kanamycin synthase gene) mRNA (extraction and conversion to cDNA see **III: 2-1-2-2**) in these lines was verified additionally by PCR (**III: 2-1-6**). The *ISPS* PCR reaction was performed with the primers “IS.sequ.1” (for) and “IS.sequ.2” (rev) and the NPTII reaction with the primers “nptII-for” and “nptII-rev”(see Table 1). The presence of the expected fragment in size of 846 bp for *ISPS* and 402 bp for *NPTII* was proved by gel electrophoresis.

To verify that the *PcDXR* gene was introduced into the Arabidopsis genome, mRNA was extracted (**III: 2-1-2-2**) from the plants that showed dark green phenotype on selective agar. The RNA was converted to cDNA (**III: 2-1-2-2**) and a PCR with the primers “Dxr3f” and “Dxr2r” (see Table 1) was performed (**III: 2-1-6**). The expected fragment in size of 309 bp was visualized by gel electrophoresis (**III: 2-1-3**).

2-4 Quantification of gene transcript levels by Quantitative Reverse Transcription-PCR (RT-PCR)

Quantitative Reverse Transcription (RT)-PCR is a sensitive technique for mRNA detection and quantification. For performing RT-PCR, SYBR^{TM}Green[7] that provides a staining method to quantify RT-PCR products was used. This dye binds to double-stranded DNA giving a green emission when the molecule became excited by blue light. Thus, when double-stranded DNA accumulates during amplification the fluorescence signal increases.

To quantify the mRNA copy numbers of the genes *PcISPS*, *PcDXR*, phytoene synthase (*PcPSY*) and β-tubulin (*PcTUB*, EMBL AY 353093) from poplar cDNA, the total mRNA was extracted and quantified spectrophotometrically (NanoDrop® ND-1000, described in **III: 2-1-4**). In the next step total RNA was reverse transcribed into cDNA (**III: 2-1-2-2**). For amplification of the *PcISPS*-gene

the primer set “Isoprensyn.For.spez.” and “Isoprensyn.Rev.spez” was used resulting in a PCR segment length of 197 bp. For the *PcDXR* gene the primer set “Dxr4f” and “Dxr2r” resulting in a fragment length of 66 bp, and for *PcPSY* the primer set “5104-Psy-RT155F” and “5105-Psy-RT241R” resulting in a fragment length of 379bp, were used. In addition the transcription levels of the *PcTUB* gene were quantified with the primer set “tub-150-for” and “tub150-rev” resulting in a fragment length of 151bp. Primers were designed via the software PrimerExpress™ (Version 2.0.0, ABI-Prism™). Sequences are shown in Table 1. The assays were prepared without delay on 96 wells[7] reaction plates with:

12.5 µl 2 x SYBR™Green PCR Master Mix[7]
7.5 µl of each diluted primer (1 pM μl^{-1} in ddH_20)[4]
5 µl of total cDNA (diluted 5 times in ddH_20)

From each sample or standard 3 parallels were analyzed. A non template control, in which no cDNA was loaded, and a non amplification control, in which no primers were loaded, were prepared as negative controls on each plate. The transcript levels were calculated by using a standard curve obtained with DNA with a known concentration. For preparing this standard curve, plasmids, in which the gene of interest was incorporated, were purified from existing bacterial cell cultures (**III: 2-1-1-2**). The following plasmids and inserts were used: *PcTUB* (507 bp) in pCR 2.1 (3921 bp); *PcISPS* (1659 bp) in pQE50 (3963 bp); *PcDXR* (2067 bp) in pBSK (2965 bp) and *PcPSY* (589 bp) in TOPO pCR II (3973 bp). The concentration of each plasmid purification was determined spectrophotometrically (NanoDrop® ND-1000, described in **III: 2-1-4**) and the copy number ml^{-1} was calculated as follows:

$$N_{pl} = (M*C_{pl})/(N_{na}/W_{na})$$

N_{pl} = copies of the plasmid ml^{-1}
M = molar mass ($6.023*10^{23}$ molecules mol^{-1})
C_{pl} = plasmid concentration (µg ml^{-1})
N_{na} = number of bases in the plasmid (including insert)
W_{na} = mean molecular weight of the bases (308.95 g mol^{-1})

By using the obtained copy number a dilution curve of the plasmid was prepared. The curve started with the concentration $2x10^7$ copies μl^{-1} and was stepwise diluted with ddH_20 (through 2 x 10^5; 2 x 10^3; 2 x 10^2 copies μl^{-1}) until the lowest concentration of 200 copies μl^{-1}. The dilution curve with 3 parallels of each step was loaded on each plate. The measurements were performed with a real-time thermocycler (Sequence Detection System 5700, Applied Biosystems, Weiterstadt, Germany) as described in Loivamäki et al., 2007a (**VII: 1**). The gene transcription rate was

determined by comparing the sample signal to that of the corresponding gene dilution series under the GeneAmp^{R} 5700 Sequence Detection System software (Applied Biosystems).

2-5 Southern Blotting

With the Southern blotting technique a particular sequence of DNA, e.g. a gene, within a full genome can be detected. In a Southern blot DNA is transferred from an agarose gel onto a membrane. The gene of interest blotted onto the membrane can be visualized with two different techniques. More traditionally it is done with a radioactively labelled probe but in the present study a color staining was used. However, first a probe that would bind to the DNA of interest (*PcISPS*-gene) must be created. The probe for performing the Southern blot analysis was obtained by labelling PCR amplicons of *PcISPS* with digoxigenin (DIG) (DIG DNA labelling and detection Kit[6]). DIG is bound by a specific antibody (Anti-DIG-AP conjugate, DIG DNA labelling and detection Kit[6]). To the antibody an alkaline phosphatase (AP) is coupled that converts the soluble colorants 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT) into an insoluble dye.

The labelled *PcISPS* gene fragment was obtained with primer pair “Isoprensyn.Rev.spez” and “Is.sequ.3” (Table 1) resulting in a fragment length of 698 bp. The *PcISPS*-probe labelling was performed like normal PCR (**III: 2-1-6**) with following modifications: instead of 0.8 µl dNTPs, 0.4 µl dNTPs and 1 µl digoxigenin marked dNTPs mix (DIG DNA labelling and detection Kit[6]) were placed into the reaction mixture. The labelling of the probe was verified by gel electrophoresis by comparing the fragment size (slightly bigger) to that of non-labelled fragment.

2-5-1 Blotting of the DNA

Genomic DNA was extracted from Arabidopsis leaves (**III: 2-1-2-1**) and cut overnight with RNase[3] and restriction enzymes *Kpn*I[3], *BamH*I[3] and/or *Apa*I[3] that do not cut within the isoprene synthase sequence. The cut DNA was precipitated in ethanol for 10h on ice (**III: 2-1-5-3**) (final concentration 25 - 30 µg, max. 20 µl^{-1}) and loaded on an agarose (0.7%) gel (**III: 2-1-3**). The gel was run with 20 V for 16h. Next, the gel was denaturized with denaturizing buffer (**S1**) for 30 min and washed afterwards with 10% Buffer 1 (**S5**). The DNA was transferred on a nylon membrane (Hybond[17]) overnight by 20x SSC (**G6**) that was soaked through the gel onto the nylon membrane and further on filter papers (for details see Sambrook et al., 1989). After the DNA transfer, the nylon membrane was washed for 2 min with 6x SSC (**G6**) after which the DNA was cross-linked to the membrane at 80°C for 1h in a APT.LineBFED 53 Hybridization oven (Binder GmbH, Tuttlingen, Germany).

2-5-2 Pre-hybridization and hybridization

Pre-hybridization of the membrane was carried out by incubating the membrane at 56°C for 1h with hybridization buffer (**S4**) (20 ml per 100 cm^2 membrane area). For hybridization 5 µl of DIG-labelled probe (from **III: 2-5**) in 200 µl TE (**G5**) was denaturized for 10 min in 96°C after which it was immediately cooled down on ice. The probe was diluted into hybridization buffer (**S4**) and incubated at 65°C overnight in 2.5 ml hybridization buffer per 100 cm^2 membrane area. The unspecifically bound probe was removed by washing the membrane with washing buffer 2 (**S2**) and twice with washing buffer 3 (**S3**).

2-5-3 Immunological detection

The membrane was washed shortly in 100 ml buffer 1 (**S5**) and further incubated in buffer 2 (**S6**) (100 ml per 100 cm^2) at RT for 30 min. In the next step the Anti-DIG-AP[6] (0.3 µl ml^{-1}) was added in buffer 2 and the membrane was incubated for 30 min gently shaking. For removing of unspecifically bound antibodies the membrane was washed twice with buffer 1 (**S5**) for 15 min in a new plate at RT. Membrane was further incubated for 2 min in buffer 3 (**S7**) (20 ml per 100 cm^2 membrane area). For visualizing the *PcISPS* gene on the membrane, the membrane was transferred into a plastic bag with 10 ml staining-solution (**S8**) 100 cm^{-2} membrane area and incubated in the darkness until the gene band became visible.

3 BIOCHEMICAL ANALYSES

3-1 Protein extraction

For protein extraction from Arabidopsis leaves, 300 - 600 mg of fresh leaf material were suspended in 4 ml plant extraction buffer (PEB, **P1**) (Mayrhofer et al., 2005) and finely homogenized at 4°C using an Ultra-turrax (T25, Janke & Kunkel, IKA® Labortechnik, Staufen, Germany) and a 2 ml potter. For protein extraction from poplar leaves 200 mg frozen and finely homogenized (mortar and pestle) leaf material was suspended in 4 mL PEB. The extraction continued then similarly for both species: the suspensions were stirred for 15 min and centrifuged at 4°C and 15,000 x *g* for 10 min. During this step PD-10 (GE Healthcare, Uppsala, Sweden) columns were equilibrated by washing 5 times with 3.5 ml ISB. After centrifugation 2.5 ml of clear supernatant were put on a column and proteins were eluted with 3.5 ml ISB (**P2**).

3-2 Protein concentration determination

Protein concentrations were determined using the Bradford assay (Bradford, 1976). The samples were mixed with Coomassie color solution (**P3**) and incubated for 15 minutes in 1.5 ml microcuvettes[16]. The binding of Coomassie Blue to proteins takes approximately 15 minutes. For calibration BSA (Albumin Bovine Fraction V, pH 7, Standard grade[9]) was used in known concentration (= 0.25, 0.5, 0.75, 1 mg ml^{-1}) as standard. For each measurement 3 parallel samples

were prepared. The absorption (*A*) was detected at its maximum of 595 nm spectrophotometrically. The protein concentration was calculated by using the correlation between BSA and absorption rate at *A* 595 nm (Fig. 7).

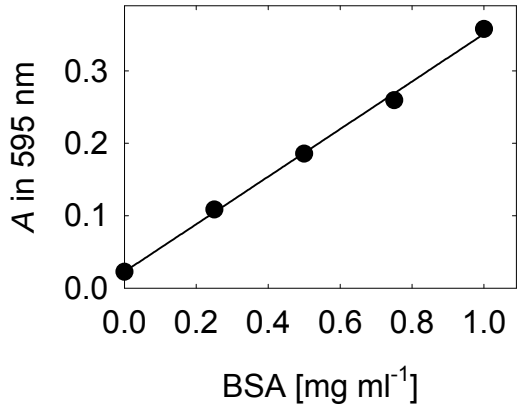

Fig 7. Bradford assay: example of a correlation between a BSA dilution series and *A* 595 nm, n = 3, mean ± SE is shown. R^2 = 0.999; y = 0.0227 + 0.3283X.

3-3 Enzyme activity measurement

Isoprene synthase activity was assayed as previously described by Lehning et al. (1999). 88 µl of the protein extract was mixed with 2 µl MgCl and 10 µl 100 mM DMADP (synthesized according to the protocol by Keller and Thompson, 1993) in gas tight 2 ml reaction vials (Supelco, Bellefonte, PA, USA). The samples were incubated in a water bath for 60 min at 30°C. For subsequent background correction of the chemical degradation of DMADP vials containing 88 µl of buffer PEB, 2 µl MgCl and 10 µl 100 mM DMADP were prepared in parallel. After incubation the aquatic solutions in the vials were removed with a gas tight syringe and the vials were washed with 150 µl ddH2O.

3-3-1 Calibration and calculation

The amount of synthesized isoprene in the head space of the vials was determined by PTR-MS as described in the chapter **III: 4-1-2**. The mean ion signal (m69) obtained from buffer controls was subtracted from that of the enzymatic samples. The apparent activity of ISPS was calculated as follows (more specific calculation is shown in attachment 1):

$$IS_{Act} = C_{isv} / (P * t_{test} * V_{test})$$

IS_{Act} = ISPS activity [nmol s^{-1} kg $protein^{-1}$]

C_{isv} = background corrected amout of isoprene in the head space of samples [nmol]

P = protein concentration in the protein extract [kg]

t_{test} = duration of the test [s]

V_{test} = test volume of enzyme assay [ml]

3-4 Western blotting

Native PAGE (10% acrylamide) was performed on pre-cast gels (Novex; Invitrogen). From each sample 20 µg protein was mixed with 5 x native sample buffer (**W5**) up to maximum 20 µl and loaded onto the gels. Native PAGE was run under constant current of 9 mA for approximately 3.5 to 4h. Protein transfer was achieved using a Millipore (Eschborn, Germany) semidry electroblot system. A sandwich of 3 x Whatman (3MM Chr, Whatman, Maldstone, England) papers, the nitrocellulose membrane (Hybond-ECL[17]), the gel and again 3 x Whatman paper was prepared. Before preparing the sandwich the Whatman paper and the membrane (for 10 min) as well as the gel (for 1 min) were equilibrated in Towbin buffer (**WE1**). The blotting was performed following the manufacturer's instructions for 30 minutes and with a current of 3 mA cm^{-2}.

The membranes were transferred into a Stuart falcon tube (Binder GmbH, Tuttlingen, Germany) and incubated in blocking buffer (**W2**) for 1h at 24°C. All incubations were carried out in a APT.LineBFED 53 Hybridization oven (Binder GmbH, Tuttlingen, Germany). Membranes were washed twice with PBS-Tween (**W3**) after which the primary polyclonal antibody (Anti-ISPS; for details and specificity of the antibody see Schnitzler et al., 2005) was applied 1:1000 in 10 ml PBS-Tween. The membranes were incubated overnight at 24°C in the hybridization oven. On the next morning the membranes were washed twice with PBS-Tween (**W3**) after which an alkaline phosphatase-conjugated secondary anti-rabbit antibody[2] was applied 1:15000 in 10 ml PBS-Tween (**W3**) on the membrane. The membrane was incubated with the antibody solution for 1h at 24°C, followed by 4 washing steps with PBS-Tween (**W3**). The PcISPS protein was detected by addition of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium i.e. BCIP/NBT-solution (**W4**). For determination of molecular weight of the PcISPS protein, a pre-stained native protein standard[9] ladder was used.

3-5 Quantification of ISPS protein by ELISA

Quantification of ISPS protein was performed as described in Schnitzler et al. (2005) with a polyclonal antibody (anti-PcISPS-IgG) generated in rabbit against purified N-terminal 6 x His-

tagged PcISPS. For use as second antibody in the ELISA anti-PcISPS-IgG was conjugated with horseradish peroxidase (HRP) (BioGenes, Berlin, Germany). For calibration purified 6 x His-tagged ISPS protein was used. For each sample and standard three parallels were measured.

3-6 Measurement of metabolic intermediates

3-6-1 DMADP

DMADP levels of Arabidopsis leaves were determined as described by Brüggemann and Schnitzler (2002b) with some modifications. The fresh weight of single leaves (approximately 50 mg) was determined immediately after harvest. Thereafter the leaves were finely homogenized under liquid N_2 and the leaf material was freeze dried (Freezedrier Alpha I-5, Christ, Osterode, Germany) in 2 ml reaction vials (Supelco, Bellefonte, PA, USA). For hydrolysis of DMADP to isoprene 100 µl of 88% H_3PO_4[4] was added on the leaf material, the glass vials were closed gas tight, mixed carefully with a vortex and incubated in a water bath for 90 min at 60°C. Within a measurement series also a standard series of known DMADP concentrations (1.25; 2.5; 3.75; 5.0 nmol DMADP $vial^{-1}$) were hydrolyzed. After incubation the samples were cooled down in an ice bath for 1 minute. The reaction mixture was neutralized and thus the hydrolysis stopped with 50 µL 4M NaOH. Isoprene in the head space was determined by PTR-MS as described in **III: 4-1-2**.

The DMADP level from Arabidopsis leaves fed with 1-deoxy-D-xylulose (DOX) was also determined in the similar way as described above. DOX is known to enter the chloroplast and further being converted into DOXP (Wolfertz et al., 2003). For feeding detached Arabidopsis leaves with DOX, DOX (final concentrations 3 and 30 nM) was added to 150 µl mineral water in which the leaves were standing in the reaction vials. In a first step the leaves were incubated for isoprene emission measurements as described in **III: 4-1-2**. After determination of isoprene emission the vials were opened and again incubated for additional 120 min on the light bench. After that 2nd step the leaves were removed and frozen rapidly in liquid N_2 for DMADP measurements.

3-6-1-1 Calibration and calculations

The PTR-MS measurements were calibrated and the isoprene concentrations (nmol $vial^{-1}$) calculated as described in **III: 4-1-2**. Initial DMADP concentration in the leaf material was calculated by correlating the released isoprene concentration from DMADP hydrolysis to that of the calibration series (Fig. 8).

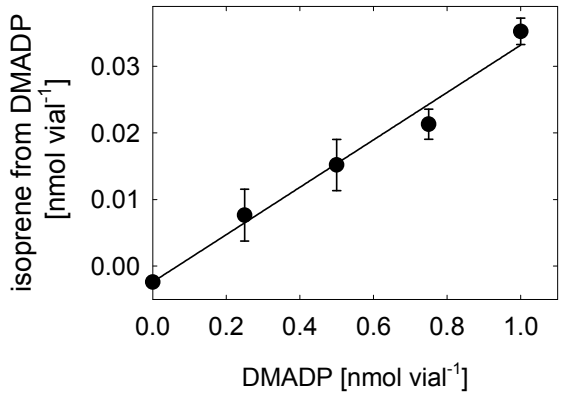

Fig. 8. Correlation between different DMADP concentrations and released isoprene from hydrolyzed DMADP, n = 3 ± SE, R^2=0.9927; y=-0.0024+0.0356x.

To correct the obtained DMADP concentrations for quenching effects of the hydrolysis in leaf material, a correction factor ("z" in the calculation below) was calculated according to a correlation between a normal calibration curve and another calibration curve in which leaf material was added in addition to the known DMADP quantities. The final DMADP concentration was calculated as follows:

$$C_{dmadp} = x * z/FW * 1000$$

C_{dmadp} = DMADP concentration [pmol FW^{-1}]
x = DMADP $vial^{-1}$ calculated from a correlation as in Fig. 8 [nmol]
z = correction value (approximately 0.87)
FW = leaf fresh weight [mg]

3-6-2 Quantification of carotenoids and chlorophylls

The determination of photosynthetic pigments (carotenoids, xanthophylls and chlorophylls) was either performed (for data in Loivamäki et al., 2007b (**VII: 2**)) using the spectrophotometrically assay described by Lichtenthaler and Wellburn (1983) or (for the screening of transgenic PcDXR over-expressed Arabidopsis) analyzed according to the method of Kirchgessner et al. (2003) using a Beckman HPLC System Gold (Beckman, München, Germany). Detection was at 440 nm with a UV/Visible diode-array detector (Beckman Model 168). System calibration was performed using purified chlorophylls (Chl*a* and Chl*b*), violaxanthin, and β-carotene isolated by flash

chromatography. Pigments were identified by their retention times, spectral properties and increased concentrations observed under dark/light transitions.

3-7 Determination of antioxidants in poplar and Arabidopsis leaves

3-7-1 Ascorbate analysis

For the extraction of ascorbate, DHA and reduced ascorbate from poplar and Arabidopsis leaves, the protocol described by Haberer et al. (2007) was applied. Frozen plant material was powdered with mortar and pestle under liquid N_2. Twenty mg of plant material was transferred to a centrifuge tube containing 0.5 ml metaphosphoric acid (5 %). The samples were well mixed and centrifuged at 12 000 x *g* and 4°C for 30 min. The supernatant (100 µl) was collected and neutralized with 20 µl 1.5 M triethanolamine and well mixed with 100 µl 150 mM sodium phosphate buffer (pH 7.4). The samples were transferred to reaction vials.

Reduced ascorbate was measured directly, but the total ascorbate content had to be determined indirectly by first reducing the oxidized ascorbate for 30 min by dithiothreitol (DTT, 50 µl, 10 mM). The samples were then treated as follows: into 100 µl leaf extraction was successively added 200 µl 10% trichloroacetic acid, 200 µl 44% orthophosphoric acid, 200 µl 44% 2.2'dipyridyl and 100 µl 3% $FeCl_3$. The samples were incubated at 37°C for 60 min. The ferric ion is reduced in acidic solution by reduced ascorbate to the ferrous ion, which is coupled with 2.2'dipyridyl. The formed complex can be detected at OD 525 nm (Haberer et al., 2007). The DHA content was determined by subtracting the determined reduced ascorbate content from that of the total ascorbate.

3-7-2 Glutathione analysis

Glutathione (GSH) was analyzed using the slightly modified method described by Schupp and Rennenberg (1988). Frozen plant material was powdered with mortar and pestle under liquid N_2. Thirty mg of leaf material was transferred to a tube containing 50 mg PVPP in 0.75 ml 0.1 N HCl, stirred and centrifuged (12,000 x *g*, 4°C, 30 min). The resulting supernatant (120 µl) was adjusted to pH 9.3 by adding 180 µl 0.2 M CHES (2-(cyclohexylamino)-ethanesulfonicacid[2]). Total glutathione content was determined after reduction of GSH with 30 µl 15 mM DTT[2] and incubating at RT for 1h. Oxidized glutathione (GSSG) was determined by blocking GSH with N-ethylmaleimide and reducing (with DTT like for total glutathione) and analyzing the remaining GSSG.

Derivatization was performed by adding 20 µl monobromobimane[18] (MBB in acetonitrile, Haberer et al., 2007) and incubating the samples for 15 minutes at RT in darkness. After centrifugation (12 000 x *g*, 4°C, 10 min) samples were analyzed by HPLC. The samples were injected to a hydrophobic column (ODS-ultrasphere, 5 µm particle size, 250 * 4.6 mm, Beckman, München, Germany) and the fluorescence of MBB-thiol derivatives at 480 nm was measured by an excitation at 380 nm. To estimate the loss of the thiols during the process, an internal standard was used.

3-7-3 Determination of malondialdehyde (MDA) content

Lipid peroxidation in the plant leaves was quantified by measuring the malondialdehyde (MDA) content, the end product of lipid peroxidation, with the thiobarbituric acid (TBA) assay (Du and Bramlage, 1992). The assay started by homogenization of 50 mg leaf material in 1500 µl TCA (**O1**) and 50 µl BHT (**O2**) solution. The homogenate was incubated in a water bath (95°C; 6A, U3, Julabo, Seelbach, Germany) for 30 minutes. The reaction was stopped by placing the reaction tubes at 4°C after which the tubes were centrifuged at 4°C, 5000 x *g* for 5 min. Part of the obtained supernatant (0.5 ml) was mixed with 0.5 ml TBA (**O3**) and other part (0.5 ml) with 0.5 ml TCA representing the zero-control. Both mixtures were incubated at 95°C for 30 minutes after which the reaction was stopped at 4°C. The absorbance of the supernatant was measured at 532, 600 and 440 nm (Perkin Elmer, Waltham, MSC, USA). The control values (supernatant with TCA) were subtracted from the values measured for the real samples (supernatant with TBA). The final MDA content was then calculated as follows:

$$MDA = \{[(A_{532} - A_{600})] - [(A_{440} - A_{600}) \times 0.057]\} / 157\,000 \times 10^6$$

MDA = malondialdehyde [nmol ml^{-1}]
A = absorbance [nm]

4 GAS EXCHANGE MEASUREMENTS

4-1 Head space analysis from individual Arabidopsis leaves by PTR-MS

4-1-1 Experimental set up

For headspace analysis, weighted leaves, flowers or roots (washed under tap-water) from Arabidopsis were placed in 2 ml vials filled with 100 µl sparkling mineral water and allowed to stabilize for 30 min on a light bench (PPFD approx. 300 µmol m^{-2} s^{-1} and temperature 35°C). After stabilization, another 150 µl of mineral water was added into the vials after which the vials were sealed gas-tight and further incubated for 180 min. Before analysis of headspace with PTR-MS, vials were kept in darkness to interrupt the light dependent isoprene formation.

Isoprene emission levels from leaves fed with 1-deoxy-D-xylulose (DOX) were determined similarly, with the exception of the mineral water amount which was decreased from the normal procedure down to 50 µl (the stabilization) and 100 µl (the incubation).

Fig. 9. Image of the incubation of Arabidopsis leaves in 2 ml reaction vials filled with 250 µl mineral water on a light bench.

4-1-2 PTR-MS set-up

The functional screening of transgenic Arabidopsis on isoprene emission was performed with a newly developed head space analysis system using on-line proton transfer reaction mass spectrometry (PTR-MS, Ionicon, Innsbruck, Austria), a combination of a proton transfer reaction drift tube and a quadrupole mass spectrometer. PTR-MS technology is based on proton transfer reaction occurring between H_3O^+-ions and any molecules whose proton affinity exceeds that of water. H_3O^+ ions are produced by a hollow cathode discharge using water vapour as a source. The produced ionized molecules are detected by ion detector i.e. the quadrupole mass spectrophotometer, which allows a fast detection of most VOCs (Hansel et al., 1995). The detection sensitivity is high being in the range of 10-100 pptv (Tholl et al., 2006). For transfer of gas samples from the 2 ml vials into the PTR-MS, the head space of the vials was first transferred with a syringe into a 10 ml injection loop by flushing the vials with 10 ml of N_2, and the samples were subsequently injected directly into the on-line MS with a flow rate of 250 ml min^{-1} (Fig. 10).

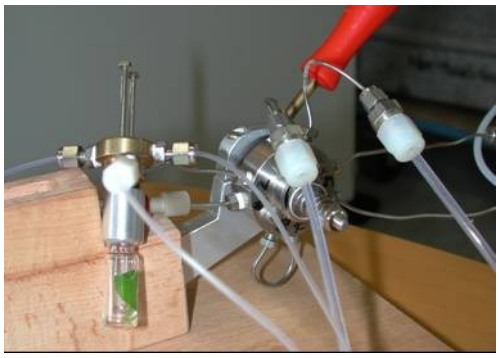

Fig. 10. The head space collected into the vials was transferred into a 10 ml injection loop by flushing the vials with 10 ml of N_2. The samples were subsequently injected into the MS.

4-1-3 Calibration of PTR-MS and calculations

The calibration of the PTR-MS was performed in the beginning of each measurement series. It was done with 2 ml gas-tight vials filled with calibration gas (10.9 ppmv or 5.8 ppmv isoprene in N_2, (Air Liquide, Griessheim, Germany or SIAD S.p.A, Bergamo, Italy, respectively)). The isoprene

concentration in at least 5 vials was measured for one calibration. The isoprene concentration in the vials was calculated as follows:

$$C_{isv} = (iS_s - iS_b) * C_{icv} / iS_{sd} / LA * 1000$$

C_{icv} = $C_{is}/V_{mol} * V$
C_{isv} = concentration of isoprene in sample vials [nmol]
iS_s = ion signal (m69) from sample
iS_b = ion signal (m69) from background
iS_{sd} = ion signal (m69) from calibration gas
LA = leaf area or leaf weight [m^{-2} or g^{-1}]
C_{icv} = concentration of isoprene in a calibration vial [mmol]
C_{is} = concentration of isoprene in the gas bottle [ppmv]
V_{mol} = mol volume in ideal gas under standard conditions
V = volume of the vial (l)

4-2 On-line measurement of isoprene emission from poplar shoot cultures by PTR-MS

4-2-1 Experimental set-up

The measurements of isoprene emission from shoot culture containers was performed with the PTR-MS as described in Loivamäki et al., 2007a (**VIII: 1**). The measurements were performed on two gas-tight culture containers in parallel, each containing 6-7 cell cultured shoots aged 6 - 8 weeks partially with a developed root system. Clean air adjusted to a dew point of 28°C was flushed at 500 ml min^{-1} into the containers and from the outlet air 100 ml min^{-1} was pumped into the PTR-MS to analyze the volatiles. The measurements were performed on each container alternatively (automatically switched each 3 min with 60 s stabilization time). Temperature was measured continuously with thermocouples inside the containers.

Isoprene emission was measured over a day/night cycle as described in Loivamäki et al., 2007a (**VII: 1**). At 22:00 of the following day one container was placed in continuous light and the other in continuous darkness (covered with aluminium foil) and emissions were measured during three virtual day/night cycles.

4-2-2 Calibration and calculations

PTR-MS was calibrated as described in Loivamäki et al., 2007a (**VII: 1**). A flow of 20 ml min^{-1} from the gas standard (including 1.05 ppmv of isoprene, Apel-Riemer, Denver, CT, USA) was diluted into the gas stream of 500 ml min^{-1} for approximately 20-30 minutes. Calibration was performed at the beginning and at the end of the experiments. Because the sensitivity of the PTR-MS went slightly down during the measuring period, the standard curve for isoprene through the experiment

was normalized to the also declining signal of the primary ion (mass 21, deuterium isotope of H_3O^+). At the end of the experiments isoprene emission from agar and roots (green material removed) was measured. Before further calculations 15 min averages (data were smoothed, not shortened) were calculated for the background ion signal of m69. So obtained smoothed background was subtracted from the data. Afterwards 15 min averages were calculated for all of the data in order to smooth the short-term scattering of the samples. The emission data were then aggregated by calculating 15 min means. By using the so obtained data and the calibration curve, the isoprene concentration was calculated as described in **III: 4-1-2** with the difference that instead of V (volume of the vial) the flow rate of the gas standard (520 ml min^{-1}) was used.

4-3 On-line gas-exchange measurement from Arabidopsis rosettes and from poplar leaves

4-3-1 Cuvette construction

New cuvettes suitable for Arabidopsis rosettes (see Loivamäki et al., 2007b (**VII: 2**)) and poplar leaves (Fig. 11) were developed to allow dynamic on-line monitoring of photosynthetic gas exchange and emission of VOC, such as isoprene. The system consists of four cuvettes (cuvette volume 530 ml for Arabidopsis and 490 ml for poplar) constructed of teflonized aluminium bodies covered with plastic glass lids. Leaf temperature can be regulated with Peltier-elements and dynamically adjusted very quickly; +5°C in ~ 130 seconds and -5°C in ~ 100 seconds to a chosen leaf temperature measured by a thermocouple within the rosette. Light is provided by five LED-lamps (DP3-W3-854, Osram, Germany) allowing light intensity to be increased up to a PPFD of 1300 µmol photons m^{-2} s^{-1}. Lamps and Peltier-elements were cooled with cooling elements and ventilators (type 8414 NGH, Epm-Papst, St. Georgen, Germany).

Fig. 11. Picture of a poplar cuvette. The leaf is placed in the cuvette so that the leaf petiole comes out from the cuvette through a small hole which is gas tightened with plastic sealing tape (Terostat II, Henkel, Düsseldorf, Germany). The cuvette functions like described in Loivamäki et al., 2007b (**VII: 2**) for the Arabidopsis cuvette.

4-3-2 Experimental set up

Synthetic air was mixed with 380 ppm CO_2 (Fig. 12, see 1) and pushed through approx. 200 ml ddH_2O to obtain higher air humidity. The extra humidity of the air was then removed by a dew point generator (Type KR-KW 12/2, Walz, Effeltrich, Germany) (Fig. 12, see 2) so that the end H_2O concentration n the air was ~ 10.5 mmol mol^{-1}.The air was pushed with a flow of two liters min^{-1} (2 l mass flow controllers (FC), Bronkhorst, Offenbach, Germany) to all the cuvettes in which air was circulated with small ventilators and pulled out to three-way valves (type NO-C-NC, Teocom, Newport, California) (Fig. 12, see 3). The three-way valves allowed the air stream to leave either as waste or for gas analysis, in our case for Fast Isoprene Sensor (FIS, Hills Scientific, Boulder, Colorado, USA) or LI-7000 CO_2/H_2O analyzer (LI-COR, Lincoln, Nebraska, USA) (Fig. 12, see 5 and 6).

The function of the FIS is based on chemiluminescence, a chemical production of light. When isoprene reacts with ozone the reaction product is in an excited electronic state and emits lights. In the FIS the sample air is pulled into a reaction sample where it reacts with ozone producing violet light that has an emission maximum at 430 nm. This light is detected by a photon detector that produces an output in photon counts.

The LI-7000 is a high performance, dual cell, differential CO_2/H_2O gas analyzer. It uses a dichroic beam splitter and two separate detectors to measure infrared absorption by CO_2 and H_2O. For analysis, a known concentration of gas is led into the reference cell (reference air, Fig. 12), and an unknown gas is led into the sample cell. The instrument software provides continuous output of the absolute concentration in the sample cell.

For gas exchange measurement the air stream from the cuvettes was divided between FIS drawing 650 ml min^{-1} and LI-7000 drawing 600 ml min^{-1}.

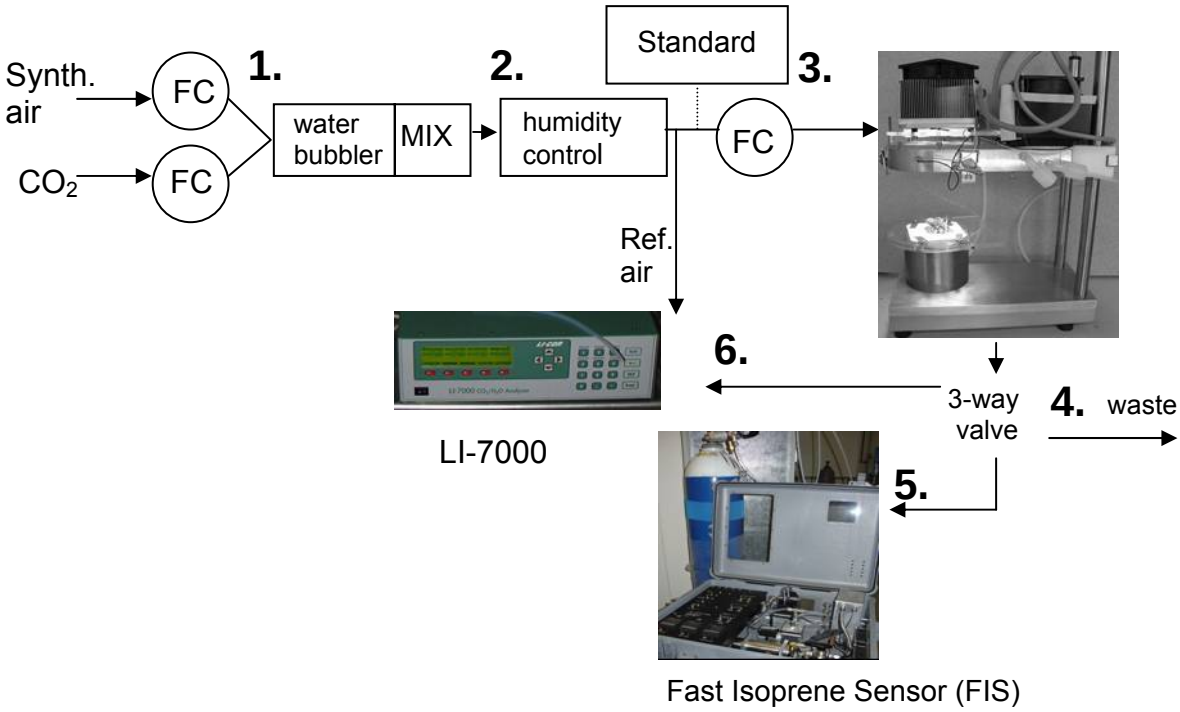

Fig. 12. Scheme of the experimental set up to measure gas-exchange from Arabidopsis rosettes. The scheme was similar when gas-exchange from poplar leaves was measured except that a cuvette bottom suitable for a poplar leaf was used. The numbers 1-6 in the scheme are explained in a detail in the text, FC: flow controller.

4-3-3 Experimental design

Changes in leaf temperature and switching between the valves (and cuvettes) were automated with a computer terminal.

For the temperature dependency studies 4 cuvettes were run in parallel. In each cuvette leaf temperature was increased in 5°C steps every 30 min from 30°C up to 45°C leaf temperature and returned similarly back to 30°C. The measurement (225 s from a cuvette at a time) was repeated two times from each of the four cuvettes at each temperature. Leaf temperature was adjusted with sliding starts before beginning measurements from the respective cuvette.

For the temperature and light stress studies only one cuvette was used. When the temperature cycles were to be applied the measurements started with 20 minutes of stabilization at 30°C and PPFD of 1000 µmol m^{-2} s^{-1}. After the stabilization six heat (temperature switched every 10 min from 30°C to 40°C and back, light stable at 1000 µmol m^{-2} s^{-1}) cycles were performed. After the cycles the initial conditions were returned and the recovery of the plants was measured for 20 min.

When the light cycles were to be applied the measurements started with 20 minutes of stabilization at 30°C and PPFD of 100 µmol m^{-2} s^{-1}. After the stabilization six light cycles (light switched every 10 min from 100 to 1300 µmol m^{-2} s^{-1} and back, temperature stable at 30°C) were performed. The recovery of the plants was measured at initial conditions for 20 min.

The combined temperature and light cycles studies were performed like described above, but applying the light and temperature cycles simultaneously. After 20 minutes of stabilization time at 30°C and 100 µmol m^{-2} s^{-1}, six simultaneous heat and light cycles (light switched every 10 min from 100 to 1300 µmol m^{-2} s^{-1} and back and temperature simultaneously from 30 to 45°C and back) were performed. After the cycles, plants were allowed to recover at the initial conditions for 20 min.

4-3-4 Calibration and calculations

4-3-4-1 Isoprene emission rate

The Fast Isoprene Sensor was calibrated always at the end of a measurement series with a calibration gas of 5.8 ppmv isoprene in N_2 (SIAD S.p.A, Bergamo, Italy) directly added into the gas stream (1 l min^{-1}) of one cuvette. The flow (2.5 to 20 ml) of the calibration gas in the cuvette gas stream was controlled by a 20 ml flow controller that is provided by FIS. Isoprene concentration in the standard was calculated as follows:

$$C = (Fs/Ff*Ci)*1000$$

C = isoprene concentration [ppb]
Fs = flow from the standard bottle [ml min^{-1}]
Ff = flow into FIS [ml min^{-1}]
Ci = isoprene concentration of the standard [ppm]

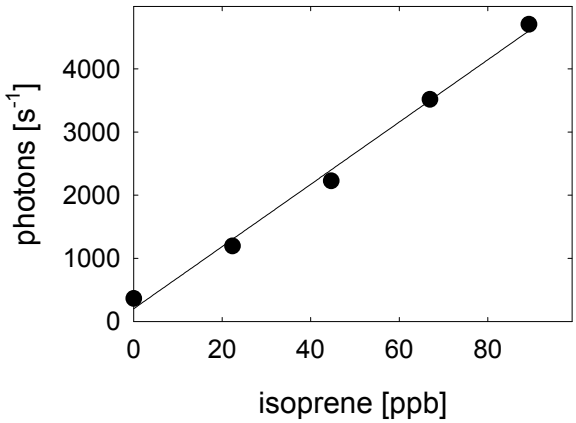

Fig 13. Example of a FIS-calibration. The single values are mean of photons during approximately 5 min calibration time in each ppb value, y = 201.86+49.3x; R^2 = 0.998.

Using the obtained calibration curve (Fig. 13), the isoprene emission rate was calculated as follows:

$$E = (x/LA)/V_{mol}*F_c$$

E = isoprene emission [nmol m^{-2} s^{-1}]

x = (S – bl)/a [photons s^{-1}]

a = photons [s^{-1}] /isoprene [ppb]

S = photons measured from sample [photons s^{-1}]

bl = photons measured from empty cuvette [photons s^{-1}]

LA = leaf area [m^{-2}]

V_{mol} = mol volume in ideal gas under standard conditions

F_c = flow in cuvette [ml min^{-1}]

4-3-4-2 Assimilation, transpiration and stomatal conductance

The LI-7000 was calibrated daily with a CO_2 gas standard (373 ppmv, Air Liquide, Griessheim, Germany) or with synthetic air (0 ppmv CO_2). Assimilation (As), transpiration (Tr) and stomatal conductance (gH2O) were calculated based on Von Caemmerer and Farquhar (1981) and Ball (1987) as follows:

$$As = (C_{out}-C_{in})/ V_{mol} *F_c/LA$$

$$Tr = [(W_{out}-W_{in})*(-1)]/ V_{mol} *F_c/LA$$

$$gH_2O = Ass*(1-W_m)/(W_l-W_{in})$$

As = assimilation [µmol CO_2 m^{-2} s^{-1}]

C_{out} = CO_2 in the outlet of a cuvette

C_{in} = CO_2 in the intlet of a cuvette

LA, V_{mol}, F_c; see **III: 4-3-4-1**

Tr = transpiration [mmol H_2O m^{-2} s^{-1}]

W_{out} = H_2O in the outlet of a cuvette

W_{in} = H_2O in the inlet of a cuvette

gH_2O = stomatal conductance [mmol m^{-2} s^{-1}]

W_m = mean H_2O; $W_m = (W_l+W_{in})/2$ [ppm]

W_l = H_2O in the leaf [ppm] *

* W_l = [dk/825*10^6]*0.000001 [ppm]

dk = 10^nk

nk = [-7.9298*(373.16/T-1)]+[5.02808*LOG10(373.16/T)]+ [1.3816*10^(-7)]+[-1+10^(11.344*(1-T/373.16))]+[8.1328*10^(-3)*(-1+10^(3.49149*(373.16/T-1)))]

T = cuvette temperature [K]

4-4 Head space analysis of Arabidopsis VOC emission rates by GC-MS

4-4-1 Experimental set up

For dynamic headspace collection 4 Arabidopsis plants of the same line and treatment were placed in 2.5 l glass jars as described in Loivamäki et al., 2008 (**VII: 3**). The plants used for this analysis were either infested with 20 first instar *P. rapae* caterpillars or left untreated as controls. The collection of plant volatiles on 200 mg Tenax TA (Grace-Alltech, Deerfield, USA) and 200 mg Carbopack X (Grace-Alltech) traps was performed as described in Loivamäki et al., 2008 (**VII: 3**).

4-4-2 GC-MS set up

Headspace samples were analyzed with a Thermo TraceGC Ultra (Thermo Fisher Scientific, Waltham, USA) as described in Loivamäki et al., 2008 (**VII: 3**). Calibration of the trapped volatiles was performed using the permeation source containing pure chemicals in individual vials in combination with a dynamic dilution system at the Research Center Jülich (ICG III), Germany as described in Schuh et al. (1997). Concentrations of the compounds (isoprene, limonene, β-caryophyllene, α-pinene, methyl salicylate (MeSA)) released from the calibration source were determined from the mass loss rates of the individual compounds and the dilution fluxes. A calibration curve is shown in Figure 14. The VOC mixing ratios were in the lower ppb to ppt range.

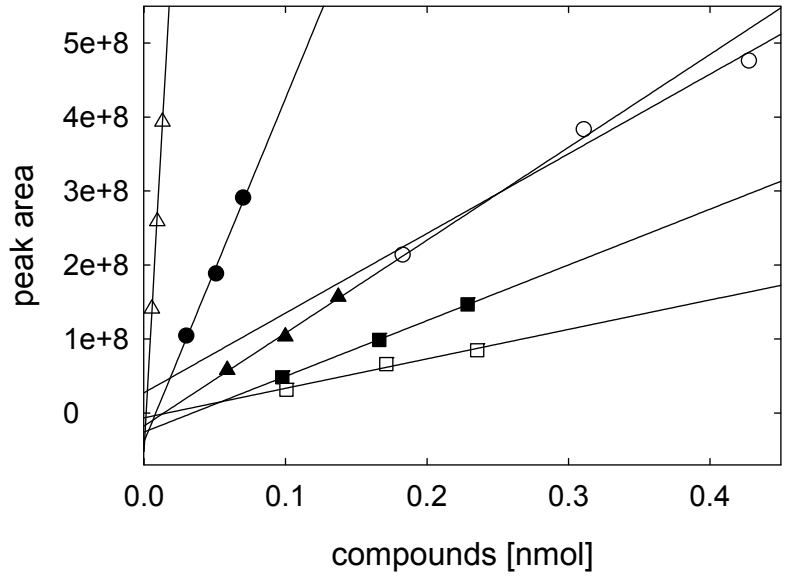

Fig. 14. GC-MS-calibration: correlation between the measured compounds (isoprene (●), limonene (Δ), β-caryophyllene (□), α-pinene (○), MeSA (■)) released from the calibration source and the detected peak area. Isoprene: $y = -3.884e^{7}+4.639e^{10}$, $R^2 = 0.992$; limonene: $y = -5{,}18e^{7}+3.3e^{10}$, $R^2 = 0.999$; β-caryophyllene: $y = -6.61e^{6}+3.98e^{10}$, $R^2 = 0.978$; α-pinene: $y = 2.727e^{7}+1.077e^{9}$, $R^2 = 0.98$; MeSA: $y = -2.585e^{7}+7.532e^{8}$, $R^2 = 0.99$.

5 ANALYSIS OF CHLOROPHYLL FLUORESCENCE

In situ chlorophyll fluorescence measurements were carried out in light-adapted leaves using the mini portable pulse amplitude modulation fluorometer (MINI-PAM, Walz, Effeltrich, Germany) to determine quantum yield (Φ_{PSII}) of photosystem II electron transport rate (ETR). At each time point a light curve with 8 light pulses (from 20 up to 367 µmol photons m^{-2} s^{-1}, duration of each pulse 10 msec) was measured and the fluorescence of the last pulse was used to calculate Φ_{PSII} and ETR according to the following formula:

(1) $$\Phi_{PSII} = (F_m - F_0)/ F_m$$

F_m = maximal fluorescence in light

F_0 = fluorescence before the start of the light curve

Since Φ_{PSII} is the yield of photochemistry, ETR and therefore the overall photosynthetic capacity could be estimated from the obtained Φ_{PSII} determination with a simple equation:

(2) $ETR = \Phi_{PSII} * PPFDa * 0.5$

PPFDa = absorbed light [µmol photons m^{-2} s^{-1}]

6 PLANT GROWTH RATE DETERMINATIONS

For measuring relative growth rate (RGR) of Arabidopsis two different methods were used, either plant weight (experiments done in climate chambers of the Research Centre Karlsruhe) or leaf area (experiments done in climate chambers of the Research Centre Jülich) were measured. In both cases Arabidopsis seeds of F3/F4, homozygously expressing *PcIPS*, were allowed to germinate as described in **III: 1-1**, but without the selection step in soil.

In the experiments in which plant mass was measured to determine the RGR, fresh weights of 12 individual plants per line were measured every second day during 12 days like described in Loivamäki et al., 2007b (**VII: 2**).

For leaf area measurements a novel technique, the so called GROWSCREEN setup (Walter et al., 2007) was used to measure projected total leaf areas and to determine their daily growth rates. The method is described in Loivamäki et al., 2007b (**VII: 2**).

7 PLANT-INSECT INTERACTION STUDIES

7-1 Behavioral studies with Y-tube olfactometer

The behavioral response of female parasitic wasps to plant volatiles was investigated with a Y-tube olfactometer like described in Loivamäki et al., 2008 (**VII: 3**). In the olfactometer (Fig. 15) (diameter: 3.5 cm, length of stem section: 22 cm) the side arms (length each: 32 cm) were both connected to 5 l Duran® glass jars (Duran, Mainz, Germany) containing the odor sources. Pressurized air was filtered over activated charcoal, entered the jars (4 l min^{-1}) at the top and left them at the bottom towards the olfactometer. The studies were performed as described in Loivamäki et al., 2008 (**VII: 3**).

In the experiments in which isoprene was added to the flow of the Y-tube olfactometer, a flow of 5 ml min^{-1} or 20 ml min^{-1} (resulting in 12.5 ppbv or 50 ppbv isoprene) was led into the airflow (4 l min^{-1}) of one of the olfactometer side arms. The isoprene flow from a gas standard of 10 ppmv isoprene in N_2 (Air Liquide, Griessheim, Germany) was controlled with mass flow controller (model 5830TR, Brooks Instr., Veenendaal, the Netherlands). In addition, another experiment was performed in which a flow of 5 ml min^{-1} was led into one olfactometer arm as described above, from a gas bottle containing 10 ppbv isoprene (SIAD S.p.A, Bergamo, Italy). This latter isoprene flow resulted in isoprene concentration of 0.0125 ppbv in one Y-tube arm. In all cases isoprene was introduced into the arms approximately 5 cm downstream from the glass jar, in which the

plants were located. This was done to prevent possible unknown effects of isoprene on other plant emitted compounds.

Fig. 15. Y-tube olfactometer set up. Photo: Hans Smid/ www.bugsinthepicture.com.

7-2 Behavioral studies of herbivore performance

The oviposing, developing and feeding performance of herbivores *P. rapae* and *Pl. xylostella* was investigated. To study herbivore performance on wild type and transgenic Arabidopsis plants in the ecotype Col-0 background, plants were individually placed in Magenta GA-7 vessels (SIGMA-Aldrich) with an insect-proof mesh lid. Plants were watered before the experiment and carefully removed from the pots. The weight of individual first instar larvae of *P. rapae* and *Pl. xylostella* was measured on a microbalance (Sartorius, Göttingen, Germany) and the caterpillars were then individually transferred to either a wild type or a transgenic Arabidopsis plant. Larvae were allowed to feed for 4 - 5 days (*Pl. xylostella*) or 7 days (*P. rapae*), and were weighed again. The period was shorter for *Pl. xylostella* because some larvae started to pupate after 4 days. The weight gain of 61 *P. rapae* and 55 *Pl. xylostella* larvae was recorded. Experiments were carried out in a climate-controlled chamber at 21 ± 1°C, a L16:D8h photoperiod, a PPFD of 80 - 110 µmol photons m^{-2} s^{-1}, and a RH of 55 ± 5%.

To investigate the feeding preference of caterpillars, a cafeteria experiment (Jermy et al., 1968; Fig. 16) was done. In the cafeteria experiment 100 first instar caterpillars of *P. rapae* and *Pl. xylostella* were individually given a free choice to feed on transgenic or wild type Arabidopsis leaves. The petiole of each leaf was placed in a 0.5 ml Eppendorf vial[16] filled with tap water. Two transgenic and two wild type leaves were placed on filter paper in a Petri dish (90 mm diameter) approximately 2 cm away from each other in a rectangular distribution. An individual caterpillar was then placed in the middle. The filter paper was moisturized throughout the experiment with tap

water. The choice of the caterpillars was recorded immediately after the start of the experiment, and also 0.5 h, 1h and 2h later. The feeding choice of 20 caterpillars was investigated simultaneously and the experiment was replicated on five different days with both species.

Fig. 16. Picture of a cafeteria-experiment set up. The caterpillar was placed in the middle and let to freely choose its food.

In two-choice experiments female *P. rapae* butterflies were given the opportunity to lay eggs on either transgenic or wild type Arabidopsis plants in a greenhouse compartment (25 - 27°C, RH 40 ± 5 %, and a L16:D8h photoperiod). At 48 h prior to the experiment freshly emerged male and female *P. rapae* butterflies were given the possibility to mate for 24 h, after which a single untreated Brussels sprouts leaf was placed in the cage as an oviposition substrate. After 6 h the leaf was removed. One male and one female were then transferred into individual experimental cages (67 x 50 x 75 cm), 16 ± 2 h before the start of the experiment. The butterflies were provided with a 10% sucrose solution during the whole experiment. In addition to natural daylight the cages were illuminated by sodium vapor lamps (SON-T, 500W, Philips, Eindhoven, the Netherlands) from 10:00 - 16:00. One wild type and one transgenic plant were placed into the cage, approximately 15 cm from each other. The orientation of the plants was changed in every second cage so as to compensate for unforeseen directional bias in the experiment. The number of eggs deposited during 4 h (10:00 – 14:00) on either of the offered plant lines (wild type or transgenic) was counted. The oviposition behavior of 10 to 12 butterflies was investigated simultaneously. The experiment was replicated 8 times on different days with new plants and new butterflies.

7-3 Electrophysiology of insect antennae

Electroantennogramme (EAG) recordings were performed as described in Loivamäki et al., 2008 (**VII: 3**). At the beginning of each measurement a dilution series was prepared. The compounds were let to evaporate from the filter paper inside of the Pasteur pipettes (like in Loivamäki et al., 2008 (**VII: 3**)) during the insect preparation. When the head of the insect was placed between the electrodes the achieved signal was let to stabilize for approximately 5 minutes. After stabilization of the EAG signal each dilution series was performed in the following order:

1. hexadecane
2. (*Z*)-3-hexen-yl acetate
3. 0.1% isoprene
4. 1% isoprene
5. hexadecane
6. 1% isoprene
7. 10% isoprene
8. hexadecane
9. (*Z*)-3-hexen-yl acetate

Between application of each compound a break of 0.5 min was introduced. In total the performance of one series took approximately 5 minutes. The mV response of an insect preparation did not significantly decrease during a series. The response was calculated as follows:

$$R_{\%} = (R_{mV} - R_{mVhexad})/(R_{mVhexy} - R_{mVhexad})$$

$R_{\%}$ = response compared to the response to positive control [%]
R_{mV} = response [mV]
$R_{mVhexad}$ = response to hexadecane i.e. background. The mean of 2 nearest responses (in relation to the RmV) was used for calculations [mV]
R_{mVhexy} = response to the positive control [mV]

8 STATISTICS

The statistical tests were performed with SPSS for Windows NT (release 8.0) except the binominal tests with Microsoft Excel and the independent and paired samples *t*-tests with Sigmaplot 2000 for Windows (Version 6.10). The correlation analyses were performed with Sigmaplot 2000 for Windows. Each statistical test applied is mentioned in the legend or/and in the text of individual results.

9 MEDIUM, BUFFERS, CHEMICALS AND OTHER LABORATORY CONSUMABLES

9-1 Origin of the chemicals and other laboratory consumables

The origin of the chemicals is indicated in the publications for each chemical by the name and location of the company. In the summary of the thesis the origin of the chemicals is indicated with numbers in subscript as follows:

1. Bayerische Gärtnerei-Genossenschaft, Munich, Germany
2. Sigma-Aldrich, Taufkirchen, Germany
3. Invitrogen (Gibco), Carlsbad, CA, USA
4. Carl Roth GmbH, Karlsruhe, Germany
5. Qiagen, Hilden, Germany
6. Boehringer Mannheim, Mannheim, Germany
7. PE Applied Biosystems, Weiterstadt, Germany
8. Merck, Eurolab, Darmstadt, Germany
9. Serva, Heidelberg, Germany
10. Fluka Chemie AG, Taufkirchen, Germany
11. Colgate-Palmolive GmbH, Hamburg, Germany
12. Roche Pharma AG, Grenzach-Wyhlen, Germany
13. Lehle Seeds, Round Rock, Texas, USA
14. Duchefa, Haarlem, Netherlands
15. Macherey-Nagel, Düren, Germany
16. Sarstedt, Nümbrecht, Germany
17. Amersham Biosciences, Buckinghamshire, United Kingdom
18. Calbiochem, La Jolla, CA, USA

9-2 Mediums and Buffers

C1 Poplar culture medium		
Macroelements (**C1A**)	10	ml
Microelements (**C1B**)	2	ml
MS-vitamins (M7150)[2]	200	µl
Fe-EDTA (**C1C**)	1	ml
Sucrose[8]	2	g
Bactoagar[16]	1.6	g
ddH_2O	200	ml
L-glutamin (20g l^{-1})[8]	1.6	ml

C1A: Macroelements		
KNO_3[8]	19	g
NH_4NO_3[8]	16.5	g
$CaCl_2 \cdot 2H_2O$[8]	4.4	g
$MgSo_4 \cdot 7H_2O$[8]	3.7	g
KH_2PO_4[8]	1.7	g
ddH_2O	1	l

C1B: Microelements		
H_3BO_4[8]	620	mg
$MnSo_4 \cdot H_2O$[8]	1.69	g
$ZnSo_4 \cdot H_2O$[8]	1.06	g
KI[8]	83	mg
$Na_2MoO_4 \cdot 2H_2O$[8]	35	mg
$CuSo_4 \cdot 5H_2O$[8]	2.5	mg
$CoCl_2 \cdot 6H_2O$[8]	2.5	mg
ddH_2O	100	ml

C1C: Fe-EDTA		
Na_2EDTA[8]	3.73	g
$FeSO_4 \cdot 7H_2O$[8]	2.78	g
ddH_2O	500	ml

C2: Arabidopsis medium		
MS with Gamborg's vitamins[2]	2.2	g
Sucrose[8]	5	g
Phytagar [2]	5	g
ddH_2O	500	ml

C3: LB-medium		
Pepton from Casein[4]	10	g
Yeast extract[4]	5	g
NaCl[8]	5	g
ddH2O	1	l

C4: LB-medium (agar)		
Like LB medium		
Agar-Agar[4]	15	g

E1: 2x CTAB		
Tris-HCl pH 8[8]	100	mM
CTAB	2	%
NaCl[8]	1.4	M
PVP 40[10]	1	%
EDTA[10]	20	mM

E2: BD1		
Glucose[8]	50	mM
EDTA[10]	10	mM
Tris-HCl[4] pH 8	25	mM
Steril filter		
and add lysozyme[6]	4	mg ml^{-1}

E3: BD2		
NaOH[8]	0.2	M
SDS[4]	1	%

E4: BD3		
Sodiumacetat[2]	3	M
with acetic acid[4] pH 5-5.2		

G1: TAE (50x)		
Tris-HCl[4]	2	M
EDTA[10]	50	mM
pH 8 with acetic acid[10]		

G2: Bromphenolblue		
Bromphenolblue-Na-salt[4]	14	mg
TES-buffer (**G7**)	10	ml
Glycerin[4] (v:v)	10	ml

G3: Ethidiumbromide solution		
Ethidiumbromide[2]	200	mg
ddH_2O up to	20	ml

G4: 1 kb standard		
1 kb standard	100	µl
Bromophenolblue (**G2**)	200	µl
ddH_2O	700	µl

G5: TE (10x)		
Tris-HCl[4] pH 8	10	mM
EDTA[10] pH 8	1	mM

G6: SSC (20x)		
NaCl[8]	3	M
Tri-NaCitrat[8]	0.3	M

G7: TES		
Tris-HCl[4] pH 8	10	mM
EDTA[8] pH 8	1	mM
NaCl[8]	100	mM

S1: Denaturizing buffer		
NaOH[8]	0.5	M
NaCl[8]	1.5	M

S2: Wash buffer 1		
2x SSC (**G6**)		
SDS[4] (w:v)	0.1	%

S3: Wash buffer 2		
0.2x SSC (**G6**)		
SDS[4] (w:v)	0.1	%

S4: Hybridizing buffer		
5x SSC (**G6**)		
Blocking reagent (w:v)[6]		
(from 10% blocking stock solution)	1	%
SDS[4] (w:v)	0.12	%
65°C ca. 1h, mix		

S5: Buffer 1		
Tris-HCl[4] pH 7,5	1	M
NaCl[8]	1.5	M

S6: Buffer 2		
Blocking reagent[6]		
(from 10% blocking stock solution)	1	%
Buffer 1		
65 °C ca. 1h		

S7: Buffer 3		
Tris-HCl[4] pH 7.5	100	mM
NaCl[8]	100	mM
$MgCl_2$[4]	50	mM
with HCl pH 9.5, not autoclv.		

S8: Staining-solution		
Buffer 3	10	ml
NBT/BCIP[6]	200	µl

P1: PEB		
Tris-HCl[4] pH 7	100	mM
$MgCl_2$[4]	20	mM
Glycerin[4] (v:v)	5	%
Triton X-100[4] (v:v)	2	%
DTT[4] (add just before use)	20	mM

P2: ISB		
Tris-HCl[4] pH 8.5	50	mM
$MgCl_2$[4]	20	mM
Glycerin (v:v)	5	%
DTT[4] (add just before use)	20	mM

P3: Bradford-solution		
Coomassie Brilliant Blue		
G-250[9] (w:v)	0.01	%
Solve in Ethanol[4] (v:v)	5	%
o-Phosphoricacid[4] 85 %	10	%

W1: Towbin		
Tris-HCl [4], pH 8.3	25	mM
Glycin	0.192	M
MeOH[4]	20	%

W2: Blocking Buffer		
Blocking reagent[6]	5	%
1x PBS (**W3**)		
Tween[8]	0.1	%

W3: 5x PBS		
NaPi, pH 7.2	50	nM
NaCl[8]	140	mM
Prepared from W31 and W32 so that W32 is added to W31 until pH 7.2 is reached.		

W31: Buffer 1		
Na_2HPO_4[8]	250	mM
NaCl[8]	700	mM

W32: Buffer 2		
NaH_2PO_4[8]	250	mM
NaCl[8]	700	mM

W4: BCIP/NBT		
BCIP	10	mg
NBT	5	mg
1M Tris-HCl pH 8[4]	20	ml
$MgCl_2$[4]	1	mM

W5: 5x Sample buffer native		
SDS (w:v)	25	%
Glycerol[4]	50	%
2-ME	0.25	%
Tris-HCl pH 6.8[4]	1.25	M

O1: TCA (10%)		
trichloroacetic acid	10	%
ddH2O	90	%

O2: BHT		
butylated hydroxytoluene	0.15	%
MeOH[4]		

O3: TBA		
thiobarbituric acid	0.5	%
10% TCA (**O1**)		
heat for dissolving		

IV RESULTS AND DISCUSSION

1 CIRCADIAN REGULATION OF ISOPRENE BIOSYNTHESIS IN GREY POPLAR

1-1 Endogenously regulated transcript rate of *PcISPS* and related genes

Transcript levels of the four genes *PcISPS*, *PcDXR*, *PcPSY* and *PcTUB* were determined at different time points over three virtual 24h day/night cycles in leaves of sterile shoot culture poplar plants. The plants were exposed to long day (LD), darkness (DD) or light (LL) conditions in the climate chamber. It was obvious that under day/night regime expression of all genes showed low levels during the night time and higher levels during the day. However, each examined gene presented a distinct diurnal pattern (Loivamäki et al., 2007a (**VII: 1**)). The results show that *PcISPS* transcript levels appeared to peak in the morning whereas *PcDXR* expression peaked later in the afternoon. The expression levels of *PcPSY* peaked in the morning, but in addition exhibited a second, less intense, peak in the evening. The expression of the 'housekeeping gene' or normalization gene *PcTUB,* whose expression was not supposed to fluctuate, was shown to peak in the evening under LD conditions but with smaller amplitude than the other genes.

Putting plants under continuous darkness the first observable striking feature was the dramatic decrease of transcript levels for *PcDXR*, *PcISPS* and *PcPSY*. Hardly any transcription was observed for these three isoprenoid genes under darkness and thus no rhythm could be determined for any of them. The result is similar with the result shown by Mayrhofer et al. (2005) who proved that *PcDXR* and *PcISPS* transcription rates are low under darkness. The results suggest that for all of the studied genes, primarily light is the triggering factor of the transcription rate.

Under continuous light two main rhythmic patterns of expression were observed for the examined genes. Transcript levels of *PcDXR* did not vary significantly, but rhythmic fluctuations with an amplitude of approx. 24 hours for both *PcISPS* and *PcPSY* testified for circadian regulation on the transcript level for these two genes. The circadian rhythm of *PcISPS* and *PcPSY* genes was verified by normalizing (minimum value brought to 0, maximum to 1) the individual experiments and calculating a mean of the six parallels. These normalized data were tested against sinusoidal fluctuations and the correlation analysis proved for a 24h period defining thus circadian rhythms (Loivamäki et al., 2007a (**VII: 1**)). Therefore, even if light triggers and is essential for the expression of *PcISPS* and *PcPSY* it is not the only regulating factor of gene transcription. A similar circadian and also light dependent regulation of gene expression was shown earlier for a germin like protein *SaGLP* in mustard (*Synapsis alba*) by Heinzen et al. (1994).

Transcript levels of β-tubulin (*PcTUB*) were also measured under continuous darkness and light. The transcript levels of this 'control' gene fluctuated less regularly than the expression of the other

examined genes. However, higher transcript levels were observed to appear in the evening. The result proves that this gene does not have 'housekeeping' properties under these conditions and should not be used as such, at least when studying different light regimes or expression variations within a day. In addition to diurnal variation shown here, a recent analysis in different developmental stages showed that the gene may not be the most appropriate choice of internal control (Brunner et al., 2004). Indeed it is important part of a RT-PCR to carefully select the proper reference gene(s) for each experimental design to be able to precisely quantify the transcription rates of the genes of interest. Especially when different gene expression rates are compared between different developmental stages or tissue or cell types care should be taken when choosing an internal control (Brunner et al., 2004).

Phytoene synthase (PSY) is the first dedicated and regulating enzyme of the carotenoids pathway (Von Linting et al., 1997). Carotenoids functions are really diverse ranging from primary metabolites involved in photosynthesis to secondary ones as antioxidants or as vitamin and hormone precursors (Britton, 1995; Armstrong and Hearst, 1996). Consistent with the role of PSY in synthesizing carotenoids for photosynthesis, this gene is highly expressed under light and repressed in the dark. The circadian pattern observed under light conditions testifies for a role of the circadian clock in the regulation of *PcPSY* and may reflect the essential role of carotenoids for photosynthesis.

Interestingly when diurnal fluctuations were investigated *PcPSY* transcription levels showed in addition to the morning peak a less intensive increase in mRNA copy numbers in the evening. The highest peak of transcript levels within the circadian fluctuations hit the less intense evening peak. It can be assumed that the morning peak of gene expression is rather light regulated whereas the evening peak would be endogenously regulated. The main peak of *PcPSY* expression observed early in the morning under LD-conditions could be related to the need of enzyme proteins of the photosynthesis-related carotenoids biosynthesis in the morning when sun comes up. However, only a further analysis of the downstream genes /proteins involved in this pathway could confirm this hypothesis.

Being the first committed step of the MEP-pathway *DXR* is proposed to be one of the rate limiting steps of isoprenoid biosynthesis (Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2002). Indeed in peppermint (*Mentha* x *piperita*) over-expression of *DXR* leads to a higher accumulation of essential oil, and co-suppression of this gene limits growth and leads to abnormal pigmentation (Mahmoud and Croteau, 2001), indicating a limiting and non-replaceable role of *DXR* in the MEP-pathway for this species. However, as *PcDXR* levels appear in the present thesis to be neither synchronized with *PcISPS* nor with *PcPSY* fluctuations and as it does not show circadian regulation, *PcDXR* may not be the key in the daily regulations of the MEP-pathway and

subsequent plastidic isoprenoids biosynthesis. This assumption is supported by Rodríguez-Concepción et al. (2001) who showed that fluctuations of carotenoid biosynthesis in tomato do not require similar fluctuations of *DXR* gene. It is likely that in Grey poplar no direct regulation of isoprene biosynthesis by *PcDXR* occurs. However, *DXR* transcript levels are shown to be highest in leaves of young plants and during inflorescence development (Carretero-Paulet et al., 2002; Guevara-García et al., 2005). Thus a possible significant role of *DXR* on the MEP-pathway could take place in special stages of plant development.

1-2 PcISPS protein content and enzyme activity level

From the investigation of PcISPS protein concentration and enzyme activity in poplar leaf extracts it became evident that ISPS protein concentration as well as its activity were lower under continuous darkness than continuous light. The protein concentration reached a maximum of ~6 ng mg protein^{-1} and the activity ~0.45 µkat kg protein^{-1} in darkness whereas the same values in the light were 12 ng mg protein^{-1} and 0.8 µkat kg protein^{-1}, respectively (Loivamäki et al., 2007a (**VII: 1**)). However, such differences were not observed between dark and light values from leaves under LD conditions. Thus nor circadian neither diurnal fluctuations in protein level or enzyme activity could be demonstrated. The discrepancies between the fluctuations in mRNA and protein level can be due to post-transcriptional regulation of the PcISPS protein as earlier suggested by Mayrhofer et al. (2005) who observed similar discrepancies in seasonal variation of isoprene synthase activity, protein level and isoprene emission. The tendency in the present experiments, however, was that the protein level and enzyme activity were higher under light than under darkness. Thus it might be possible that with a much higher number of replicates the fluctuation at least under LD conditions becomes ascertainable.

1-3 Isoprene emission fluctuates in circadian manner in poplar

Under LD conditions isoprene emission rate from poplar shoot cultures followed a clear diurnal pattern, verifying the result of Mayrhofer et al. (2005). As it was expected from light-off experiments (e.g. Kreuzwieser et al., 2002; Magel et al., 2006) isoprene emission decreased very fast from the original to a very low level when the shoots were placed in continuous darkness. Since isoprene emission is closely linked to the light-dependent supply of photosynthetic intermediates (e.g. Loreto and Sharkey, 1993; Magel et al., 2006) it is supposed that under darkness in the photosynthetic active parts of the poplar shoots there is no substrate available for isoprene emission and thus the emission rates should be near zero. Remarkably, isoprene emission of the shoot cultures was measurable at a low rate (also after subtracting the background value from medium and jar, which was approx. 10% of the over-all emission) under darkness. It might be hypothesized that the carbon supply sustaining this isoprene emission stems from the growth medium that contains sucrose.

Although no rhythms in tune could be observed in ISPS protein or enzyme activity levels under continuous light, the isoprene emission rates fluctuated with a 24 hours period. Isoprene emission was as its strongest approx. 25% higher than the emission as its lowest rate on the 3rd and 4th days of the measurements (Loivamäki et al., 2007b (**VII: 1**)). Relative values (minimum value brought to 0, maximum to 1) of fluctuation were tested against sinusoidal curve, but because of the decline in isoprene emission during the first two days, it was only possible to fit a curve on the data of the last two days. The fluctuation of isoprene emission during these days was highly significant clearly testifying for switched circadian rhythm of the isoprene emission whose highest rate occurred always in the subjective afternoon. Similarly, switched (12h delayed when compared to diurnal rhythm) circadian rhythm was shown for isoprene emission in Oil Palm (*Elaeis guineensis*) under continuous light by Wilkinson et al. (2006). The authors were able to measure isoprene fluctuating in circadian manner in broad range of temperatures and light intensities suggesting for a clock element that is pre-adapted to work also in higher temperatures, a feature which was not previously observed for any other plant species (Wilkinson et al., 2006). Also Dudareva et al. (2003) show circadian emission for monoterpenes myrcene and (*E*)-β-ocimene from inflorescences of snapdragon flower (*Antirrhinum majus*). The group was able to show the clock controlled fluctuations for the compounds also in the darkness which was not possible for isoprene (Wilkinson et al., 2006; Loivamäki et al., 2007a (**VII: 1**)) that is not stored in the leaf organs like monoterpenes can be (Kesselmeier and Staudt, 1999).

Dudareva et al. (2003) showed that the emissions of myrcene and (*E*)-β-ocimene are partly regulated by monoterpene synthase expression rate. However, the authors do not exclude the possibility of regulation also by other means, e.g. by the rate of substrate supply or by translational or post-translational modifications. In the present study it is somewhat disturbing that *PcISPS* expression and isoprene emission rates fluctuated in circadian manner, whereas PcISPS protein level and enzyme activity level did not show clearly similar rhythms. Thus neither the circadian nor even the diurnal rhythms observed in isoprene emission level seem to be due to the transcription rate of *PcISPS* gene. It must be assumed that the isoprene emission rate should be regulated in another way. Indeed, it is not known to which extend the two forms of isoprene synthase, membrane bound and soluble (Wildermuth and Fall, 1998; Schnitzler et al., 2005), are actually active under *in vivo* conditions in the leaves. It is possible that one of these forms is post-translationally regulated in a circadian manner. Besides, the observed circadian rhythm of isoprene emission might primarily occur because of in a similar rhythm fluctuating substrate supply. As it is well documented that several genes involved in photosynthesis are clock-regulated (Harmer et al., 2000) such a regulation of the MEP-pathway might occur rather for providing substrate for primary metabolism (e.g. for synthesis of photosynthetically active pigments like carotenoids) than for

isoprene biosynthesis. Therefore, assuming that formation of photosynthesis and thus the DMADP-pool undergo to a certain extent a circadian change, it might be hypothesized that the circadian change of isoprene emission is due to a slightly enhanced metabolic flux within the MEP-pathway. However, the substrate supply from the MEP-pathway can also occur in circadian manner due to other reasons e.g. due to cytosolic and chloroplastic processes competing from phospho*enol*pyruvate (PEP), as was suggested by Wilkinson et al. (2006). Phospho*enol*pyruvate is indeed needed in cytosolic processes e.g. for nitrate assimilation that undergoes at the level of nitrate reductase in the evening/early morning peaking circadian rhythms (Yang and Midmore, 2005). Such a competition from PEP would support the suggested hypothesis about isoprene emission as a metabolic safety valve (Rosenstiel et al., 2004).

2 TRANSGENIC ARABIDOPSIS OVEREXPRESSING *PcISPS*

2-1 Successful transformation of *PcISPS* into Arabidopsis

In the F1 generation after transformation 1.83% of the Arabidopsis plants were green on selection medium therefore carrying the resistance against kanamycin. From these green plants 56 were selected and screened for the presence of isoprene emission (Fig. 17), and from those plants 11 lines (the bars in black in Figure 17) were selected for further use.

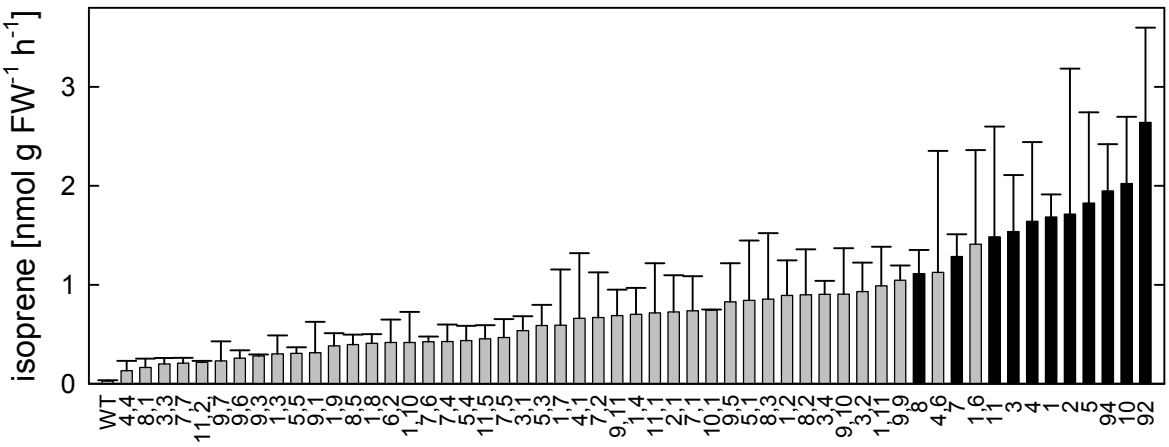

Fig. 17. Leaf isoprene emission from isoprene emitting *Arabidopsis thaliana* plants in the first generation (F1). Mean + SE is shown, n = 3 ± 1. The bars in black represent positive lines that were selected for further screening in the second (F2) generation.

When the seeds of F2 (obtained following self-pollination of F1-transformed plants) were plated on MS with kanamycin, the lines appeared to be in mean 80.9% antibiotica resistant proving for a single gene insertion in the plant genome. Expression of *PcISPS*, *NPTII* (codes for an aminoglycoside (neomycin) phosphotransferase conferring resistance to antibiotics such as kanamycin or neomycin) and for comparison actin 2 (*AtACT2*) genes from total RNA of F1

generation were measured and testified for the successful transformation of Arabidopsis with the poplar *ISPS* gene (Loivamäki et al., 2007b (**VII: 2**)).

Following several isoprene emission measurements from single leaves of the transgenic lines in F2 generation (age of rosette from 3 to 5 weeks), five of the lines were selected for further experiments: three of so-called strong isoprene emitting lines i.e. lines 10, 92 (later called as line 9) and 8 emitted 3-10 fold more isoprene than the two other so-called low-emitting lines i.e. lines 3 and 5 (Fig. 18). Isoprene emission rates were in general higher in F2 generation than in F1 generation (Fig. 17 and 18).

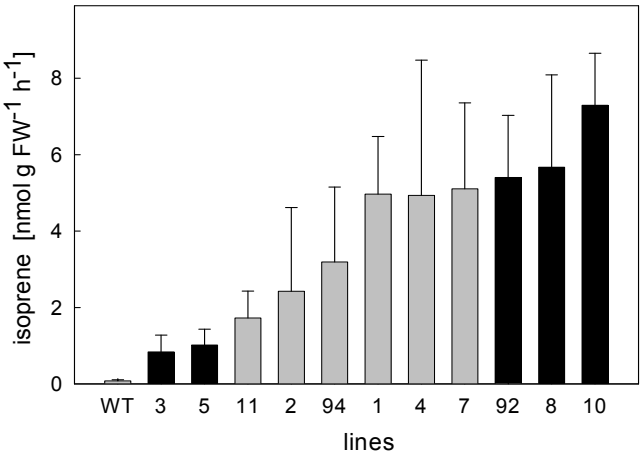

Fig. 18. Leaf isoprene emission rates from the selected isoprene emitting *Arabidopsis thaliana* lines in the F2 generation. Mean + SE is shown, n = 5. Bars in black represent the lines that were selected for further experiments.

In addition to emission measurements, protein and ISPS activity level measurements further proved that in the lines 8 and 9 isoprene biosynthesis was highest. ISPS protein concentration and enzyme activity correlated with isoprene emission levels, being strongest in lines 8 and 9 and lower in the other lines (Loivamäki et al., 2007b (**VII: 2**)). Being introduced into the Arabidopsis genome under the regulation of a constitutive promoter, *PcISPS* was expressed in all organs of Arabidopsis. Line 9 had approximately two fold higher transcript levels in all plant organs than other lines, within which the expression levels were comparable to each other (Loivamäki et al., 2007b (**VII: 2**)).

Leaves, roots and flowers (with ~ 1 cm stem) of transgenic Arabidopsis emit isoprene (Loivamäki et al., 2007b (**VII: 2**)). Isoprene emission from leaves and flowers was 4 - 6 nmol g FW^{-1} h^{-1} from strong isoprene emitting lines 8 and 9, and 1.5 nmol g FW^{-1} h^{-1} from line 10. Emission from line 3 was 0.2 nmol g FW^{-1} h^{-1}, being approximately four fold higher than from line 5 or from wild type (Loivamäki et al., 2007b (**VII: 2**)). The emission rates are relatively low compared to real isoprene emitting species (Sharkey et al., 2005; Behnke et al., 2007) but in similar range with other

transgenic isoprene emitting Arabidopsis plants carrying either the *ISPS* gene from white poplar (*Populus alba,* generated by Sasaki et al., 2007) or the *ISPS* gene from kudzu (generated by Sharkey et al. (2005)). The plants from Sasaki et al. (2007) were shown to emit approximately 0.2 - 1 nmol g DW^{-1} h^{-1} (= approximately 0.4 - 2 nmol g FW^{-1} h^{-1}, if plants' dry weight is considered to be 50% of the fresh weight (JP Schnitzler, personal communication)) and the plants from Sharkey et al. (2005) approximately 1.32 nmol isoprene m^{-2} s^{-1} (= approximately 12 nmol g FW^{-1} h^{-1}, calculated by using the correlation between Arabidopsis leaf fresh weight and leaf area in Loivamäki et al., 2008 (**VII: 3**)).

Even if the wild type plants showed a very low emission rate, neither *PcISPS* expression nor PcISPS activity or protein signal in wild type could be detected. Similar low emission rate was shown by Sasaki et al. (2007) from their wild type Arabidopsis plants. The isoprene emission from wild type plants might be explainable e.g. by chemical degradation of DMADP, since this molecule is unstable already under neutral pH conditions (Brüggemann and Schnitzler, 2002b).

2-2 Intermediates of MEP-pathway and substrate dependency of isoprene emission in *PcISPS* expressing Arabidopsis

To find an explanation for the low level of isoprene emission from transgenic Arabidopsis the DMADP availability within the MEP-pathway for isoprene biosynthesis was examined. Even if some substrate is used for isoprene production, total DMADP contents in transgenic plants were similar in range to wild type, namely around 10 pmol mg FW^{-1} (Loivamäki et al., 2007b (**VII: 2**)). In addition measurement of total carotenoid and chlorophyll levels showed no difference between transgenic lines and wild type indicating that photosynthetic pigment concentrations were not affected by the introduction of the *PcISPS* gene (Fig. 19).

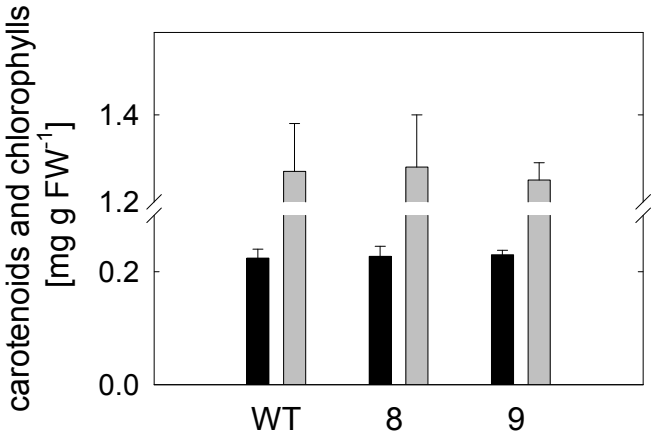

Fig. 19. Total carotenoid (black bars) and chlorophyll (grey bars) content in leaves of 5 week-old wild type (WT, Col-0) and transgenic isoprene emitting Arabidopsis lines 8 and 9. Mean + SE is shown, n = 4 ± 1, no significant differences were found between the lines (Kruskall-Wallis-test).

Consistent with higher presence of DXS and DXR in developing parts, in light-grown seedlings and in the inflorescence (Carretero-Paulet et al., 2002), the results in the present thesis revealed the highest DMADP contents and isoprene emission rates in young, developing Arabidopsis leaves (Loivamäki et al., 2007b (**VII: 2**)). A similar feature was found by Lücker et al. (2001) in transgenic petunia plants that constitutively expressed a linalool synthase gene. Enzyme activity could not be detected from old petunia leaves despite the relatively easy detection of activity in young leaves. The present results collectively support the idea of developmental regulation of the MEP-pathway in Arabidopsis and suggest that it is very difficult to predict isoprene emission from a whole Arabidopsis rosette at a certain developmental stage.

Isoprene emission from transgenic Arabidopsis lines could be enhanced by feeding with 1-deoxy-D-xylulose (DOX). DOX externally provided to cut leaves via the transpiration stream can be taken up by the chloroplast and bypasses DXS activity and its regulative role on the MEP-pathway, providing substrate for the MEP-pathway (Wolfertz et al., 2003). Feeding of leaves with 30 nM DOX enhanced total DMADP contents by a factor of two combined with two-fold higher isoprene emission rates ($P < 0.01$, independent samples *t*-test) compared to water-supplied control leaves, whereas feeding with a lower concentration of DOX (3 nM) did not produce any significant changes (Loivamäki et al., 2007a (**VII: 2**)). This result indicates that isoprene emission in Arabidopsis indeed depends on the substrate availability from the MEP-pathway. Moreover, in the light of this result, the overall different isoprene emission rates within different lines might not be due to different transcription rates but rather due to different substrate supply for isoprene biosynthesis. With

respect to the present data it is likely that the relatively low isoprene emission rate from transgenic Arabidopsis could be enhanced by co-over-expressing a gene upstream on the MEP-pathway, e.g. *DXS* or *DXR*. Whether such a co-transformation would have negative consequences for the overall fitness of the plants remains to be elucidated.

2-3 Relative growth rates of the *PcISPS* expressing Arabidopsis

The introduction of *PcISPS* into Arabidopsis could have led to a re-direction of isoprenoid precursors, and thus isoprene emission competing with formation of other isoprenoids synthesized downstream of the MEP-pathway. So far, many experiments aiming to cause over-expression of isoprenoids or related genes showed altered phenotypes of transgenic plants being restricted in growth due to depletion of precursors (Fray et al., 1995; Aharoni et al., 2003, 2006). For example, introduction of (S)-linalool and linalool derivatives synthase encoding gene into Arabidopsis (Aharoni et al., 2003) or into potato (Aharoni et al., 2006) led to altered phenotypes when higher linalool levels were detected.

Thus, to understand the influence of isoprene emission for overall plant fitness, experiments to measure shoot relative growth rate (RGR) under normal (23°C) and altered temperature (29°) conditions were set up. The altered temperature conditions were chosen because isoprene is thought to protect against high temperature stress (Singsaas et al., 1997; Sharkey et al., 2001; Behnke et al., 2007; for review see Sharkey et al., 2008). Two different methods were used to quantify growth rates, using either leaf area- or biomass measurements. It was surprising to observe that isoprene emission did not have a negative impact on the plant fitness, on the contrary, under thermal stress, isoprene emitting plants even grew faster than wild type (Loivamäki et al., 2007b (**VII: 2**)).

Based on leaf area measurements, with the GROWSCREEN imaging system (Walter et al., 2007) the leaf growth of isoprene emitting Arabidopsis plants (lines 8, 10 and 3) was significantly faster (ANOVA and Tukey's post hoc analysis, $P < 0.05$, $P < 0.001$ and $P < 0.01$, respectively) compared to wild type or to the very low isoprene emitting line 5. The differences in RGR (relative growth rate) were higher at the beginning of the experiment than at the end when rosettes became fully developed. In addition the differences in the growth rates were higher under higher temperature (29°C) than in lower temperature (23°C) (Loivamäki et al., 2007b (**VII: 2**)).

When biomass was used to calculate RGR, differences between isoprene emitting lines and wild type were of the same order of magnitude as those based on non-invasively measured leaf area data. However, variability was higher due to destructive harvests of different populations for data acquisition at different time points. Thus, based on the fresh weight data, only line 3 showed a

significantly faster growth (ANOVA and Tukey's post hoc analysis, $P < 0.001$) compared to wild type or line 5 at higher temperature.

Interestingly, the transgenic Arabidopsis plants grew fastest at the beginning of rosette development when also DMADP levels as well as PcISPS activity and isoprene emission rates were observed to be at their highest. Supporting the present observations, Carretero-Paulet et al. (2002) showed that the highest metabolic flux through the MEP-pathway occurred in an early stage of plant development. Finding the highest isoprene emission, DMADP and enzyme activity levels together with highest growth rates in young plants during their vegetative growth phase indicate that enhanced growth of transgenic plants under moderate thermal stress is indeed likely due to introduced *PcISPS*. In addition comparable results, proving that the transgenic isoprene emitting Arabidopsis rosettes can grow larger than the wild type plants, was recently shown by Sasaki et al. (2007). The authors proved that transgenic plants had larger leaves and gained more weight than the wild type Arabidopsis plants in the same age. In addition they revealed a tendency that in isoprene emitting Arabidopsis plants the cell size was larger than in the respective wild type. Sasaki et al. (2007) suggested that the larger leaf size in isoprene emitting Arabidopsis might be due to higher substrate supply to biosynthesis of other products of the MEP-pathway, i.e. hormones like ABA or gibberellins.

2-4 Other volatile organic compounds emitted by Arabidopsis

Introducing the isoprene synthase gene from Grey poplar into Arabidopsis could lead to altered volatile profile due to substrate limitation in these plants. However, VOC analysis verifies that the emission from untreated or herbivore infested wild type and transgenic plants do not differ from each other. Isoprene was the predominant volatile compound emitted by uninfested transgenic plants, composing up to 91.3% of all detected volatiles. When the emission from the plants was induced by herbivore infestation (*Pieris rapae* (Small Cabbage White butterfly) or *Plutella xylostella* (Diamondback moth; Fig. 20)), isoprene emission tend to be lower than from uninfested plants (Loivamäki et al., 2008 (**VII: 3**)). Thus, a small metabolic shift of carbon from isoprene to other herbivore induced volatiles in transgenic lines might be visible. The result further verifies (1) that isoprene emission is substrate dependent and (2) that the emission does not have overwhelming effect on the plant; isoprene biosynthesis is enhanced only when its substrate is available.

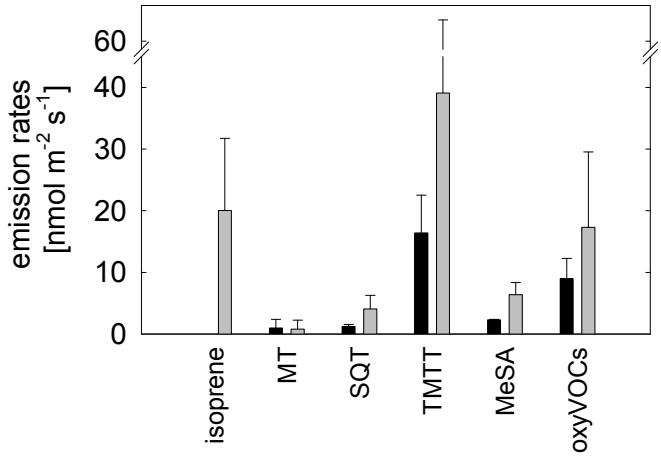

Fig. 20. Volatile organic compounds emitted from *Plutella xylostella* infested wild type and transgenic isoprene emitting plants. Black bars: wild type, grey bars: transgenic plants. MT: monoterpenes (α- and β-pinene, (*E*)-β-ocimene, limonene, 2 unidentified MT); SQT: sesquiterpene (α-farnesene); TMTT: (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetradiene; MeSA: methylsalicylate; oxyVOCs: other oxygenated volatile organic compounds (21 compounds). Two independent samples per treatment were analyzed and means + SE are presented.

Infestation of Arabidopsis rosettes with Small Cabbage White larvae (*P. rapae*) induced a significant release of the monoterpene linalool, homoterpene (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetradiene (TMTT), and the sesquiterpene α-farnesene (main effect tested by Kruskall-Wallis-test and significant differences by Mann-Whitney *U*-test, $P < 0.05$). Also the emission of some individual oxygenated volatile compounds like methylsalicylate (MeSA), hexanal, dimethyltrisulfide and two other, yet unidentified compounds were induced.

Pl. xylostella and *P. rapae* induced volatile blends were similar except for linalool whose emission was found to be *P. rapae*-specific and was neither observed for uninfested nor for *P. xylostella*-infested rosettes. Surprisingly, an emission of linalool emission from Arabidopsis was not observed in the previous studies (Van Poecke et al., 2001; Chen et al., 2003) with the same ecotype Col-0. However, the discrepancies between different studies can be explained by different developmental stages of the experimental plants or by variations within the same ecotype between different laboratories.

3 TRANSGENIC ARABIDOPSIS OVEREXPRESSING *PcDXR*

3-1 Introduction of *PcDXR* into Arabidopsis

In the F1 generation after transformation 0.6% of the plants, grown on selective MS agar containing hygromycin as selective antibiotic, showed a green phenotype indicating the presence of hygromycin resistance. From these plants 14 were chosen in order to generate the second generation. In the F2 generation at least 80% of the plants were green under selective conditions.

The seedlings showing the normal dark green phenotype and carrying at least one copy of the DNA of interest were transferred to soil and used for further measurements.

Analysis of *PcDXR* gene from total RNA of F2 generation plants confirmed the successful introduction of this gene into Arabidopsis. Naturally Arabidopsis contains its own *DXR*. However, the primers did not bind to wild type Arabidopsis cDNA, due to the mismatches between the designed primers (forward 5 bp and reverse 6 bp differences) and the Arabidopsis cDNA sequence. In Figure 18 an example is shown of *PcDXR* gene accumulation in transgenic Arabidopsis. From 13 lines (A1, A2, B1, C2, D1, D2, F1, F2, G1, G2, K1, K2, K3; the same letter indicates a common parent in F0 generation) *PcDXR* expression could be successfully detected in mRNA level. Thus those lines were selected for further analysis.

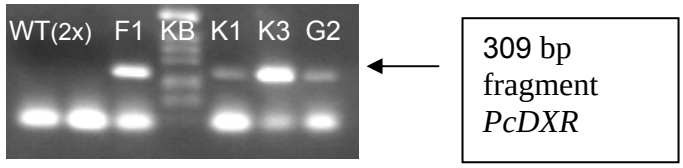

Fig. 21. Accumulation of *PcDXR* cDNA fragments in some of the transgenic lines. In wild type no cDNA-fragment was detected.

3-2 Functional screening of transgenic Arabidopsis over-expressing *PcDXR*

Functional screening of transgenic lines expressing *PcDXR* was performed similar to the work of Estévez et al. (2001) and Carretero-Paulet et al. (2006) using the accumulation of carotenoids as functional selection marker assuming that over-expression of *PcDXR* results in an enhanced metabolic flux through the MEP-pathway.

In addition, total leaf DMADP concentrations were determined in transgenic and wild type plants. As shown in Figure 22, analysis of DMADP levels revealed no differences (main effect tested with Kruskall-Wallis-test) between the different transgenic lines and wild type plants.

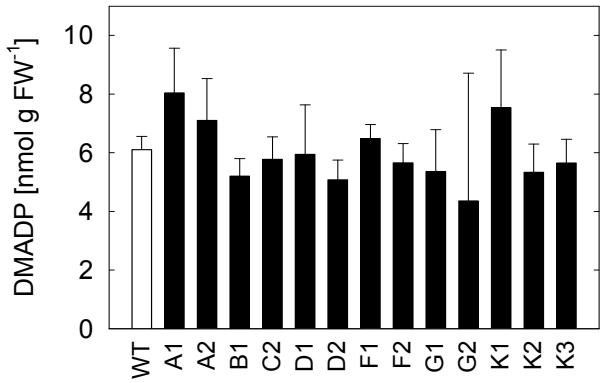

Fig. 22. Total leaf DMADP concentration in transgenic *PcDXR* over-expressing Arabidopsis and in wild type (WT) in 4 weeks old plants. Mean + SE is shown, n = 5 ± 1.

The fact that DMADP level in the transgenic lines was not altered compared to wild type does not necessarily mean that the flow through the MEP-pathway would not be enhanced. DMADP is an intermediate that can be fast converted further to higher isoprenoids and end-products of MEP-pathway. Estévez et al. (2001) proved that over-expression of a DXS gene in Arabidopsis can lead to an accumulation of carotenoids, chlorophylls and abscisic acid (ABA) which are, among others, products of the MEP-pathway. Thus the carotenoid and chlorophyll levels were determined in the F3 generation of *PcDXR* transformed plants (Fig. 23).

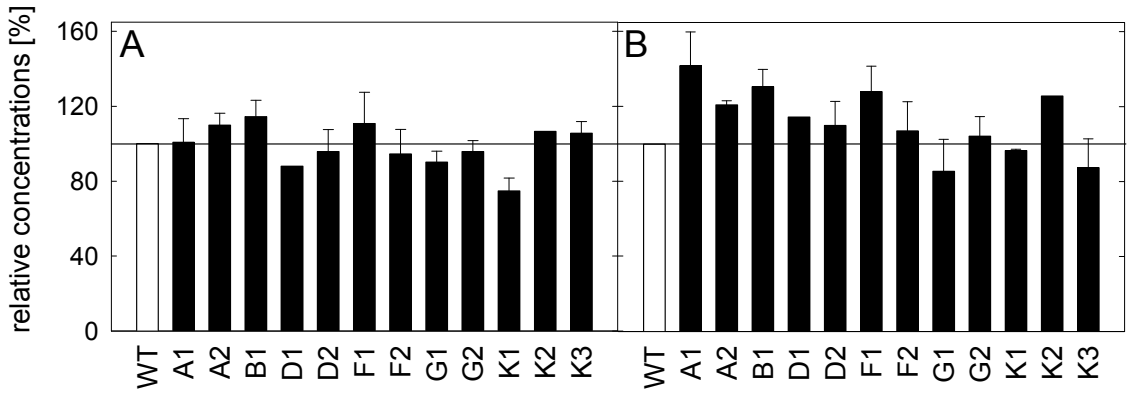

Fig. 23. Relative concentrations of carotenoids (A) and chlorophylls (B) in 4 weeks old transgenic, isoprene emitting lines (black bars) and wild type (white bars) Arabidopsis plants. The raw values were related to the mean of corresponding values of wild type. WT: Wild type n = 12; transgenics n = 4 ± 2. No significant differences were found between the lines.

Although in some transgenic lines a tendency for a relative accumulation of total chlorophyll was observed, no significant differences were found, as for carotenoids (main effect tested with Kruskall-Wallis-test). This observation is in consistence with previous results showing that *DXR*

levels do not correlate with the accumulation of carotenoids in chloroplasts of ripening tomato (*Lycopersicon esculentum*) plants (Rodríguez-Concepción et al., 2001). However, Carretero-Paulet et al. (2006) were able to transform Arabidopsis *DXR* cDNA to *Arabidopsis thaliana* itself and observed that the gene expression level can indeed limit the biosynthesis of isoprenoids from MEP-pathway. In this specific case over-expression of *DXR* led to a moderate accumulation of carotenoid and chlorophyll levels (approximately 25% more than in wild type). However, in the same work transformation of Arabidopsis with *DXS* showed much higher increase of plastidial isoprenoids (approximately 70% more than in wild type) (Carretero-Paulet et al., 2006). Taken together the previous results from other groups working on MEP-pathway related genes *DXS* (Estévez et al., 2001; Enfissi et al., 2005), *HDR* (Botella-Pavía et al., 2004) and *DXR* (Mahmoud and Croteau, 2001; Carretero-Paulet et al., 2002) it is likely that several enzymes share the regulation task on the MEP-pathway. Whether over-co-expression of several MEP-pathway regulating genes would lead to higher accumulation of end products from MEP-pathway remains to be solved. In the present particular case future analysis of the transgenic lines should involve ^{13}C-labelling, e.g. by CO_2 fumigation, to quantify possible alteration of metabolic fluxes within the MEP-pathway. In addition to chlorophylls and carotenoids, possible accumulation of other end products (e.g. that of ABA or tocopherol (Estévez et al., 2001)) could be studied. Furthermore the tendency of accumulating chlorophylls might become significant when simply more parallel experiments were performed. In addition the gene expression of *PcDXR* should be studied under stress conditions. Under stressful conditions, when more end-products from MEP-pathway are needed, the *PcDXR* over-expressing Arabidopsis plants might show advantage to wild type. For example high light stress would increase the need of photoprotective pigments (Havaux and Niyogi, 1999) and herbivore or pathogen infestation would increase the need of defence for example by terpenes (Dudareva et al., 2006). To evaluate whether the *PcDXR* was, or was not, really successfully introduced into Arabidopsis, more detailed investigations with the *PcDXR*-expressing lines should be done in the future.

In the present thesis no further studies with the *PcDXR* over-expressing lines were performed since transformation with *PcISPS* resulted in isoprene emitting lines useful for functional studies of isoprene emission. However, the *PcDXR* transformed lines can be used in future studies to enhance isoprene emission of the existing *PcISPS* expressing Arabidopsis lines by crossing of these lines or by a new transformation of the isoprene emitting lines with the available *PcDXR* binary vector system.

4 PHYSIOLOGICAL STUDIES PERFORMED WITH TRANSGENIC AND WILD TYPE ARABIDOPSIS AND POPLAR PLANTS

4-1 Testing the thermotolerance hypothesis

4-1-1 High temperature stress application on Arabidopsis

To analyze dynamically photosynthetic gas exchange and VOC emissions from whole Arabidopsis rosettes or individual poplar leaves, the newly developed cuvette system was used. The dependency of isoprene emission and photosynthetic parameters on temperature was tested in transgenic (lines 8 and 9) and wild type Arabidopsis with a temperature program increasing leaf temperature in 5°C steps from 30°C until 45°C and back lasting for 30 min at each temperature plateau (for details see Loivamäki et al. (2007b) (**VII: 2**)). Isoprene emission from the transgenic lines and from wild type correlated positively with temperature ($P < 0.01$, Pearson's correlation) whereas net assimilation decreased with increasing leaf temperature, as it was expected from previous studies (Monson et al., 1992; Singsaas et al., 1997; Velikova and Loreto, 2005). Even if stomatal conductance (gH_2O) decreased with increasing leaf temperature, leaf transpiration increased ($P < 0.05$, Pearson's correlation). Line 9 showed significant difference in isoprene emission rate, net assimilation, transpiration and gH_2O compared to wild type and line 8 ($P < 0.05$, Tukey's post hoc analysis) (Loivamäki et al., 2007b (**VII: 2**)). This suggests that the ability to emit isoprene has changed the physiology of this highest isoprene emitting line, which appear more temperature resistant. The observation is supported by several previous studies showing thermotolerance effect for isoprene (Singsaas et al., 1997; Sharkey et al., 2001; Velikova and Loreto, 2005; Velikova et al., 2005), the first one done by Sharkey and Singsaas (1995) who showed that isoprene fumigation increases the thermotolerance of isoprene emitting species. Besides isoprene certain higher isoprenoids, in addition to their several other roles e.g. in biotic defence, also can protect against heat stress. Loreto et al. (1998) showed for the first time that fumigation with monoterpenes can enhance the photosynthetic performance under high temperature. Later this observation was confirmed for *Quercus* species by Delfine et al. (2000) and Copolovici et al. (2005), who studied thermotolerance by fumigating with exogenous monoterpenes either isoprenoid non-emitting species (Delfine et al., 2000) or monoterpene emitting species treated with FSM (Copolovici et al., 2005). However, these studies investigating isoprenoids function under high temperature concentrate on fumigation with exogenous isoprenoids. The present study for the first time investigated transgenic isoprene emitting species and analyzed whether they also can show better performance of photosynthesis than respective wild type under high temperature treatment. The result shows that the highest isoprene emitting Arabidopsis line 9 might have advantage from the isoprene emission capacity compared to the wild type.

However, in the present experiments the temperature applied was too high or lasted too long, because the recovery of the plants from the relatively high thermal stress was often not complete (= visible damage of plants). Indeed, isoprene is suggested to play rather a role particularly in

recovery from suddenly occurring temperature stress (Singsaas and Sharkey, 1998; Sharkey et al., 2001; Sharkey et al., 2008) than surviving to continuous high temperature. Also for monoterpenes it has been shown that the protective role is more evident when plants were given more than one high term temperature treatment (Delfine et al., 2000). Thus, to examine the physiological function of isoprene in a more reasonable and realistic manner so called transient temperature treatment was applied in the following experiments.

4-1-2 Transient temperature stress application on Arabidopsis

Temperature experiments to test possible functions of isoprene have previously been performed in a number of different ways. To test the hypothesis about isoprene protecting from damage caused by rapid and transient high temperature events (Sharkey and Singsaas, 1995; Singsaas and Sharkey, 1998) a comparable experimental design as previously used by Sharkey et al. (2001) was chosen. These authors assessed thermotolerance as recovery of photosynthesis from short-term treatments at 46°C. In the present case with transgenic and wild type Arabidopsis plants isoprene emission, net assimilation and transpiration were analyzed (Loivamäki et al., 2007b (**VII: 2**)) during similar short-term treatments.

The gas exchange from the plants was measured before, during and after a transient temperature stress created with rapidly cycling leaf temperature (from 30°C to 40°C and back) under constant PPFD of 1000 µmol photons m^{-2} s^{-1}. Before start of the 6 heat cycles, significantly higher isoprene emissions were detected in lines 8 and 9 compared to wild type. Transpiration and gH_2O were higher, but net assimilation showed no differences in line 8 compared to wild type. In line 9, however, transpiration and gH_2O were globally lower than in other lines before the start of heat cycles and through the whole experiment (Loivamäki et al., 2007b (**VII: 2**)).

Each increase in leaf temperature up to 40°C caused a rapid transient reduction in net assimilation and gH_2O, accompanied by an increase in transpiration in all the lines. Within 30 min both wild type and the isoprene emitting line 8 recovered completely from heat stress cycles without significant reductions in net assimilation or transpiration. Wild type plants even tend to have higher net assimilation rates after the heat stress than before whereas in line 8 stomatal conductance and transpiration slightly increased compared to the initial values. However, the initially lower assimilation and isoprene emission level of the transgenic line 9 decreased after the transient thermal stress treatment indicating that from transient thermal stress the highest isoprene emitting line survived worse than wild type or the lower isoprene emitting line 8 (Loivamäki et al., 2007b (**VII: 2**)).

While isoprene emission from plants naturally emitting isoprene appears to play a significant role in plant survival, at least under stress conditions (Loreto et al., 2001; Sharkey et al., 2001; Affek and Yakir, 2002; Velikova and Loreto 2005; Velikova et al., 2005; Wiberley et al., 2005; Behnke et al.,

2007), the present results with Arabidopsis indicate that isoprene protection against damage from transient thermal stress may not be widely applicable to isoprene non-emitters transformed to emit isoprene. Photosynthetic gas exchange studies showed no negative effect from transient but still moderate temperature stress in wild type compared to transgenic isoprene emitting Arabidopsis plants. Temperature stress created with rapid heat cycles did not have a negative effect on the recovery of net assimilation rate of wild type plants, indicating that Arabidopsis does not need isoprene to protect itself against transient thermal stress. In fact, net assimilation of the highest isoprene emitting line 9 seems to be more affected by the transient temperature stress than that of wild type. A similar observation was shown for the non-isoprene emitting leaves of bean plants (*Phaseolus vulgaris*) that showed irreversible thermal damage during dark fluorescence experiments in higher temperature than isoprene emitting oak (*Quercus alba*) and kudzu leaves (Singsaas et al., 1997). However, Sasaki et al. (2007) showed recently that isoprene emitting Arabidopsis, carrying the *Populus alba ISPS*-gene, can survive a high temperature treatment better than the corresponding wild type plants. The authors applied 60°C temperature for 2.5 h (PPFD 2 µmol photons m^{-2} s^{-1}) and recorded the recovery of the plants 7 days after the stress. The photographs shown by Sasaki et al. (2007) prove for better recovery of the transgenic isoprene emitting Arabidopsis plants. The results suggest that isoprene does protect against high temperature stress also in Arabidopsis when the temperature applied is high and lasts long enough. Possibly the fact that contradicting results were observed in the present study was due to the art of temperature treatment application that was lower and shorter than in the applications done by Sasaki and co-authors.

Moreover, contradictory to the present data is also the observation of Delfine et al. (2000) showing that isoprenoid non-emitting species can have advantage from exogenous monoterpenes. The group showed that thermotolerance of non-isoprene emitting oak-species (*Quercus suber*) was enhanced and that their photosynthetic capacity was higher when the plants were fumigated with exogenous monoterpenes. Delfine et al. (2000) suggested that isoprenoid emitting species might enhance also the well-being of neighbouring plants under high temperature conditions.

It seems likely that the protecting effect of isoprenoids against stresses depends on each plant species' initial stress tolerance capacity. In Arabidopsis, in addition to heat shock proteins (Howarth and Ougham, 1993), at least calcium, ABA, ethylene and salicylic acid play independently roles in the protection against thermal stress (Larkindale and Knight, 2002; Larkindale et al., 2005). Furthermore, Larkindale and Knight (2002) showed that differences also exist in thermotolerance of different ecotypes of Arabidopsis: Columbia, for example, was more thermotolerant in their studies than e.g. Landsberg ecotype.

4-1-3 Transient light stress application on Arabidopsis and poplar

Plants in the nature, however, likely rarely face transient temperature stress without concomitantly enhanced light intensities (Leakey et al., 2005). Thus an aim in the thesis was to combine both transient temperature and light stresses to create even more naturally occurring stress conditions. Before combining both stresses, transient light stress alone was studied. Indeed in temperate climate regions from which many isoprene emitting species are native (e.g. Salicaceae and *Populus*) (Ellenberg,1963), transient light stress in tree canopies might rather likely occur than a transient temperature stress reaching up to 45°C. Even in tropical rain forest transient leaf temperature reaches maximum 38°C with the maximum PPFD 1600 µmol photons m^{-2} s^{-1} during a day (Leakey et al., 2005).

The gas exchange from Arabidopsis plants and in addition wild type and transgenic, isoprene non-emitting, poplar (for details see Behnke et al., 2007) leaves was measured. Wild type and transgenic poplar plants were included in the present study in order to compare the physiological responses of this natural isoprene emitting species to those obtained with Arabidopsis plants under similar stress conditions. The experiments were performed comparable to the transient heat cycles, but instead of temperature light intensities varied from PPFD 100 to 1300 µmol photons m^{-2} s^{-1} while leaf temperature was held constant at 30°C. Each of the six high light periods lasted 10 minutes after what low light was applied for another 10 minutes. Initial values and recovery were measured at 30°C and PPFD 100 µmol photons m^{-2} s^{-1} for 20 minutes.

Under constant conditions (30°C and 100 µmol photons m^{-2} s^{-1}) just before start of the light cycles, isoprene emission rates from Arabidopsis lines 8 and 9 were higher than that from wild type. However, at the end of the experiment all lines showed comparable low levels of isoprene emission. During the light stress treatment isoprene emission rates decreased constantly without following the up and down of light intensity (Fig. 24A). Net assimilation rates were enhanced rapidly and simultaneously with increasing light intensity (Fig. 24B) whereas stomatal conductance stayed rather constant (Fig. 24C) over time. In summary net assimilation and stomatal conductance of transgenic and wild type Arabidopsis did not differ from each other. Especially in the case of net assimilation astonishing similar values with low deviation were measured from all the plants used in the experiments.

Contrary to Arabidopsis, isoprene emission rates from wild type poplar fluctuated simultaneously with transient light cycles (Fig. 24D). During each light peak isoprene emission rates were increased by a factor of two compared to dim light conditions. In addition to the transient increases of emission during the high light phases isoprene emission rates became stimulated from cycle to

cycle. Isoprene emission from both transgenic *ISPS* knock-down poplar lines was very low as previously described by Behnke et al. (2007).

In all the lines of both species net assimilation rate was rapidly up-regulated by a factor of 3 during the high light phases and again down-regulated under dim light (Fig. 24E). Stomatal conductance did not follow these rapid changes of net assimilation in either of the species (Fig. 24F). Similar to Arabidopsis, presence or absence of isoprene emission in poplar leaves had no effect on net assimilation or stomatal conductance.

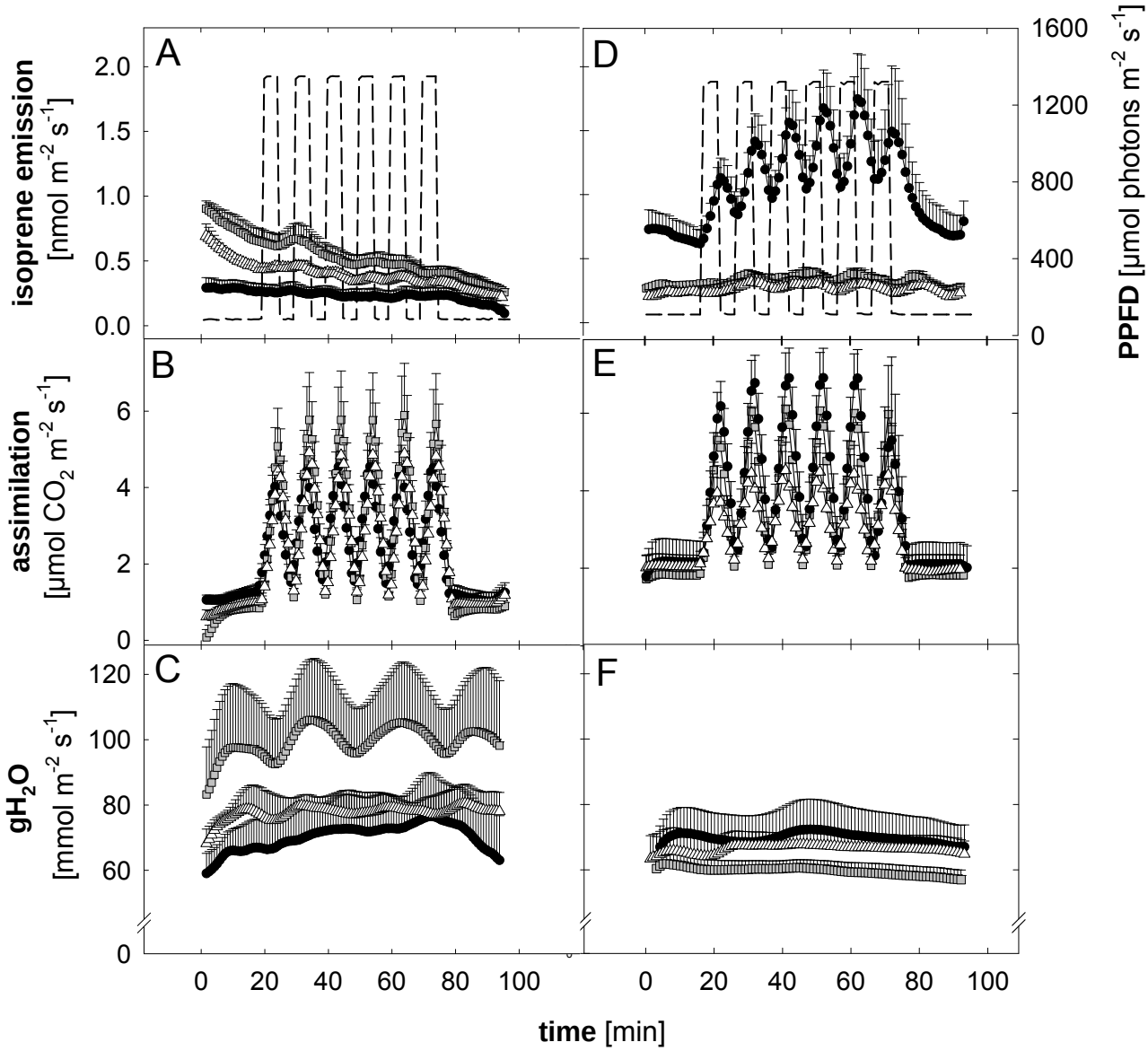

Fig. 24. Isoprene emission, net assimilation and stomatal conductance (gH_2O) of Arabidopsis wild type (●), line 8 (Δ) and line 9 (■) (A), (B) and (C), respectively and Grey poplar wild type (●), line RA22 (Δ) and line RA1 (■) (D), (E) and (F), respectively, before, during and after the light stress treatment. After 20 min stabilization at 30°C and 100 µmol m^{-2} s^{-1}, six light cycles (light increased from 100 to 1300 µmol m^{-2} s^{-1} and

back) were performed. Recovery of the plants at 30°C and 100 µmol m^{-2} s^{-1} was measured during 20 min following the last cycle. The given values are 60 s means of 3-4 individual plants + SE.

In summary the results suggest that transient light cycles do not cause a physiological stress neither for isoprene emitting or non-emitting Arabidopsis nor for isoprene emitting or non-emitting poplar plants when assimilation is used as an indicator. The observed increase of isoprene emission from light cycle to light cycle in wild type poplar is probably due to higher carbon fixation rate under high light and lower rate under dim light (Muraoka et al., 2003; Leakey et al., 2005), which consequently may lead to an increase of pool sizes of photosynthetic intermediates feeding the MEP-pathway. Probably the higher carbon fixation rate during each light cycle was the reason for the fact that isoprene emission from poplar was not only rapidly up-regulated inside of one light cycle, but also globally enhanced from the beginning of the cycles until the end of the cycles.
Even if the isoprene emission cycled according to the cycling light in the natural isoprene emitter, the emission from Arabidopsis did not show similar pattern. According to the data it seems that fluctuations in Arabidopsis isoprene emission do not exist indicating that the flux of photosynthetic intermediates in the MEP-pathway in Arabidopsis and Grey poplar is different.
The results support previous studies (Evans, 1956; Chazdon and Pearcy, 1991; Leakey et al., 2005) showing that plants are used to face changing light intensities. Up to a certain level (up to 800 µmol photons m^{-2} s^{-1} in a cool-temperature broadleaf forest) flecking light intensity do not necessarily create a stress due to the excess PPFD; indeed leaves of broad leaf trees can even orientate their leaves so that they are able to maximize the experience of sunflecks (Muraoka et al., 2003). Muraoka et al. (2003) show in their study that high light flecks indeed improve photosynthesis in cool-temperature broadleaf forest. The observations in the present study suggest that also Grey poplar and Arabidopsis plants are able to improve their photosynthesis when their leaves are exposed to short-term high light intensities. However, short-term high light periods may have other negative impact on plants and therefore, as an example, the relationship between oxidative stress, isoprene emission and flecking light should be studied in the future. It might be that to gain higher photosynthetic performance, plants have to trade-off with oxidative stress that is induced by high light intensity (Niyogi, 1999). In such a trade isoprene might play an important role by protecting photosynthetic apparatus against oxidative damage (Loreto and Velikova, 2001; Affek and Yakir, 2002). In wild type poplar plants isoprene emission is indeed increased concomitantly with the light flecks and assimilation, indicating that the compound may play a protective role by functioning as antioxidant under flecking light intensity and possible oxidative conditions.

4-1-4 Combined transient temperature and light stress application on Arabidopsis and poplar

In the next experiments the physiology of wild type and transgenic Arabidopsis and wild type and transgenic poplar plants was investigated under both transient temperature and light cycles, combining the temperature and light cycles as described above. The experiments started with constant leaf temperature of 30°C and a light intensity of 100 µmol photons m^{-2} s^{-1} for 20 minutes. After this so called stabilization period, six heat/light cycles (temperature increased in 10 min from 30°C to 45°C and back; light simultaneously from 100 to 1300 µmol photons m^{-2} s^{-1} and back) were performed. Recovery of the plants was measured during 20 min at 30°C and 100 µmol photons m^{-2} s^{-1} following the last cycle.

Under combined transient temperature and light stress situation isoprene emission rates of Arabidopsis plants did not remarkably differ from that observed under constant light and variable leaf temperature (see Loivamäki et al., 2007b (**VII: 2**)). Each increase in leaf temperature and light intensity caused a transient increase in isoprene emission rate (Fig. 25A).
Simultaneously, combined light and temperature increase caused a rapid up-regulation of net assimilation rate (Fig. 25B) combined with decrease in stomatal conductance (Fig. 25C).
When the recovery of the Arabidopsis plants was compared to the initial values it became obvious that net assimilation tend to be lower than in the beginning in wild type (75% less) and in line 8 (38% less), whereas net assimilation of transgenic line 9 was not altered. On the other hand stomatal conductance of wild type, line 8 and line 9 tend to increase 28%, 20% or 74%, respectively, until the end of the experiment compared to the initial values. Thus the Arabidopsis line 9 showing highest isoprene emission rates had more open stomata and higher assimilation rates under the stress conditions than wild type and line 8. These results, however, were not significant. Comparable values were obtained with other Arabidopsis plants grown under another temperature regime i.e. at 30°C (instead of 20°C) during the light phase. The assimilation and stomatal conductance of the plants grown at 30°C were comparable to that observed from plants grown at 20°C, with the exception that assimilation rate tend not to decrease, but rather increase over the experiment (Fig. 25E). Indeed by the end of the experiment the assimilation rate and stomatal conductance increased for all lines, clearly indicating that the plants were not stressed during the light and temperature cycles. Previous observations have shown that the plants growth conditions e.g. experiences of high temperature periods positively affect the isoprene emission capacity (Sharkey and Loreto, 1993). Furthermore Wiberley et al. (2005) proved recently that Kudzu plants that have previous experience at higher temperature (30°C) start to emit isoprene earlier and can also develop more rapidly than the plants that had grown at 20°C. Thus, it is likely that also the Arabidopsis plants that had grown at 30°C were better adapted to high temperature than plants grown at 20°C.

Like expected from previous experiments (**IV: 4-1-2** and **4-1-3**) isoprene emission from Grey poplar leaves responded rapidly and simultaneously to each combined transient temperature and light cycle. Each increase in leaf temperature and light intensity caused a rapid increase in isoprene emission rate (Fig. 25G). Contrary to previous temperature fleck experiments (Behnke et al., 2007) but similar to light fleck experiments (**IV: 4-1-3**, for poplar), each increase in temperature and isoprene emission was here accompanied by rapid up-regulation of net assimilation rate (Fig. 25H). The stomatal conductance was simultaneously decreased (Fig. 25I). The impaired recovery of transgenic poplar leaves from transient heat/light cycles compared to that of wild type was obvious. Net assimilation in both isoprene non-emitting lines decreased rapidly already after the first cycle and was significantly lower by the end of the experiment compared to initial values ($P < 0.05$, Wilcoxon's signed ranks test). In general net assimilation of transgenic lines at the beginning and at the end of the recovery phase stage were lower in both transgenic lines than in wild type leaves ($P = 0.011$ for RA1 and $P = 0.016$ for RA22, Mann Whitney *U*). Similarly stomatal conductance was lower in non-isoprene emitting poplars compared to wild type at the end of the experiment ($P = 0.0083$ for RA1 and $P = 0.018$ for RA22, Mann Whitney *U*). Moreover, in line RA1 stomatal conductance was lower at the end of the experiment than at the beginning ($P = 0.043$, Wilcoxon's signed ranks test).

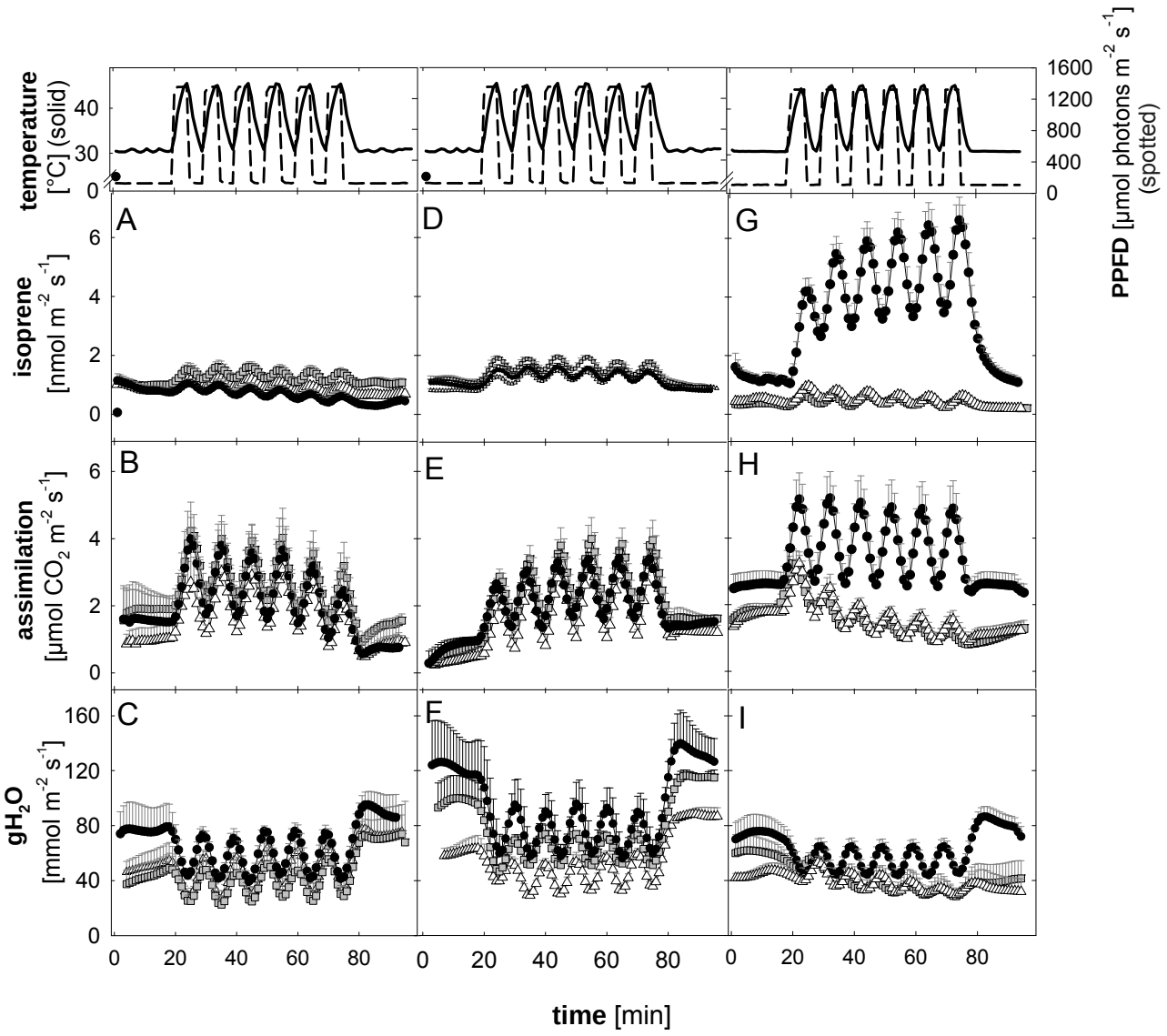

Fig. 25. Isoprene emission (A, D, G), net assimilation (B, E, H) and stomatal conductance (gH_2O) (C, F, I) of Arabidopsis rosettes and Grey poplar leaves (leaf no. 9 below the apex). The gas exchange is shown for Arabidopsis wild type, line 8 and line 9 plants grown at 20°C (A, B and C) or 30°C (D), (E), (F), before, during and after the temperature and light stress treatment. Similarly the values are shown for Grey poplar (G, H, I) wild type, line RA22 and line RA1. After 20 min stabilization at 30°C and 100 µmol m^{-2} s^{-1}, six heat/light cycles (temperature increased in 10 min from 30°C to 45°C and back; light simultaneously from 100 to 1300 µmol m^{-2} s^{-1} and back) were performed. Recovery of the leaves was measured during 20 min at 30°C and 100 µmol m^{-2} s^{-1} following stress. The given values are 60 s means of 3-4 individual plants + SE. Wild type Arabidopsis (●), line 8 (∆) and line 9(■); wild type poplar (●), line RA22 (∆) and line RA1 (■).

Isoprene emission rates from transgenic Arabidopsis rosettes in the experiment were somewhat lower than that observed under constant light and cycling temperature (see Loivamäki et al., 2007a (**VII: 1**)), probably due to an unsaturated carbon fixation rate under the low initial light intensity of

100 µmol photons m^{-2} s^{-1}. The up-regulation of net assimilation during each light and temperature pulse was globally similar as observed under cycling light for both of the species (Fig. 24B). The result emphasizes the importance of light for carbon fixation. The fluctuations in stomatal conductance were likely due to cycling temperature as they were not present under constant temperature and transient light cycles (**IV: 4-1-3**). In addition, it seems that the observed global decrease of net assimilation in experiments performed with rosettes grown at 20°C was rather due to initial temperature conditions (30°C) in the cuvettes than due to the temperature cycles as plants grown at 30°C overcame the additional thermal stress without complications.

Contrary to transgenic Arabidopsis plants for the natural isoprene emitter poplar the capacity to emit isoprene is essential to sustain photosynthesis under transient temperature and light stress. Net assimilation of both non-isoprene emitting poplar lines became significantly impaired by transient light and temperature cycles. The effect was even stronger (net assimilation in transgenic lines down to approximately 20% of that of wild type) than observed by Behnke et al. (2007) under temperature fleck stress only (net assimilation in transgenic lines down to approximately 55% of that of wild type). The observed stronger down-regulation of photosynthesis and stomata closure by the non-isoprene emitting poplar plants might be mainly due to higher intensity in the cycling temperature (from 30°C up to 45°C whereas Behnke et al. (2007) applied only from 30°C up to 38°C) than to simultaneous light cycles, as it was shown in **4-1-3** that light flecks alone do not impair assimilation. Another difference to the results of Behnke et al. (2007) is the fact that in their experiments the net assimilation decreased by each increasing temperature whereas in the experiments here it temporarily increased, even if the global tendency was a decrease. The different results are likely due to different light regimes; plants under dim light increase their photosynthesis when light flecks are experienced (Figs. 24B, H and 25B, E, H) as their carbon fixation rate may be limited under low light (Leakey et al., 2005).
Similarly to wild type poplar, transgenic isoprene emitting Arabidopsis line 9 tend also to have somewhat higher net assimilation rates and more open stomata than wild type plants or the lower isoprene emitting line 8 under transient temperature/light stress. The tendency was observed in plants grown both at 20°C and 30°C. However, net assimilation of wild type or line 8 was not impaired significantly and when the plants were previously grown at 30°C, net assimilation even increased under stress.

In summary it seems evident that Arabidopsis is an extremely thermotolerant species and does not need isoprene emission for maintaining photosynthesis during short-term episodes of temperature and light stress, at least not to recover from the kind of temperature or/and light stresses that were applied here. Contrary to the results obtained here, Sasaki et al. (2007) showed that their isoprene emitting Arabidopsis plants could survive better than wild type plants under long-term thermal

stress (2.5 h) at 60°C and PPFD of 2 µmol s^{-1} m^{-2}, whereas no impairment in overall survival of the plants was observed up to 50°C. However, such a high, long lasting temperature stress would rarely be observed in nature, at least under practical darkness. In addition, the dim light hints that the plants isoprene emission capacity is remarkably low during the experiment. In future more detailed experiments are necessary to find a biochemical explanation for the finding of Sasaki et al. (2007).

Arabidopsis might be a useful model plant in several occasions but for studying the physiology of isoprene emission it seems not to be the best one. Opposite to Arabidopsis, the present data with Grey poplar again prove the importance of isoprene emission for the protection of photosynthesis. However, it becomes clear that isoprene emission in poplar positively influences membrane localized processes of photosynthetic electron transport and energy dissipation keeping net assimilation stable under transient and moderate thermal and light stress. Future experiments with these lines should try to answer the question how isoprene influences biophysical procedures in the thylakoid membrane and more globally if the experimental triggered temperature and light effects are of relevance under natural conditions representing really a positive adaptive trait for isoprene producing leaves.

4-1-5 Recovery of Arabidopsis and poplar from combined temperature and light stress

4-1-5-1 Electron transport rate (ETR) in poplar leaves

Electron transport rate and thus the overall photochemical efficiency, was followed in transgenic and wild type poplar leaves. The fluorescence analysis was performed directly before the transient temperature and light flecks (**IV: 4-1-2**), immediately after taking out the leaves from the cuvette and during a recovery period of 10h. In all lines application of transient temperature and light stress negatively impaired ETR compared to the initial values before stress (Fig. 26). The general impairment was significant directly and 0.5h after the stress compared to initial values ($P < 0.05$; paired *t*-test). In leaves of the transgenic lines (the results of the both lines pooled together) ETR was reduced directly and 0.5 after the stress application, compared to the initial value ($P < 0.05$ for each time point, Wilcoxon`s signed ranks test). For wild type ETR tend to be reduced directly after the stress, but already after half an hour it reached back its initial level. In addition, the ETR of line RA22 is significantly lower ($P = 0.031$, ANOVA and Dunnet-T3), and for line RA1 almost significantly lower ($P = 0.061$) than that of wild type directly after the stress (Fig. 26).

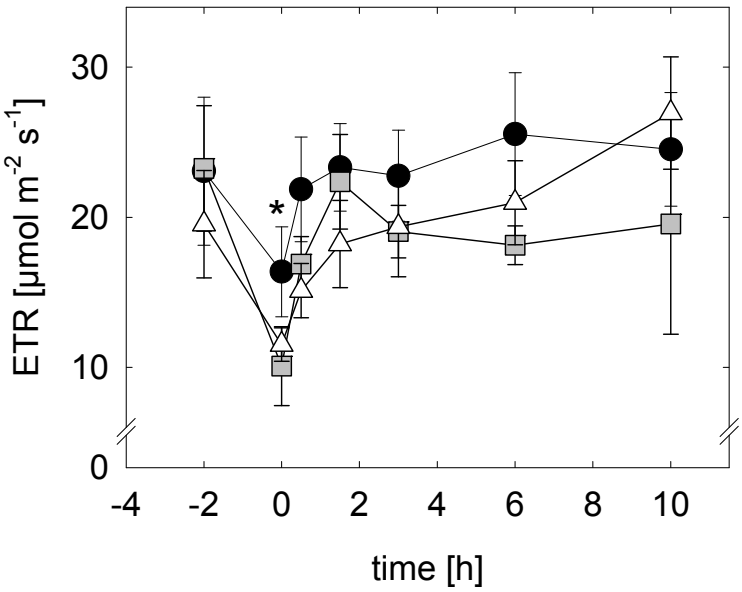

Fig. 26. Electron transport rate (ETR) in Grey poplar leaves (leaf no. 9 below the apex) that faced transient temperature and heat cycles between the hours -2 to 0. The ETR was measured directly before (-2) and after (0) the stress phase and further followed until 10h after the stress. Mean ± SE is shown. Wild type: (●), n = 5; RA1: (∆), n = 3; RA22: (■), n = 3. The differences between wild type and line RA22 line were significant directly after the stress, **P* < 0.05 (ANOVA and Dunnet-T3).

The recovery from stress was slower in transgenic isoprene non-emitting lines than in wild type. Knowing that net assimilation of the transgenic lines was shown to be significantly impaired in gas exchange studies whereas net assimilation of the wild type did not show impairment (Fig. 25H; **IV: 4-1-2**) the slower recovery of the transgenic lines from the stress is not surprising. Indeed Behnke et al. (2007) showed in their studies with transgenic isoprene non-emitting poplar plants that the ETR in these plants decreases rapidly already after the first temperature cycle (that reached 38°C) and continues decreasing by each further temperature cycle. The present results show that the detected impairment in ETR was not permanent. The transgenic lines recovered indeed relatively fast and the ETR was fully capable latest 1.5 h after the stress phase. In the isoprene emitting wild type poplar the recovery of ETR from the high transient temperature treatment was, however, faster than that of the transgenic lines. The observation supports the previous studies suggesting isoprene protecting the photosynthetic machinery against damage by high temperature episodes (Velikova and Loreto, 2005; Behnke et al., 2007).

4-1-5-2 Antioxidant content in Arabidopsis and poplar leaves

To assess the effect of combined temperature and light cycles (**IV: 4-1-2**) on the antioxidant status of poplar and Arabidopsis leaves 10h after application of transient temperature and light stress concentrations of reduced ascorbate (ASC), dehydroascorbate (DHA), reduced glutathione (GSH) and oxidized glutathione (GSSG) were compared.

No differences in ascorbate content between stressed sample and unstressed control poplar leaves were observed (Fig. 27A) indicating that transgenic and wild type lines had either recovered from the stress or were not initially stressed.

Interestingly, however, total and reduced ascorbate contents were enhanced by approx. 35 % in both non-isoprene emitting poplar lines compared to wild type (Fig. 27A, $P < 0.05$, ANOVA and Dunnet-T3). In Arabidopsis leaves, neither in plants grown at 20°C nor in plants grown at 30°C, such differences between wild type and transgenic lines were observed (Fig. 27B). Furthermore the redox ratio (DHA/reduced ascorbate) did not show any differences in any of the treatments neither in poplar nor in Arabidopsis leaves (data not shown).

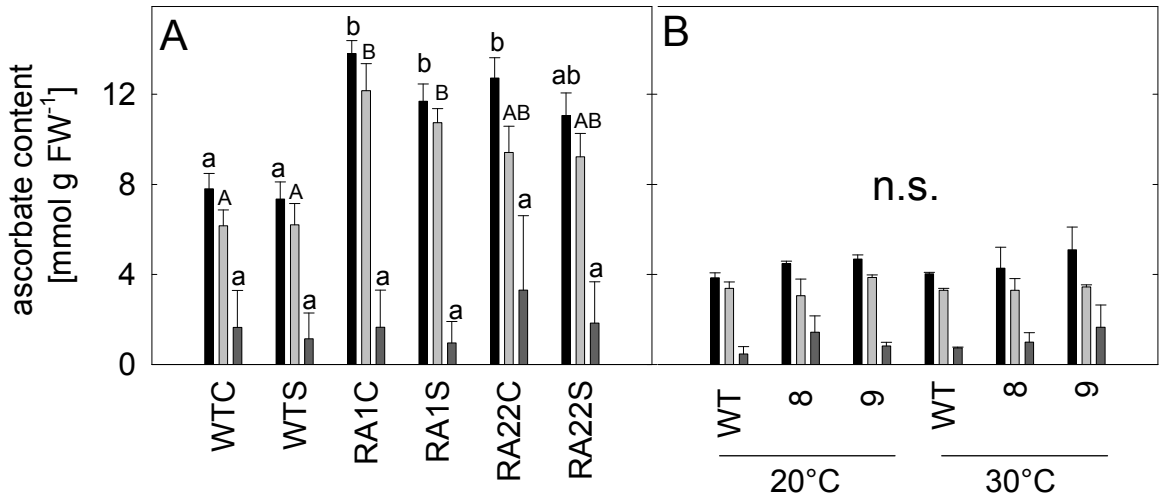

Fig. 27. Ascorbate content in transgenic and wild type Arabidopsis and Grey poplar leaves 10h after the transient thermal stress application. Total ascorbate (black bars), reduced ascorbate (light grey bars) and DHA (dark grey bars) in leaves of: (A) wild type (WT) and 2 (RA1; RA22) non isoprene emitting poplars and (B) wild type (WT) and transgenic (lines 8 and 9) Arabidopsis. For Grey poplar; S in the end of the sample name = samples faced transient light and thermal stress 10h before sampling, C in the end of the sample name = control leaves. The experiments with Arabidopsis were performed with plants grown at 20°C and with plants grown at 30°C. Mean + SE is shown, n = 5-6. Different letters above columns indicates statistically significant differences (within each color) ($P < 0.05$; ANOVA and Dunnet-T3); n.s.: no significant differences.

Similar to the ascorbate analysis no treatment effect was observed for glutathione concentrations and ratios of reduced and oxidized forms in Grey poplar leaves (Fig. 28A). In addition, neither a line effect nor a treatment or growth temperature effect was observed for Arabidopsis leaves (Fig. 28B).

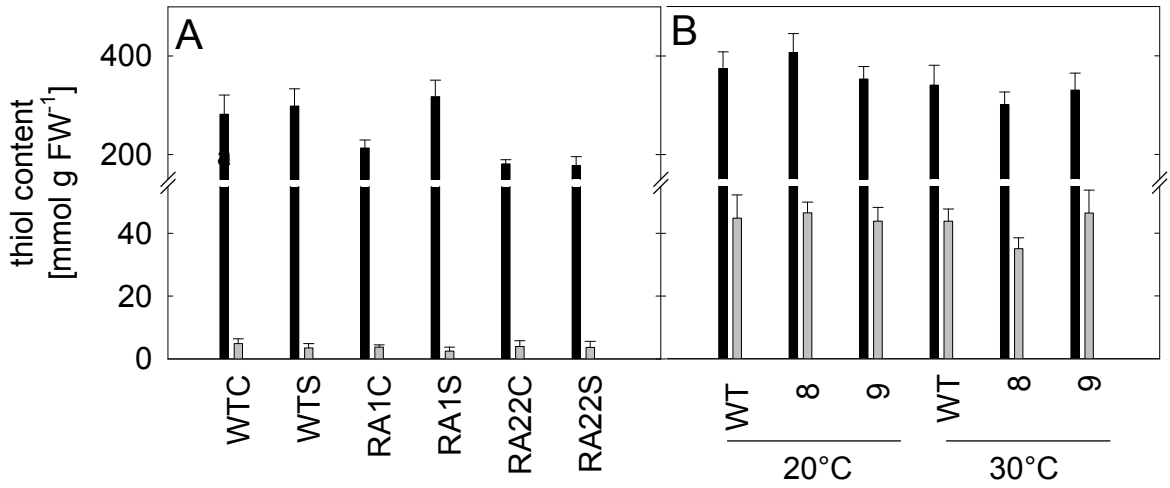

Fig. 28. Thiols content in transgenic and wild type Arabidopsis and Grey poplar leaves 10h after the transient thermal stress application. Reduced (GSH; black bars) and oxidized (GSSG; grey bars) glutathione were determined in leaves of: (A) wild type (WT) and transgenic poplar (RA1; RA22) and (B) wild type (WT) and transgenic (8; 9) Arabidopsis. For Grey poplar; S in the sample name: samples faced transient light and thermal stress 10h before sampling, C in the sample name: control leaves. The experiments with Arabidopsis were performed with plants grown at 20°C or 30°C. Mean + SE is shown, n = 5 - 6, no statistically significant differences were found (ANOVA and Dunnet-T3).

Ascorbate and glutathione are not consumed in the antioxidative cycle, but their proportion varies according to the need of antioxidative defence and has been observed to increase under stress conditions (Noctor and Foyer, 1998; Hofer et al., 2007). In the present experiments no differences between treated and control poplar leaves were observed. It might be possible that alterations in antioxidant content or redox ratios due to the temperature and light flecks were already diminished and thus not anymore detectable at the time of the measurements. Interestingly, however, the total content of ascorbate was higher in both non-isoprene emitting lines compared to wild type. It might be that in transgenic lines the antioxidant content was higher to compensate the antioxidative capacity of isoprene. However, if the plants were fully capable to compensate isoprene biosynthesis capacity no alteration in MDA should have been found (see **IV: 4-1-5-3**).

In Arabidopsis no differences in ascorbate concentrations between transgenic lines and wild type or between plants grown at 20°C or 30°C were found. It is possible that an alteration in ascorbate content was also already diminished after 10h recovery from the stress. On the other hand these data and the gas exchange analysis indicate that Arabidopsis leaves do not need extra protection in form of isoprene to recover from the combined temperature and light cycles.

4-1-5-3 Malonylaldehyde (MDA) content in Arabidopsis and poplar leaves

Malonylaldehyde (MDA) is a general indicator of lipid peroxidation state and membrane damage. The present analysis of this parameter showed that MDA in unstressed poplar leaves was not

different from that of leaves that had faced the combined light and temperature stress 10h before sampling (Fig. 29A). Striking, however, is the observation that in leaves of transgenic isoprene non-emitting poplar lines the MDA content was significantly higher than in wild type ($P < 0.05$; ANOVA and Tukey`s HSD) (Fig. 29A). Contrary to this line effect in poplar, in Arabidopsis leaves (Fig. 29B) no differences in MDA contents were found between wild type and transgenic lines or between plants grown at 20°C or 30°C. However, compared to poplar MDA concentrations in Arabidopsis were significant lower.

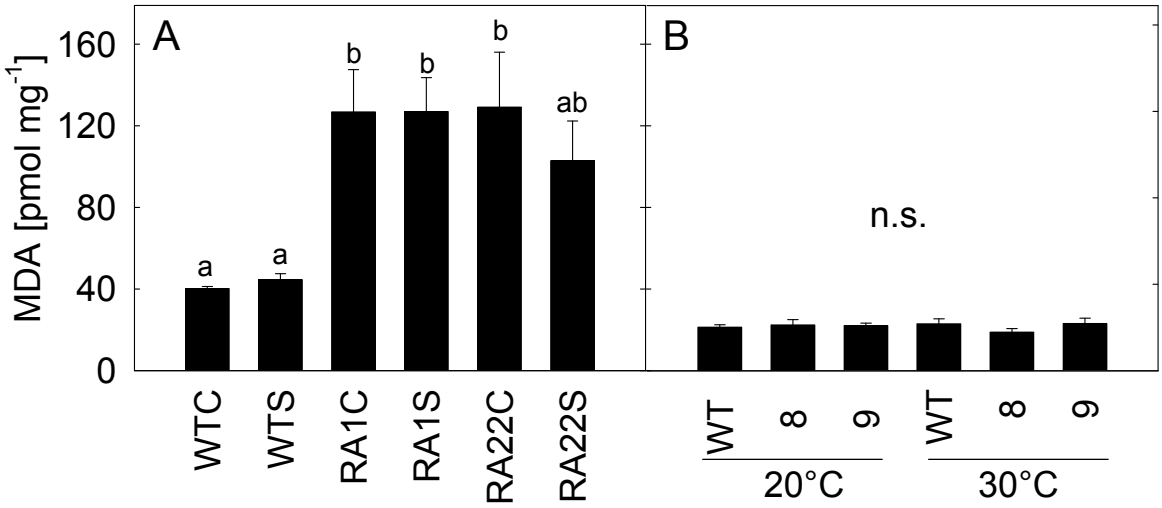

Fig. 29. Lipid peroxidation rate in Grey poplar wild type (WT) and transgenic Grey poplar lines (RA1 and RA22) (A) and in Arabidopsis wild type (WT) and transgenic lines (8 and 9) (B). For Grey poplar (A): S in the end of the sample name: samples faced transient light and thermal stress 10h before sampling; C in the end of the sample name: control leaves. For Arabidopsis (B) the experiment was performed with plants grown at 20°C or 30°C. Mean + SE is shown, n = 5-6. Different letters above columns within a graph indicate significant differences ($P < 0.05$; ANOVA and Tukey's HSD), n.s.: no significant differences.

Experiments by Velikova and Loreto (2005) demonstrated a protective effect of isoprene emission on membrane damage (measured as MDA) when heat stressed leaves of reed plants (*Phragmites australis*) were fumigated by isoprene compared to leaves that were not treated with isoprene. The present results indicate that 10h after the temperature/light flecks the lipid peroxidation state is not affected due to the applied stress in isoprene emitting or non-emitting leaves of poplar and Arabidopsis. However, the transgenic non-isoprene emitting poplar leaves had higher lipid peroxidation state initially than wild type leaves. The result suggests that the knock-down of isoprene synthase gene expression can change the oxidative status and cause higher lipid peroxidation and membrane damage in Grey poplar leaves. This observation together with higher total ascorbate content in transgenic lines (**IV: 4-1-5-2**) supports the previously suggested role for isoprene as an antioxidant (Loreto et al., 2001; Loreto and Velikova, 2001; Affek and Yakir, 2002) protecting the membranes from membrane lipid denaturation (Siwko et al., 2006). It seems that the

enhanced total ascorbate content in transgenic lines (**IV: 4-1-5-2**) was not fully able to compensate the antioxidative capacity of isoprene. Further studies should be conducted to determine why knock-down of the isoprene synthase gene results in initially higher antioxidant content and higher membrane damage in poplar leaves.

For Arabidopsis similar increase in membrane damage was not observed in wild type compared to transgenic isoprene emitting lines. Taken together the biochemical and gas exchange data (**IV: 4-1-4**) of Arabidopsis confirm that this species do not need isoprene to protect itself against short-term transient temperature or light stress. However, longer treatment of very high temperature (up to 60°C) was shown by Sasaki et al. (2007) to be stressful for the wild type Arabidopsis plants whereas the isoprene emitting transgenic Arabidopsis survived better. In future determining the MDA and antioxidant contents directly after the stress phase would give more information about the direct effect of the temperature stress and the possible role of isoprene for recovering of Arabidopsis.

5 ISOPRENE INTERFERES TRITROPHIC INTERACTIONS

5-1 Choice of parasitic wasps in Y-tube olfactometer

The isoprene emitting Arabidopsis plants were used as tools to investigate plant-insect interactions in two well-studied systems (Van Poecke and Dicke 2004; Barker et al., 2007). Firstly, the behavior of the Small Cabbage White butterfly *Pieris rapae* and its endoparasitic wasp *Cotesia rubecula* that is attracted by the volatile blend of *P. rapae*-infested Arabidopsis plants (Van Poecke et al., 2001; Van Poecke and Dicke, 2002) was investigated. As a second model system the behavior of the larval parasitoid wasp *Diadegma semiclausum* and its natural host *Plutella xylostella* (Diamondback Moth) was studied. *P. xylostella* is not commonly observed to feed on Arabidopsis, but the herb was recently shown to be a suitable host for the herbivore (Barker et al., 2007).

Throughout the experiments *D. semiclausum* preferred wild type Arabidopsis plants compared to the isoprene emitting transgenic ones. When a choice between untreated transgenic isoprene emitting and respective wild type plants were offered in Y-tube olfactometer assays, the wasps preferred the volatile blend of wild type Arabidopsis (binominal test, P=0.019, n=84). The bioassays with infested Arabidopsis plants further proved the observation: 62% of 84 responding parasitoids preferred the *P. rapae* induced VOC blend of wild type to the respective blend of transgenic isoprene emitting plants (P = 0.019). In consistence to that 60% of 82 responding parasitoids preferred the VOC blend of *P. xylostella* infested wild type to that of respectively infested transgenic plants (P = 0.048) (Loivamäki et al., 2008 (**VII: 3**)).

Cotesia rubecula, in contrast, was rather ignorant in the presence of isoprene. Although *C. rubecula* preferred volatiles of uninfested, isoprene emitting transgenic plants to uninfested wild type plants (P = 0.023, n = 74), the parasitoids showed no preference to any of the odor sources when the corresponding plants were either *P. rapae* or *P. xylostella* infested (Loivamäki et al., 2008

(**VII: 3**)). The controversial behaviors of the two parasitic wasp species are in consistence with previous results obtained by Mumm and co-workers (personal communication, Wageningen University, the Netherlands) showing that *C. rubecula* and *D. semiclausum* respond differently to plant volatile blends which differ in their isoprenoid profiles.

The effects of isoprene on the behavior of the parasitoid wasps was further examined by adding 12.5 ppbv isoprene (from an isoprene-standard with 10 ppmv isoprene in N_2) into the odor flow of the uninfested wild type Arabidopsis rosettes. The obtained volatile blend and comparable blend without external isoprene was used to observe the behavior of the parasitoids in the Y-tube olfactometer. This independent, external control gave similar results as isoprene emitting plants: again *D. semiclausum* wasps preferred the wild type plants without isoprene over those whose odor blend was supplemented with isoprene (P = 0.036) whereas *C. rubecula* showed no preference between the two odor sources (Loivamäki et al., 2008 (**VII: 3**)).

Knowing that uninfested wild type Arabidopsis do not emit large quantities of VOCs (Fig. 17; Loivamäki et al., 2008 (**VII: 3**); Chen et al., 2003) it was of interest to investigate the natural plant - herbivore - parasitoid - combination *Brassica oleracea* - *P. xylostella* - *D. semiclausum*. Similar to wild type Arabidopsis, *B. oleracea* do not emit any isoprene. The volatile blend from infested *B. oleracea* i.e. Brussels sprouts is quite more prominent than that of Arabidopsis (Chen et al., 2003; Vuorinen et al., 2004). In the experiment the effect of isoprene was tested by adding either 12.5 or 50 ppbv isoprene to the natural odor to one of the two *P. xylostella*–infested cabbage plants of the Y-tube olfactometer simulating a ‘low’ and ‘high’ isoprene concentration in the environment. When the plant volatile mixture was enriched with 50 ppbv isoprene, *D. semiclausum* preferred the odor source without isoprene (binominal test, P = 0.029), however, 12.5 ppbv enrichment did not influence the choice of parasitic wasps (Fig. 30).

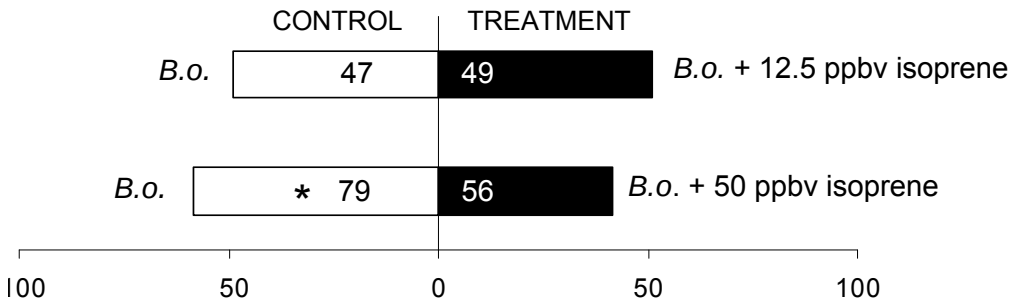

Fig. 30. Response of naïve *Diadegma semiclausum* females to volatiles released by wild type *P. xylostella*-infested *Brassica oleracea* (*B.o.*) in a Y-tube olfactometer. The isoprene concentrations indicate the concentration added to one of the Y-tube hands. Bars represent the overall percentages of wasps choosing either of the odor sources, numbers in bars are the total numbers of wasps choosing that odor source. Choices between odor sources were analyzed with binominal test (*;$P < 0.05$).

An explanation for this dose-dependent insect response might be that the low isoprene concentration becomes masked in the VOC blend of *P. xylostella* infested white cabbage whose mono- and sesquiterpenes blend is approximately 100 fold higher (0.15 - 0.25 µg h^{-1} 100 cm^{-2}) (Vuorinen et al., 2004) than that of Arabidopsis rosettes (maximum 0.0015 µg h^{-1} $plant^{-1}$ (Chen et al., 2003), one average rosette is approximately 50 - 100 cm^2). Therefore, 12.5 ppbv isoprene might not affect the response of the wasps to the volatile blend of *P. xylostella*-infested Brussel sprouts, whereas it does so when mixed with the odor from infested Arabidopsis plants. Given that isoprene emission by poplar leaves can result in concentrations up to 100 ppbv close to the emitting leaves (Behnke et al., 2007), the isoprene doses used here are realistic. Once emitted, plant volatiles become rapidly diluted. However, atmospheric isoprene concentrations up to 12 ppbv are possible within mixed forest canopies with a high proportion of isoprene emitters (Fuentes et al., 2007).

The parasitoid-repellent effect of isoprene in tritrophic interactions is surprising, since higher terpenes such as monoterpenes, homoterpenes and sesquiterpenes are rather observed to function as attractants than repellents to herbivore-enemies (Kappers et al., 2005; Schnee et al., 2006; Mumm et al., 2008). To answer the question why isoprene repels *D. semiclausum* is not simple. In fact the function of isoprene in the environment may not only include the host plant, host and parasitoid, but also a neighboring isoprene emitting species. The research on tritrophic interactions so far has mainly focused on isolated systems without including the effects of background volatiles (but see Kessler et al., 2004; Gols et al., 2005; Mumm and Hilker, 2005). The present results warn that orientation of plants bodyguards might differ in the fields and forests sites

(in which herbivores host plants are surrounded by isoprene emitters) from that observed in laboratories. At least it seems unprofitable for a cruciferous plant to place its roots under a poplar tree as the present results indicate that herbaceous plants which do not emit isoprene are compromised in indirect defence when they are in an isoprene rich environment.

5-2 Electrophysiology shows that insect antennae recognize isoprene

Isoprene evoked electroantennographic (EAG) responses in the antennae of *D. semiclausum* parasitoid females in a dose-dependent manner reaching a significant threshold when 1% (v/v) isoprene was applied in hexadecane (Wilcoxon's signed ranks test, $P < 0.05$). Higher concentrations of isoprene (10%) evoked higher response in the insect antennae than lower concentration (0.1%) ($P < 0.05$) (Loivamäki et al., 2008 (**VII: 3**)). The experiment clearly shows that the olfactory receptor neurons in the olfactory lobe of the wasp's insect antennae recognize isoprene and respond to it. The result is the mechanistic evidence that the clear behavioral responses of the wasp in the y-tube olfactometers assays to isoprene have a basis in the chemosensory apparatus of the antennae. Given that isoprene concentrations in atmosphere are higher than those of mono- or sesquiterpenes (Guenther et al., 1995), the ability to recognize isoprene can have bigger consequences on plant-insect interactions than so far understood.

5-3 Herbivore performance on isoprene emitting Arabidopsis

Although the behavior of the larval parasitoid wasp *D. semiclausum* was affected by isoprene emission of Arabidopsis, the performances of its herbivore host *P. xylostella* and that of *P. rapae* were not affected. After five days (*P. xylostella*) or one week (*P. rapae*) of infestation and feeding on either wild type or transgenic Arabidopsis plants the larvae of both species had gained equal weights. Moreover, when the larvae were given a free choice in a cafeteria test to feed either on wild type or on transgenic *Arabidopsis* leaves they did not prefer either plant type in the beginning, 0.5h or 2h after the beginning of the experiment. Nevertheless, *P. xylostella* preferred to feed on transgenic-plants at one time point: 1h after the beginning of the experiment ($P < 0.05$, Loivamäki et al., 2008 (**VII: 3**). Moreover, the overall tendency in the experiments investigating *P. xylostella* behavior showed that the herbivore may prefer to eat on isoprene emitting transgenic Arabidopsis than on wild type plants.

However, ovipositing *P. rapae* females did not discriminate between wild type and isoprene emitting transgenic plants: when 81 butterfly females were allowed individually a choice between transgenic and wild type Arabidopsis, they laid on average 22.7 ± 1.8 eggs on WT and 22.9 ± 1.6 eggs on transgenic plants in 4 h, respectively (Loivamäki et al., 2008 (**VII: 3**)).

In conclusion, the present results suggest that high isoprene concentration in the air indeed gives an advantage to herbivores over their parasitoids. In isoprene rich environment parasitic wasps

searching for hosts might be misled (**IV: 5-2**), whereby herbivores' feeding seems not to be disturbed. Therefore plant-herbivore-parasitoid interactions are dependent on so far overlooked environmental aspects, such as shown here for isoprene. There is a need to investigate the role of isoprene in tritrophic interactions of a real isoprene emitter, e.g. poplar or willow in the future. For studying the plant-insect interactions within poplars, the existing isoprene knock-down lines, here used for studying temperature and light effects, represent an interesting tool.

V CONCLUDING SUMMARY AND OUTLOOK

Several new findings in the field of isoprene emission research were achieved within the frames of this thesis:

Firstly, it could be shown that gene expression of isoprenoid genes and isoprene emission is regulated in a circadian manner, emphasizing the importance of isoprene emission capacity for its emitting species. The investigations proved that the previously detected diurnal rhythms of *PcISPS*-gene (Mayrhofer et al., 2005) are not only due to day/night changes, but also linked to the internal circadian clock. Moreover, the isoprene emission rate under continuous irradiation displayed circadian changes. However, no fluctuations were observed on PcISPS protein and enzyme activity levels even if both appeared to become reduced under constant darkness, while under constant light the protein level and activity was high. The result suggests that post-transcriptional regulation for isoprene exists. It is surprising that the first level of regulation of isoprene emission, namely the expression rate of the isoprene synthase gene, and the emission itself present circadian rhythms, when neither the PcISPS protein level nor its activity display significant diurnal or circadian variations. Therefore, the observed fluctuations of emission do not seem to be due to fluctuating *PcISPS* transcription rate.

Measurement of other isoprenoid genes revealed that carotenoids related *PcPSY* transcription rate displays circadian fluctuations whereas *PcDXR,* the possible first committed step of the MEP-pathway, does only show a light regulation. Gene expression of *PcDXR* and *PcISPS* are not synchronized which raises the question about the pool of DMADP, substrate of isoprene. Interestingly, DMADP pools were previously shown to fluctuate diurnally in different species (Fisher et al., 2001; Brüggemann and Schnitzler 2002b), including poplar (Magel et al., 2006; Nogués et al., 2006). Assuming that photosynthetic activity undergoes to a certain extent also a circadian change it may be hypothesized that the circadian change of isoprene emission is due to a slightly enhanced metabolic flux within the MEP-pathway. Indeed it is well documented that numerous genes and proteins involved in photosynthesis are clock regulated, as is fixed carbon allocation itself (Harmer et al., 2000). As recently fixed carbon is the major pool used to produce isoprene in poplar (Schnitzler et al., 2004), isoprene emission variations under continuous light may result from circadian fluxes of fixed carbon into the MEP-pathway. In future experiments $^{13}CO_2$ feeding could help to prove whether the carbon supply rate results in similarly fluctuating rate of isoprene emission in poplar. However, the substrate supply could also be regulated by competition of PEP between cytosolic and chloroplastic processes, as was suggested by Wilkinson et al. (2006). Thus, in which manner the PEP supply in chloroplasts occurs should be investigated in more detail. Moreover, in the future it should be studied how widespread the circadian fluctuating isoprene emission is in the plant kingdom in order to take this phenomenon into account when producing global scale isoprene emission inventories.

Secondly, the studies performed within this thesis proved that isoprene emission capacity can be successfully transformed in a natural non-emitter. However, the emission rates did not reach the level of natural isoprene emitters suggesting possible different regulation of the isoprene biosynthesis and/or lack of substrate. Even if the isoprene emission was low, over-expression of poplar *ISPS* in Arabidopsis resulted in isoprene emitting Arabidopsis plants that show enhanced growth rates compared to wild type under thermal stress. The fact that highest growth rates, higher DMADP levels and isoprene synthase enzyme activities were detected in young developing plants indicates that enhanced growth of the transgenic plants under thermal stress is due to the introduced *PcISPS* gene.

Dynamic measurements of photosynthetic gas-exchange of Arabidopsis applying long-term or transient cycles of heat and light stress to wild type and transgenic plants indicate that Arabidopsis does not need isoprene to protect net assimilation from thermal or light stress. Indeed, Arabidopsis is already well enough thermotolerant and does not need isoprene under natural or close to natural conditions. To find a biochemical explanation for the findings of Sasaki et al. (2007), who showed isoprene protecting against very high thermal stress, more detailed experiments are necessary. In addition, to find a reason for the observed higher growth rate of the transgenic plants compared to wild type under high temperature conditions further biochemical experiments are needed. It will for example be interesting to study whether the higher growth rates were due to an indirect effect of introduced *ISPS* gene, i.e. higher substrate supply from MEP-pathway for plant hormones like ABA and gibberellin (Barta and Loreto, 2006) as was suggested by Sasaki et al. (2007).

Temperature and light cycles applied to leaves of transgenic non-isoprene emitting Grey poplar showed that in a natural isoprene emitter the ability to emit isoprene is crucial to maintain photosynthesis under stress. The results suggest that reduction of net assimilation was primarily due to cycling temperature as cycling light alone did not affect the gas-exchange of the studied lines. However, the results should not be directly transferred to other species or be considered as a general phenomenon: even if isoprene can be essential under temperature and light stress for its natural emitter, for other species under other environmental conditions isoprene may present different advantage, e.g. higher growth rate in the case of Arabidopsis. In addition more natural studies of the protective role of isoprene under and after sunflecks should be conducted. It would be interesting e.g. to bring the poplar plants outside and investigate whether the wild type poplar photosynthetic machinery is more flexible than that of an *ISPS* knock-down poplar when sunshine and shadow change fast.

Isoprene emission from transgenic Arabidopsis is very low probably due to substrate limitation as indicated by DOX-feeding. If the metabolic flux through the MEP-pathway was enhanced by over-coming some of the limiting steps on the MEP-pathway the isoprene emission level from transgenic Arabidopsis would probably be higher. Over-expressing *DXS* (Estévez et al., 2001),

DXR (Carretero-Paulet et al., 2006) or *HDR* (Botella-Pavía et al., 2004) genes could lead to higher substrate supply for isoprene biosynthesis. In the frame of this thesis *PcDXR* was transformed into Arabidopsis in order to later cross *PcDXR* and *PcISPS* transformed Arabidopsis plants and thus obtain "a super isoprene emitter". However, even when the transformation was successful the phenotypes of the selected lines did not show significant increases on the level of carotenoid end-products.

Thirdly, the investigations with the isoprene emitting Arabidopsis plants add a new ecophysiological component to the previously proposed biological roles of isoprene. It could be demonstrated that isoprene is perceived by a parasitic wasp's (*D. semiclausum*) chemoreceptors and interferes with its attraction to volatiles from herbivore-infested plants. The repellent effect on parasitic wasps was verified by adding external isoprene to the volatile blend of wild type plants. In contrast, the performance of two herbivores (*P. rapae* and *Pl. xylostella*) was not affected by isoprene emission. The results indicate that isoprene emitting plants, like many tree species, might have to "trade-off" with higher herbivore infestation for gaining a protection against e.g. thermal or oxidative stress (Sharkey and Singsaas, 1995; Loreto and Velikova, 2001; Behnke et al., 2007). Moreover herbaceous plants that do not emit isoprene may be compromised in indirect defence when they are in an environment with isoprene emitters. The results indicate that orientation of 'plant bodyguards' may indeed differ in open field and forest sites from what observed in laboratory. How common is isoprene recognition in the nature and what are its real roles for the plant itself but also for insect species needs urgently to be elucidated. Future work should focus on the role of isoprene in tritrophic interactions of a real isoprene emitter, using e.g. the existing isoprene emission knock-down Grey poplar lines. It might be that the very common defoliation of isoprene emitting trees, e.g. poplar trees by gypsy moths (*Lymantria dispar*) (Russell et al., 2004), could be related to the repellent nature of isoprene for parasitoids/predators benefiting to the herbivores.

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VII PUBLICATIONS

Publikation (1)

LOIVAMÄKI M, LOUIS S, CINEGE G, ZIMMER I, FISCHBACH RJ, SCHNITZLER JP (2007)

Circadian rhythms of isoprene biosynthesis in grey poplar leaves.

Plant Physiology **143**: 540-551

http://www.plantphysiol.org/cgi/content/abstract/143/1/540

Publication (2)

LOIVAMÄKI M, GILMER F, FISCHBACH RJ, SÖRGEL C, BACHL A, WALTER A, SCHNITZLER JP (2007)

Arabidopsis, a model to study biological functions of isoprene emission?

Plant Physiology **144**: 1-13

http://www.plantphysiol.org/cgi/content/abstract/144/2/1066

Publication (3)

LOIVAMÄKI M, MUMM R, DICKE M, SCHNITZLER JP (2008)

Isoprene interferes with the attraction of bodyguards by herbaceous plants

Proc Natl Acad Sci USA **105**: 17430-17435

http://www.pnas.org/content/105/45/17430.abstract

VIII ATTACHMENT

Attachment 1: Calculation of isoprene synthase activity from GC data

*Calculation of isoprene synthase activity from GC data

**********!!!!enter variables!!!!!**********

**********1. calculation!!!!! (calculate one time and then exchange values in the spreatsheet)***

*********Freshweight [g] and projected lea area [cm2] and Protein in [mg/ml) of the leaf material used*******

la_blatt: projected leaf area**

```
COMPUTE la_blatt = 43.72.
EXECUTE.
```

fg_blatt: fresh weight****

```
COMPUTE fg_blatt = 1.
EXECUTE.
```

fresh weight extracted in PEB*

```
COMPUTE fg = 0.35.
EXECUTE.
```

```
COMPUTE protein1 = 0.553.
EXECUTE .
COMPUTE protein2 = 0.554 .
EXECUTE .
COMPUTE protein3 = 0.555.
EXECUTE .
```

*********correction factor for total leaf area****

```
COMPUTE la_fakt = 2.00 .
EXECUTE.
```

*********!!!!GC-calibration [in µV*sec/ppm] ADD allways the actual calibration**********

```
COMPUTE m = 220 .
EXECUTE.
```

**********!!!!background values of isoprene formation from DMADP ADD allways the actual values (in µV*sec]**********

```
COMPUTE blind = 22.6.
EXECUTE.
COMPUTE blindsd = 2.19.
EXECUTE.
```

```
**********************************************************************************************************

**********2. calculation step!!!!!(only calculate once)**************************************************


COMPUTE tla_fg = la_blatt*la_fakt*0.0001 / fg_blatt .
EXECUTE.

COMPUTE la = tla_fg * fg .
EXECUTE.

**********Protein in kilogram*****************************************************************************


COMPUTE protein4 = protein1 / 1000 .
EXECUTE .
COMPUTE protein5 = protein2 / 1000 .
EXECUTE .
COMPUTE protein6 = protein3 / 1000 .
EXECUTE .

COMPUTE protein = MEAN(protein4,protein5,protein6).
EXECUTE.
COMPUTE protsd = SD(protein4,protein5,protein6).
EXECUTE.
**********************!!!!!Extraktion volume (PEB) at the beginning [in
mL]*****************************

COMPUTE peb1 = 4.0 .
EXECUTE.

*****!!!!volume of protein extract applied to the PD10-column (after centrifugation) [in mL]???***

COMPUTE peb2 =  2.5 .
EXECUTE.

***************!!!!elutuion volume of the PD10-column [in mL]???**************************

COMPUTE isb1 = 3.5 .
EXECUTE.

***************!!!!volume of protein extract in the assay [in mL]???**********************

COMPUTE isb2 =  0.088 .
EXECUTE.

***************!!!!which assay time [in Sekunden]?????*********************************************

COMPUTE test =  7200 .
EXECUTE.
```

**********CALCULATION**********

********calculation of GC raw data from the enzymatic reaction (subtraction of background)**********

```
COMPUTE fe_dif = (fe_mwis - blind).
EXECUTE.
COMPUTE fe_difsd = fe_dif * sqrt((fe_sdis/fe_mwis)**2 + (blindsd/blind)**2).
EXECUTE.
```

*****calculation of isoprene concentration in the head space in the GC-Vial in ppm***

```
COMPUTE ppmvial = fe_dif / m .
EXECUTE .
COMPUTE ppmsd = fe_difsd / m .
EXECUTE .
```

******calculation of isoprene amount in the vial**********

```
COMPUTE nmolvial = ppmvial * 1.944 / 22.414 .
EXECUTE .
COMPUTE nmolsd = ppmsd * 1.944 / 22.414 .
EXECUTE .
```

******calculation of isoprene synthase activity per total leaf area**********

```
COMPUTE nmolm2s = nmolvial / (test * la) * (isb1 / isb2) * (peb1 / peb2) .
EXECUTE .
COMPUTE nmolm2sd = nmolsd / (test * la) * (isb1 / isb2) * (peb1 / peb2) .
EXECUTE .
```

******calculation of specific enzyme activity in µmol/s/kg total protein **********

```
COMPUTE spezakt = nmolvial / (test * protein * isb2) .
EXECUTE.
COMPUTE spezaksd = spezakt * sqrt((nmolsd/nmolvial)**2 + (protsd/protein)**2) .
EXECUTE.
```

XI ACKNOWLEDGEMENTS

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Kaikkein suurimmat kiitokset ansaitsee perheeni, äiti, isä ja Tommi, Mari sekä rakas Philip.

Garmisch-Partenkirchen, April 2008

Maaria Loivamäki