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ISOPRENE: FROM THE CELL TO THE ENVIRONMENT

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Abstract

One of the most relevant aspects reported in the last Intergovernmental Panel on Climate Change is represented by the projections of global warming as well as the consequent possible effect of rising temperature on ecosystems. The expected negative impacts of global climate change makes it very important to generate crop and forest plants that will have enhanced resistance and tolerance to environmental stress conditions and, in particular, to the combined stress caused by sunlight, and rising temperature and drought. Drought and high temperatures are often concurrent factors in nature, and the combined effect of these two environmental constraints should be determined. Moreover, current global change models predict that lower frequency of rainfall days and longer dry intervals associated with atmospheric warming will affect large areas of the globe. Thus, it is of paramount importance to study the acclimatory responses of plants to the interactive effects between water stress and rising temperature.

Drought and temperature represent the main rising variables of the global warming, which could affect not only the plant response to environmental stress conditions, but also the atmospheric chemistry. This is particularly true if we apply it to the forest ecosystems. Terrestrial vegetation, especially tropical rain forest, releases vast quantities of carbon as volatile organic compounds (VOC) into the atmosphere of which the most important is isoprene. The emissions of these volatile isoprenoids are strictly dependant on environmental

factors and they are highly reactive with correspondingly short lifetimes. Volatile isoprenoids can be removed by oxidation reactions; this reactions chain is mainly initiated by hydroxyl radicals (OH), primarily formed through the photo-dissociation of ozone. The consequence of forest degradation, especially in the Amazon region, would be to reduce the VOC emissions, with a subsequent impact on ozone levels at the surface. As pointed out by Betts et al. (2008) “Isoprene emissions have a significant impact on the projected future surface ozone levels. Ignoring vegetation changes has meant that future simulated ozone levels were greater by 5-10 ppbv, owing to larger isoprene emissions. This may have implications for air quality in the region, with potential implications for the health of humans, animals, ecosystems and crops”. All together, these considerations reveal the possible impact of volatiles isoprenoids, and in particular isoprene, on the ecosystems, especially on forest ecosystems.

These metabolites are involved in numerous biological processes, such as electron transport, photosynthesis, hormonal regulation, membrane fluidity, and plant defense responses, or communication with other organisms. *In planta*, isoprene is considered an important molecule for ameliorating abiotic stresses. Isoprene may increase thermotolerance particularly when leaves are exposed to transiently high temperatures. Despite the large convincing experimental evidences, the mechanism by which isoprene exerts its protective action is not completely understood. The impact of drought on isoprene emission has also been studied, since drought is a major limitation for plant growth worldwide, and the intensity and frequency

of drought conditions is going to increase with current and future climate change. The available evidence suggests that isoprene biosynthesis is relatively unaffected by drought. Episodes of severe drought stress may lead to suppression of photosynthesis, while isoprene emission is only slightly reduced.

While numerous isoprenoids are formed in and emitted by leaf chloroplasts, where they likely exert their protective action, plants also emit volatile compounds from their flowers. The (*E*)-β-caryophyllene synthase (TPS27) catalyzes the formation of (-)-(*E*)-β-caryophyllene the major sesquiterpene emitted from Arabidopsis floral tissues. It has been hypothesized that (*E*)-β-caryophyllene can play a role in the attraction of floral visitors, leading to low levels of cross-pollination and increasing reproductive fitness in natural populations. However, given the low levels of volatile emission, it is quite possible that the (*E*)-β-caryophyllene biosynthesis have a function besides, or in addition to, pollinator attraction. Though recent studies support that constitutive (e.g. isoprene, monoterpenes and sesquiterpenes) terpene volatiles released from vegetative tissues of different plant species exposed to abiotic stress could serve as mediators of thermotolerance or in protecting cells against oxidative stress, there is limited information available about the possible roles of sesquiterpenes in the physiological response of plants to environmental stress conditions.

Many questions about the biological functions of isoprenoids are still open. Even when considering the large amount of studies on the ecological and physiological impact of the isoprenoids release into

the atmosphere from vegetation, there is only partial and incomplete information about function, biosynthesis, and regulation of these compounds in the plant cell. It is important to fill this “gap” especially when considering the important role played by these compounds in the defense system against environmental stress. In particular isoprene seems to protect leaves from high temperature and oxidative damages. Given the specific and active function of isoprene in the plant defense system, it is expected that isoprene biosynthesis be specifically and strongly regulated. The goal of this work is to provide an answer to some of these points not yet clarified. Using different plant species and different experimental approaches we will try to elucidate the regulation of isoprene function in the nature; it will be shown that isoprene is able to enhance thermo-tolerance also in plants which naturally do not emit isoprene (like Arabidopsis), and that this protective role is not only directly played, scavenging ROS excess or helping membrane stability, but has also an indirect component, priming the defense system against thermal and oxidative stress. New biophysical evidences supporting the hypothesis that isoprene may also be able to directly interact with chloroplastic membranes increasing their stability during the stress are also presented.

Similarly to isoprene, many questions regarding another class of isoprenoids, the sesquiterpenes, are still open. Genetically engineered model plant species, such as *Arabidopsis thaliana*, overexpressing enzymes that regulate sesquiterpene biosynthesis in plants, may constitute a suitable tool to study the physiological role of

sesquiterpenes in plants. Following this line, a characterization of the plants overexpressing the *TPS27* gene will be shown, and how those plants respond to environmental stress, compared to wild-type plants, will be assessed. We used this tool to demonstrate that (*E*)-β-caryophyllene is able to enhance plant thermotolerance, and also that this protective role is triggered by similar mechanisms to those suggested for isoprene. Finally, all together these results reveal a pattern that could be assimilated to that also obtained with the Arabidopsis plants transformed to overexpress the isoprene synthase gene. We can surmise that all volatile isoprenoids exert a similar protective action increasing plant resistance to high temperatures. Looking beck the results obtained with isoprene and (*E*)-β-caryophyllene, looking on the recent variety of works on these and other isoprenoids, and also on the evolutionary history of isoprenoid biosynthesis, it is possible that plants emit volatile compounds, as a part of the secondary metabolism, combining them to release a bland of molecules with specific functions, independently from the plant species. Plants have been developed evolving their capacity to regulate more and more finely the biosynthesis of even more specific family of isoprenoids to better respond to specific environmental. Therefore, filogenetically distant compounds, like isoprene (an hemiterpene) and (*E*)-β-caryophyllene (a sesquiterpene), synthesized and emitted by the same plant produce similar effect, showing the same biological function of thermal protection. This ‘merging’ hypothesis brings beck the explanations, not only the thermo-tolerance, for the isoprenoid emission to a

unique multiple biological function: protection of plants against the environmental stress conditions, and enhancement of the plant fitness. The ‘merging’ hypothesis overlap isoprenoids coming from different plant species suggesting that the biosynthesis and utilization or not of a specific volatile compound in two different species should not be attributable to the fact that this compound may be or not ‘waste of carbon’, but because that plants have evolved in a different way, adapting their emission pattern to different environments, and, consequently, selecting different isoprenoids to respond to similar phenomenon.

Abstract – Italian version

Uno degli aspetti più rilevanti riportato nell'ultimo IPCC è rappresentato sia dalle proiezioni sul riscaldamento globale come dal possibile effetto sugli ecosistemi dell'innalzamento della temperatura. L'atteso impatto negativo del cambiamento climatico globale evidenzia l'importanza di poter generare piante d'interesse agro-alimentare e forestale in grado di tollerare condizioni più aspre di stress ambientale, in particolare dagli effetti combinati di alta temperatura e siccità. Tali fenomeni, in natura, sono spesso interconnessi, e i loro effetti dovrebbero essere attentamente monitorati. Oltretutto, è da tenere in considerazione il fatto che gli attuali modelli sui cambiamenti climatici predicono che la compartecipazione tra la riduzione della frequenza di eventi piovosi e l'allungamento degli intervalli di siccità, associati al riscaldamento atmosferico, potrebbero colpire vaste aree del globo. Ciò considerato, è di primaria importanza studiare la risposta delle piante agli effetti interattivi dello stress idrico e delle alte temperature.

Siccità e temperatura rappresentano, infatti, le variabili descriventi il riscaldamento globale che più delle altre potrebbero incrementare, e il cui incremento colpirebbe non solo la risposta delle piante a condizioni di stress, ma anche la chimica dell'atmosfera. Questa previsione è particolarmente vera se applicata agli ecosistemi forestali. La vegetazione terrestre, specialmente le foreste pluviali tropicali, rilasciano nell'atmosfera larghe quantità di carbonio sotto forma di composti volatili organici (VOC), il cui maggior

rappresentante è l'isoprene. L'emissione di questi composti è strettamente legata a fattori ambientali, ed essi sono tanto rettivi quanto hanno una ridotta emivita. Gli isoprenoidi volatili possono essere rimossi da reazioni di ossidazione, le quali sono principalmente iniziate da radicali idrossilici (OH), di solito formati dalla foto-dissociazione dell'ozono. Una conseguenza della deforestazione, specialmente nella regione amazzonica, potrebbe essere una ridotta emissione di VOC, con un conseguente impatto sui livelli superficiali di ozono. Come evidenziato da Betts et al. (2008) <Le emissioni di isoprene hanno un impatto significativo sulle proiezioni future dei livelli superficiali di ozono. Ignorare i cambiamenti della vegetazione ha significato che i livelli di ozono in seguito simulati furono superiori di circa 5-10 ppbv, a causa delle maggiori emissioni di isoprene. Questo può avere implicazioni sulla qualità dell'aria in quella regione, con implicazioni potenziali sulla salute di uomini, animali, ecosistemi e raccolti>. Tali considerazioni ci rivelano il possibile impatto degli isoprenoidi volatili, in particolare l'isoprene, sugli ecosistemi, in particolar modo su quelli forestali.

Questi metaboliti sono coinvolti in numerosi processi biologici, quali il trasporto elettronico, la fotosintesi, la regolazione ormonale, la fluidità di membrana, i sistemi di difesa delle piante, come anche la comunicazione con altri organismi. Nelle piante l'isoprene in particolare è considerato un'importante molecola nel migliorare la risposta a stress abiotici. L'isoprene, infatti, può incrementare la termo-tolleranza, in particolare quando le foglie sono esposte a transienti periodi di alte temperature. A dispetto delle numerose e

convincenti evidenze sperimentali a supporto di tale ipotesi, il meccanismo con cui l'isoprene esercita il suo ruolo protettivo non è stato ancora del tutto chiarito. Anche l'impatto della siccità sull'emissione d'isoprene è stato affrontato, considerando che tale stress rappresenta uno dei maggiori fattori limitanti lo sviluppo delle piante nel mondo, e che l'intensità e la frequenza di condizioni di siccità andrà a incrementare in risonanza all'attuale e futuro cambiamento climatico globale. Le attuali conoscenze suggeriscono che la sintesi d'isoprene non sia influenzata dalla siccità; cicli di forte stress idrico, che possono indurre una notevole diminuzione della fotosintesi, riducono solo parzialmente l'emissione d'isoprene.

Sebbene numerosi isoprenoidi siano formati ed emessi dai cloroplasti a livello fogliare, dove essi esercitano la loro azione protettrice, altri composti volatili, come i sesquiterpeni, sono emessi anche dagli organi floreali. L'(*E*)-β-cariofillene sintasi (TPS27) catalizza la formazione del (-)-(*E*)-β-cariofillene, il maggior sesquiterpene emesso dall'apparato floreale di Arabidopsis. È stato ipotizzato che l'(*E*)-β-cariofillene possa giocare un ruolo rilevante nell'attrarre insetti, contribuendo al mantenimento di un basso livello di impollinazione incrociata ed incrementando la fitness riproduttiva nelle popolazioni naturali. Tuttavia, considerando i bassi livelli di emissione degli isoprenoidi volatili, è possibile che la biosintesi di (*E*)-β-cariofillene possa avere una funzione nella o in aggiunta all'attrazione d'insetti impollinatori. A dispetto dei recenti studi a supporto dell'idea che la sintesi di terpeni volatili costitutivi (come isoprene, monoterpeni e sesquiterpeni) rilasciati dai tessuti vegetativi

di differenti specie vegetali esposte a stress abiotico, possa mediare la termo-tolleranza o la protezione delle cellule dallo stress ossidativo, ci sono solo limitate informazioni disponibili sui possibili ruoli dei sesquiterpeni nella risposta fisiologica delle piante a condizioni di stress ambientale.

Molte delle questioni riguardanti le funzioni biologiche degli isoprenoidi sono ancora aperte. Pur considerando la cospicua mole di studi fatti sull'impatto ecologico e fisiologico del rilascio in atmosfera degli isoprenoidi dalla vegetazione, attualmente è disponibile solo una parziale ed incompleta informazione riguardo la funzione, la biosintesi e la regolazione di tali composti a livello cellulare. Sarebbe importante poter riempire questo "gap" specialmente considerando il ruolo primario giocato da questi composti nel sistema difensivo delle piante contro stress ambientali. Particolarmente rilevante sembra essere, infatti, il ruolo dell'isoprene nel proteggere le foglie dai danni provocati dalle alte temperature e da condizioni di stress ossidativo. Data la specifica e attiva funzione dell'isoprene nel sistema difensivo delle piante, è ipotizzabile che la sintesi dell'isoprene sia regolata in modo altamente specifico. L'intento di questo lavoro è di fornire una risposta ad alcuni di questi punti ancora non del tutto chiariti. Utilizzando differenti specie vegetali e differenti approcci sperimentali abbiamo cercato di far luce sulla regolazione della funzione dell'isoprene in natura. Sarà mostrato che l'isoprene è in grado di incrementare la termo-tolleranza anche in piante che naturalmente non lo emettono (come piante di *Arabidopsis thaliana* ingegnerizzate per esprimere il gene

dell'isoprene sintasi). Sarà dimostrato che tale ruolo protettivo non è giocato solo direttamente, detossificando l'eccesso di specie reattive dell'ossigeno (ROS) o aiutando la stabilità di membrana, ma anche in modo indiretto, attivando, già prima dell'accorrere di uno stress, il sistema di difesa specifico contro alte temperature e stress ossidativo. Saranno presentate inoltre nuove evidenze biofisiche a supporto dell'ipotesi che l'isoprene può essere in grado di interagire direttamente con le membrane cloroplastiche incrementandone la stabilità durante lo stress.

Come per l'isoprene, molte domande su di un'altra classe d'isoprenoidi, i sesquiterpeni, sono ancora senza risposta. L'uso di specie modello geneticamente modificate, come Arabidopsis, per esprimere in modo costitutivo gli enzimi che regolano la biosintesi degli isoprenoidi nelle piante, può rappresentare un valido strumento d'indagine del ruolo fisiologico anche dei sesquiterpeni vegetali. Seguendo questo percorso, sarà presentato in questo lavoro una caratterizzazione di piante di Arabidopsis ingegnerizzate per esprimere in modo costitutivo il gene *TPS27*, e come tali piante rispondono agli stress ambientali rispetto a piante selvatiche. Abbiamo utilizzato questo strumento per dimostrare che (*E*)-β-cariofillene è in grado di incrementare la termo-tolleranza delle piante, ed anche che questo ruolo protettivo è innescato da meccanismi simili a quelli suggeriti per l'isoprene. Infine, l'analisi d'insieme dei risultati ottenuti, ci ha suggerito la possibilità che ci possa essere un percorso tanto funzionale quanto regolativo assimilabile a quello attenuto analizzando i dati raccolti con le piante

di Arabidopsis ingegnerizzate per esprimere il gene dell'isoprene sintasi. Possiamo quindi ipotizzare che tutti gli isoprenoidi volatili esercitino un'azione protettiva simile, incrementando la protezione contro le alte temperature. Ripercorrendo i risultati ottenuti con l'isoprene e (*E*)-β-cariofillene, guardando anche ai recenti lavori pubblicati su questi e altri isoprenoidi, ed infine analizzando anche la storia evolutiva della sintesi degli isoprenoidi, risulta possibile, o quantomeno ipotizzabile, che le piante emettano composti volatili, come parte del loro metabolismo secondario, combinandoli per rilasciare una miscela di molecole con specifiche funzioni, indipendentemente dalla specie vegetale. Le piante si sarebbero evolute sviluppando la loro capacità di regolare via via più finemente la biosintesi di una sempre più specifica famiglia d'isoprenoidi, per rispondere sempre meglio a specifiche condizioni ambientali. Oltretutto, composti filogeneticamente distanti come l'isoprene (un emiterpene) e (*E*)-β-cariofillene (un sesquiterpene), sintetizzati ed emessi dalla stessa pianta, producono effetti simili, mostrando la stessa funzione biologica termo-protettrice. Questa ipotesi "concatenante" lega insieme le spiegazioni, non solo quella della termo-tolleranza, per spiegare l'emissione d'isoprenoidi verso un'unica molteplice funzione biologica: protezione delle piante da condizioni di stress ambientale e incremento della loro fitness. L'ipotesi "concatenante" racchiude gli isoprenoidi provenienti da differenti specie vegetali, suggerendo che sia la sintesi quanto l'uso o meno di uno specifico composto volatile da parte di due specie diverse non dovrebbe essere imputato al fatto che tale composto

possa essere o no carbonio “spazzatura”, ma perché quelle piante si sono evolute seguendo vie differenti, adattando i loro profili di emissione a differenti condizioni ambientali e, di conseguenza, selezionando differenti isoprenoidi per rispondere a fenomeni simili.

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I

Effect of Environmental Stress Conditions on Plants

Plant stress caused by extreme environmental conditions, such as drought, salinity, high temperatures, chemical toxicity and oxidative stress, is the primary cause of crop loss worldwide (Khush, 2001) (Figure 1). These different abiotic stresses are often interconnected, and may induce similar osmotic and oxidative stresses, in turn activating cell signalling pathways and cellular responses.

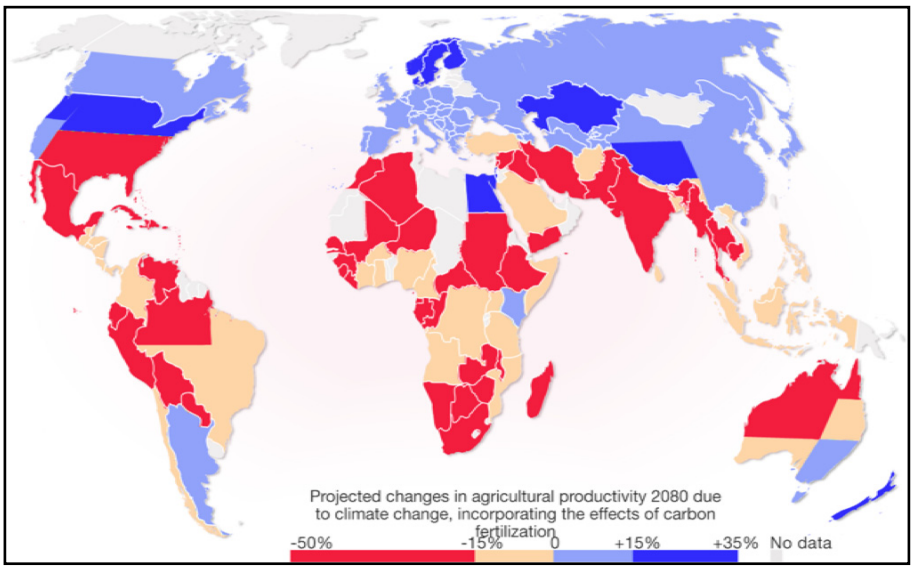

Figure 1. Projected Agriculture in 2080 Due to Climate Change.
With climate changes, we have to adapt our life to a new environment, in most cases warmer and possibly wetter and drier. Projections on the climate in the future provide some guidance for us, but how can we create models for how the human society reacts? This map presents a rough idea of changes in agricultural output from increased temperatures, precipitation differences and also from carbon fertilization for plants. Projecting climate is one thing, but agriculture adds multiple more dimensions of complexity – extreme events, crop rotations, crop selection, breeds, irrigation, erosion, soils and much more.

Oxidative stress conditions may cause de-naturation of functional and structural proteins and membranes, and disruption of osmotic and ionic homeostasis. These initial stress signals trigger a downstream process of signal transduction and gene expression control at transcriptional level which activate genes involved in the abiotic stress response, to protect and repair damaged proteins and membranes. Inadequate response at one or several steps in the mechanism of plant defense against abiotic stresses, or too strong stress conditions, may result in irreversible damages, leading to plant death.

One of the most relevant aspects reported in the last Intergovernmental Panel on Climate Change is represented by the projections of global warming as well as the consequent possible effect of rising temperature on ecosystems (Figure 2). The expected negative impacts of global climate change (IPCC, 2007) makes it very important to generate crop and forest plants that will have enhanced resistance and tolerance to environmental stress conditions and, in particular, to the combined stress caused by sunlight, and rising temperature and drought, which leads to photo-oxidative cell damages (Niogy, 1999).

In plants, several reactive oxygen species (ROS) are continuously produced as by-products of metabolic processes, via the Mehler reaction in chloroplasts (Mehler, 1951), as well as through the photorespiratory and respiratory electron transport (Halliwell and Gutteridge, 1989; Polle, 1995; Asada et al., 1998; Corpas et al., 2001). In plant cells, ROS are efficiently scavenged by the activity of

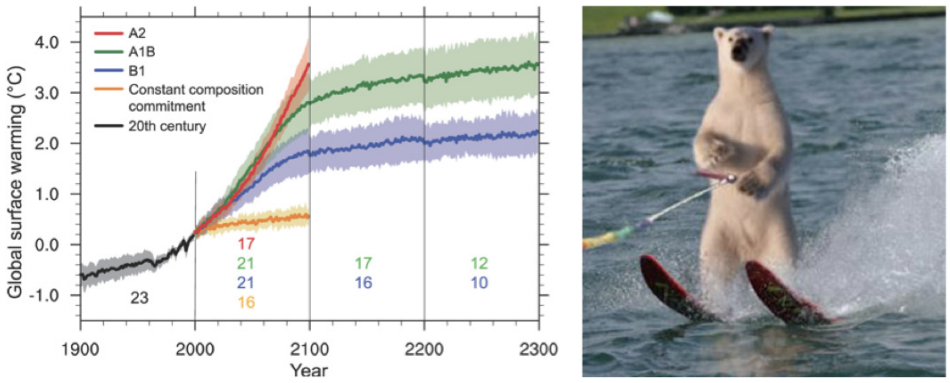

Figure 2. Projections of Global Warming and Possible Effect of Rising Temperature on Life.

Values beyond 2100 are for the stabilization scenarios. Lines show the multi-model means of surface warming for the scenarios A2, A1B and B1, shown as continuations of the 20th-century simulation, shading denotes the ±1 standard deviation range of individual model annual means. Discontinuities between different periods have no physical meaning and are caused by the fact that the number of models that have run a given scenario is different for each period and scenario, as indicated by the colored numbers given for each period and scenario at the bottom of the panel. Global Climate Projections, IPCC 2007, Cap 10.

According to the IPCC 2007, many natural resources could fall victims to climate change. Average global temperature, until 2050, should rise another 1.5 to 2.5 degrees Celsius from their 1990 levels, facing 20 to 30 percent of all species a high risk of extinction. The Arctic probably will due to the greatest relative warming, and endemic species, like polar bear, should find the way to change their behavior or become extinct.

different ROS-scavenging enzymes (Figure 3), such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (Asada, 1999; Mittler, 2002; Apel and Hirt, 2004). However, the balance between ROS production and scavenging may be perturbed by a number of abiotic and biotic stress factors, as high intensity illumination or elevated temperatures, wounding, elicitors and response to pathogens (Bolwell, 1999; Orozco-Cardenas et al., 2001) resulting in a very fast rise of their level in cells and apoplast.

When the production of ROS exceeds the capacity of the antioxidant metabolism to remove them, the unbalance leads to a

signalling response triggered by the increased H_2O_2 and NO concentration, and that includes gene expression, enzyme activation, oxidative damage to cellular macromolecules and structures, and programmed cell death.

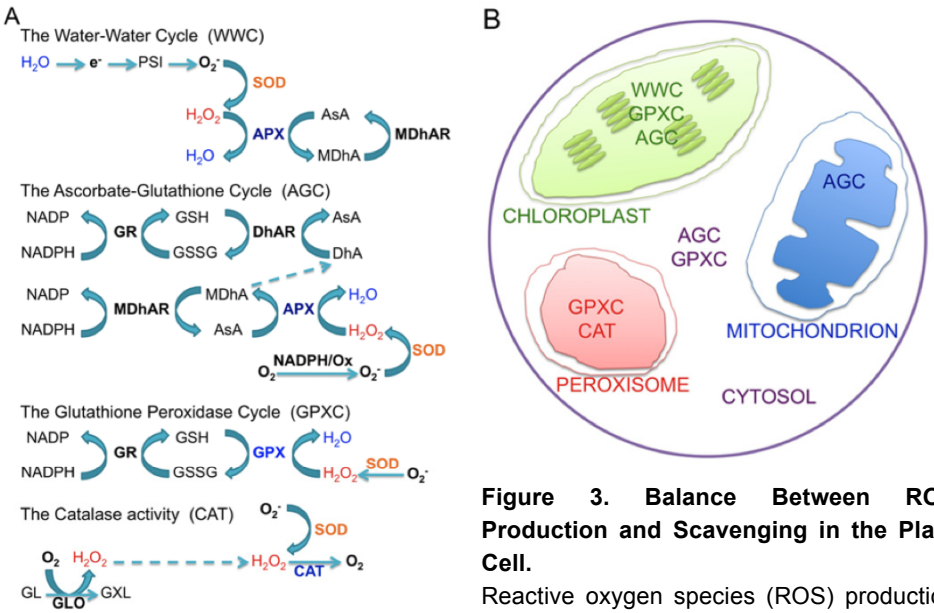

Figure 3. Balance Between ROS Production and Scavenging in the Plant Cell.

Reactive oxygen species (ROS) production and scavenging system involves a complex network of different pathways, often interconnected, localized in different compartments in plant cell.

A. Reactions involved in the ROS scavenging system: water-water cycle (WWC), ascorbate-glutathione cycle (AGC), glutathione peroxidase cycle (GPXC), and catalase pathway (CAT). Enzymes involved in these reactions are: SOD, superoxide dismutase; APX, ascorbate peroxidase; MDhAR, ferredoxin monodehydroascorbate reductase; GR, glutathione reductase; DhAR, dehydroascorbate reductase; NADPH/Ox, NADPH oxidase; GPX, glutathione peroxidase; GLO, glycolate oxidase; CAT, catalase. Note: e^-, electrons; PSI, photosystem I; O_2^-, singlet oxygen; H_2O_2, hydrogen peroxide; AsA, ascorbate; MDhA, monodehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione; GL, glycolate; GLX, glyoxylate.

B. Exemplificative picture of the localization in plant cells of ROS-scavenging pathways.

Of all ROS produced in plant cells, H_2O_2 has been implicated in numerous plant responses to either physiological and environmental stress conditions. H_2O_2 has a dual role in plant tissues (Dat et al., 2000), on one hand, its toxic accumulation leads to cellular damages (Mittler et al., 1999; Dat et al., 2003). On the other hand, H_2O_2 might function as a signalling molecule mediating responses to various stimuli in plant cells, having a lifetime long enough for free diffusion and being an uncharged molecule, able to penetrate membranes (Orozco-Cardenas et al., 2001; Neill et al., 2002). H_2O_2 was proved to mediate a variety of cellular responses to pathogen elicitation, systemic acquired resistance, programmed cell death and stomata responses (Bethke and Jones, 2001; Zhang et al., 2001).

Control of H_2O_2 level in the plant cells seems to be a key factor in activation of defence mechanisms against oxidative stress damages. High non-toxic H_2O_2 levels regulate the molecular response to heat-shock and oxidative stress conditions (Neill et al., 2002; Volkov et al., 2006). Different experimental evidences are reported in last years linking the responses to high temperature and oxidative stress. These stress conditions induce activation of similar pathways, resulting in the expression of heat-shock factors (HSF) and accumulation of heat-shock proteins (HSP) in plants (Banzet et al., 1998; Dat et al., 1999; Panchuk et al., 2002).

Drought and high temperatures are often concurrent factors in nature, and the combined effect of these two environmental constraints should be determined. Moreover, current global change models predict that lower frequency of rainfall days and longer dry

intervals associated with atmospheric warming will affect large areas of the globe. In the Mediterranean basins, for instance, the average maximum summer temperature is expected to increase by about 5.1°C and summer precipitation is expected to decrease by about 27% by the end of this century (IPCC, 2007). Thus, it is of paramount importance to study the acclimatory responses of plants to the interactive effects between water stress and rising temperature.

Drought and temperature represent the main rising variables of the global warming, which could affect not only the plant response to environmental stress conditions, but also the atmospheric chemistry. This is particularly true if we apply it to the forest ecosystems. Terrestrial vegetation, especially tropical rain forest, releases vast quantities of carbon as volatile organic compounds (VOC) into the atmosphere of which the most important is isoprene. The emissions of these volatile isoprenoids are strictly dependant on environmental factors and they are highly reactive with correspondingly short lifetimes (Kesselmeier and Staudt, 1999). Volatile isoprenoids can be removed by oxidation reactions; this reactions chain is mainly initiated by hydroxyl radicals (OH), primarily formed through the photo-dissociation of ozone. They can not only destroy, but also create ozone, depending on the levels of nitrogen oxides (NOx). When NOx levels are low, these VOC can react directly with ozone, reducing its levels. Otherwise, when NOx levels increase, net ozone production occurs. The consequence of forest degradation, especially in the Amazon region, would be to reduce the VOC emissions, with a subsequent impact on ozone levels at the surface

(Sanderson et al., 2003). The VOC emissions were calculated using the algorithms developed by Guenther et al. (1995), not including the direct effect of CO_2 on isoprene (Rosenstiel et al., 2003). With the vegetation distribution fixed at that for the 1990s, isoprene emissions were projected to increase from 550 to approximately 740 Tg yr^{-1} by 2100 (IPCC, 2007).

As pointed out by Betts et al. (2008) “Isoprene emissions have a significant impact on the projected future surface ozone levels. Ignoring vegetation changes has meant that future simulated ozone levels were greater by 5-10 ppbv, owing to larger isoprene emissions. This may have implications for air quality in the region, with potential implications for the health of humans, animals, ecosystems and crops”.

All together, these considerations reveal the possible impact of volatiles isoprenoids, and in particular isoprene, on the ecosystems, especially on forest ecosystems.

II

Isoprenoids as a Part of Plant Defence System

Vegetation emits a wide range of volatile organic compounds (VOC), of which the most substantial are isoprenoids. These metabolites, or non-volatile metabolites derived from them, are involved in numerous biological processes, such as electron transport, photosynthesis, hormonal regulation, membrane fluidity, and plant defense responses, or communication with other organisms (Monson et al., 1992; Kesselmeier and Staudt, 1999; Pichersky and Gershenzon, 2002). In higher plants, two independent pathways are responsible for the biosynthesis of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), the central five-carbon intermediates of all isoprenoids (Figure 4). The cytosolic pathway, which involves mevalonate (MVA) as a key intermediate (Quershi et al., 1981; Newman and Chappell, 1999), provides the precursor molecules for sterols, ubiquinone, sesquiterpenes (C_{15}, like caryophyllene), and triterpenes (C_{30}). On the other hand, the chloroplastic MVA-independent (or DOXP/MEP) pathway starts with a condensation of pyruvate and D-glyceraldehyde 3-phosphate (GA-3P) via 1-deoxy-D-xylulose 5-phosphate/2-*C*-methyl-D-erythritol 4-phosphate (DOXP/MEP) as an intermediate. This pathway is involved in the formation of precursors for the biosynthesis of hemiterpenes (C_5, like isoprene), monoterpenes (C_{10}), diterpenes (C_{20}), tetraterpenes (C_{40}), and products that arise from those, as carotenoids, abscisic acid, the side chains of chlorophylls,

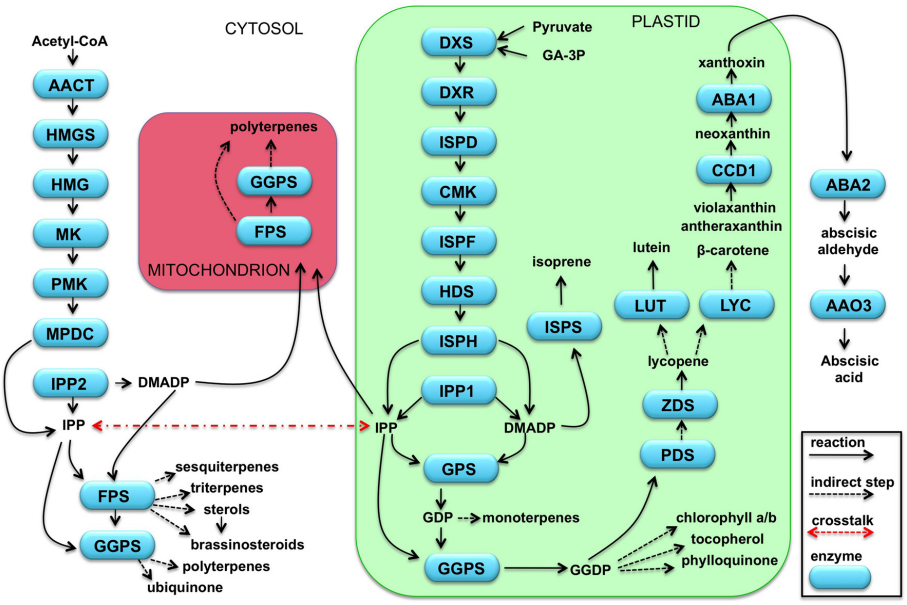

Figure 4. Plant Isoprenoid Biosynthetic Pathway.

Mevalonate (MVA) pathway is localized in the cytosol to supply IDP and DMADP for the synthesis of cytosolic and mitochondrial isoprenoids. The DOXP/MEP pathway is localized in plastids. IDP is utilized as a substrate for a variety of prenyl diphosphates of increasing size. Geranyl diphosphate (C_{10}), farnesyl diphosphate (C_{15}) and geranylgeranyl diphosphate (C_{20}) are key intermediates for the synthesis of the wide range of end products derived from the isoprenoid pathway.

AACT, acetoacetyl-CoA thiolase; HMGS, hydroxymethyl-glutaryl-CoA synthase; HMG, hydroxymethyl-glutaryl-CoA reductase; MK, mevalonate kinase; PMK, phospho-mevalonate kinase; MPDC, phospho-mevalonate decarboxylase; IPP1 and 2, isopentenyl-PP isomerase; FPS, farnesyl-PP synthase; GPS, geranyl-DP synthase; GGPS, geranylgeranyl-DP synthase; DXS, 1-deoxy-D-xylulose-5-P (DOXP) synthase; DXR, DOXP reductoisomerase; ISPD, 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; CMK, 4-(cytidine-5-diphospho)-2-*C*-methyl-D-erythritol kinase; ISPF, 2,4-*C*-methyl-D-erythritol-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-phosphate synthase; ISPH, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-phosphate reductase; ISPS, isoprene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LUT, lycopene ε-cyclase; LYC, lycopene β-cyclase; CCD1, carotenoid cleavage dioxygenase 1; ABA1, zeaxanthin epoxidase 1; ABA2, oxidoreductase 2; AAO3, abscisic aldehyde oxygenase 3.

tocopherol, and plastoquinone (Lichtenthaler, 1999). Once formed, IDP and DMADP condense to form geranyl diphosphate (GDP), farnesyl diphosphate (FDP), and geranylgeranyl diphosphate (GGDP).

These reactions are catalyzed by short-chain prenyltransferases (Koyama and Ogura, 1999). Farnesyl diphosphate is synthesized by a large family of homodimeric prenyltransferases called FDP synthases (FPS). On the other hand, the synthesis of GDP is more complex. In some cases, like in Arabidopsis (Bouvier et al., 2000), GDP is synthesized by homodimeric GDP synthases (GPS), while, in other species, like peppermint (*Mentha x piperita*), snapdragon (*Antirrhinum majus*) and *Clarkia breweri*, these are heterodimeric enzymes, each subunit being a member of the prenyltransferase protein family (Burke et al., 1999; Tholl et al., 2004). The three acyclic prenyl diphosphates (FDP, GDP, and GGDP) serve as the immediate precursors of the corresponding monoterpenoid (C_{10}), sesquiterpenoid (C_{15}), and diterpenoid (C_{20}) classes, to which they are converted by a very large group of enzymes called the terpene (terpenoid) synthases (TPS). These enzymes are often referred to as terpene cyclases, since the products of the reactions are most often cyclic.

Isoprene (2-methyl-1,3-butadiene) is the most abundant VOC emitted by plants on earth, with an annual global flux estimated at 5 x 10^{14} g yr^{-1} (Guenther et al., 1995). Isoprene is synthesized by the enzyme isoprene synthase (ISPS), using dimethylallyl diphosphate (DMADP) as a substrate.

The ISPS protein is located in plant chloroplasts (Schnitzler et al., 2005), together with a large family of terpene synthases that form different isoprenoids from products of the DOXP/MEP pathway. Isolation, expression and functional characterization of *ISOPRENE SYNTHASE* gene (*ISPS*) has only been reported from grey poplar (*Populus alba* x *tremula* syn. *P*. x *canescens*) (Miller et al., 2001; Schnitzler et al., 2005), *Populus tremuloides*, *Pueraria montana* (Sharkey et al., 2005), *Populus alba* (Sasaki et al., 2005), and *Populus nigra* (Fortunati et al., 2008a); whereas the purification and biochemical properties of isoprene synthase protein has been reported from several other plant species, including willow (Wildermuth and Fall, 1998), aspen (Silver and Fall, 1995) and European oak (Lehning et al., 1999).

In planta, isoprene is considered an important molecule for ameliorating abiotic stresses. Isoprene may increase thermotolerance (Sharkey and Singsaas, 1995; Peñuelas et al., 2005; Velikova and Loreto, 2005), particularly when leaves are exposed to transiently high temperatures (Sharkey et al., 2001; Behnke et al., 2007). A leaf in which isoprene emission was inhibited by the treatment with fosmidomycin (an inhibitor of the DOXP/MEP pathway) suffered more heat damage and recovered less if compared neither to not-treated leaves (in which was possible the endogenous isoprene biosynthesis) or treated with fosmidomycin but supplied with exogenous isoprene (Sharkey et al., 2001; Velikova and Loreto, 2005). In other experiments, exogenous isoprene treatment could restore all of the thermo protection found in leaves

emitting isoprene (Sharkey et al., 2001). Transgenic poplar trees that lack the capacity synthesize isoprene show increased damage to photosynthesis by heat spikes relative to control trees (Behnke et al., 2007). Arabidopsis plants transformed to overexpress the *ISPS* gene from *P. alba* can tolerate heat stress compared to the wild-type non-emitting plants (Sasaki et al., 2007). One the other hand, Arabidopsis plants expressing the *ISPS* gene from *Populus x canescens* did not show enhanced tolerance to heat spikes (Loivamäki et al., 2007a) and ROS (Peñuelas et al., 2005; Velikova and Loreto 2005), quenching ozone products (Loreto and Velikova 2001), or intercalating into membrane lipids, preventing membrane damage upon stress conditions (Siwko et al., 2007). It is likely that isoprene "directly" scavenges nitric oxide over-production (Velikova et al., 2005), may quench ozone levels inside leaves (Loreto and Fares, 2007) and reduce oxidative damage (Loreto and Velikova, 2001). Isoprene also reduces the formation of nitric oxide in the mesophyll, thereby indirectly modulating signaling of defense-induced biosynthetic pathways (Velikova et al., 2005). Despite the above-mentioned convincing experimental evidences, the mechanism by which isoprene exerts its protective action is not completely understood.

The impact of drought on isoprene emission has also been studied, since drought is a major limitation for plant growth worldwide, and the intensity and frequency of drought conditions is going to increase with current and future climate change (IPCC, 2007). The available evidence suggests that isoprene biosynthesis is

relatively unaffected by drought. Episodes of severe drought stress may lead to suppression of photosynthesis, while isoprene emission is only slightly reduced (Sharkey and Loreto, 1993; Pegoraro et al., 2004; Fortunati et al., 2008a). In white poplar, isoprene emission rate was about 30% of the pre-stress value even during the most severe phase of stress, when photosynthesis was totally inhibited (Brilli et al., 2007). When recovering from drought stress, isoprene emission rapidly returns to pre-stress level, and may even transiently be stimulated over these levels (Sharkey and Loreto, 1993; Fang et al., 1996; Brilli et al., 2007).

Recently, many studies have used a molecular approach to advance our knowledge about mechanisms regulating isoprene biosynthesis and emission under environmental stress conditions, such as high temperature and drought stress (e.g. Behnke et al., 2007). On one hand, the model species *Arabidopsis thaliana*, that naturally does not emit isoprene, was transformed to overexpress the *ISPS* gene (Sharkey et al., 2005; Loivamäki et al., 2007; Sasaki et al., 2007). On the other hand, the strong isoprene emitters *Populus* sp., were transformed to generate: *ISPS*-overexpressing pro35S::*ISPS* lines and, using the gene silencing technique RNA interference (RNAi), *ISPS*-RNAi lines, with a suppressed isoprene emission (Behnke et al., 2007). These studies provided relevant suggestions on the evolution of isoprene emission trait (Sharkey et al., 2005), and on the possibility to use Arabidopsis as a model to study the biological functions of isoprene (Loivamäki et al., 2007). Interestingly, Loivamäki et al. (2007) discussed that transgenic

isoprene-emitting Arabidopsis lines have improved thermotolerance, but this was not due to protection of photosynthesis. Otherwise, Behnke et al. (2007) showed that poplar pro35S::*ISPS* lines were not particularly different, compared to the wild-type plants, in the response to high temperature treatments. The same authors also showed that *ISPS*-RNAi lines exhibited greater sensitivity to thermal stress, compared to the wild-types, displaying a slower recovery of photosynthesis.

While numerous isoprenoids are formed in and emitted by leaf chloroplasts, where they likely exert their protective action, plants also emit volatile compounds from their flowers. These volatiles belong primarily to three major groups of compounds: phenylpropanoids, fatty acid derivatives and terpenes (Dudareva & Pichersky, 2000; Knudsen et al., 1993). Although *Arabidopsis thaliana* is considered to be mainly self-pollinating, the flowers of this species release a complex mixture of volatile monoterpenes (C_{10}) and sesquiterpenes (C_{15}), with (*E*)-β-caryophyllene as the dominant component (Chen et al., 2003). Arabidopsis, with its extensive genetic and genomic resources, provides an ideal model system to study the fundamental roles of isoprenoid volatiles *in planta*. These resources allow for the relatively easy localization of the sites of expression of the biosynthetic genes, identification of mutant lines lacking volatile emission, and the comparison of volatile profiles in different environmental conditions. Moreover, the information available for Arabidopsis is a helpful instrument to elucidate the molecular processes responsible for regulation and variability of

volatile organic compound biosynthesis and emission. The Arabidopsis genome contains a large family of 32 predicted terpene synthase (At*TPS*) genes, of which several are exclusively or almost exclusively expressed in the flowers. Arabidopsis, that naturally does not emit isoprene, makes a large spectrum of different politerpenes, more complexes than isoprene, and of which the most abundant are sesquiterpenes. The (*E*)-β-caryophyllene synthase (TPS27) catalyzes the formation of (-)-(*E*)-β-caryophyllene the major sesquiterpene emitted from Arabidopsis floral tissues. It has been hypothesized that (*E*)-β-caryophyllene can play a role in the attraction of floral visitors, leading to low levels of cross-pollination and increasing reproductive fitness in natural populations (Köllner et al., 2008). However, given the low levels of volatile emission, it is quite possible that the (*E*)-β-caryophyllene biosynthesis have a function besides, or in addition to, pollinator attraction. Though recent studies support that constitutive (e.g. isoprene, monoterpenes and sesquiterpenes) terpene volatiles released from vegetative tissues of different plant species exposed to abiotic stress could serve as mediators of thermotolerance or in protecting cells against oxidative stress (Loreto and Velikova, 2001; Sharkey and Yeh, 2001), there is limited information available about the possible roles of sesquiterpenes in the physiological response of plants to environmental stress conditions. Recently, the two terpene synthases responsible for the biosynthesis of the major part of Arabidopsis floral volatile blend (At5g23960 and At5g44630) were characterized (Chen et al., 2003; Tholl et al., 2005). The At5g23960

enzyme, referred as TPS27, is able to convert the farnesyl diphosphate into the sesquiterpene products (-)-α-copaene, α-humulene, and (-)-(*E*)-β-caryophyllene.

III
What to Know About Isoprene

Many questions about the biological functions of isoprenoids are still open. Even when considering the large amount of studies on the ecological and physiological impact of the isoprenoids release into the atmosphere from vegetation, there is only partial and incomplete information about function, biosynthesis, and regulation of these compounds in the plant cell. It is important to fill this “gap” especially when considering the important role played by these compounds in the defense system against environmental stress. In particular isoprene, the most abundant volatile organic compound emitted by plants, seems to protect leaves from high temperature and oxidative damages. Given the specific and active function of isoprene in the plant defense system, it is expected that isoprene biosynthesis be specifically and strongly regulated. The isoprene synthase (*ISPS*) gene is responsible for the production of the ISPS enzyme, by which isoprene formation is catalyzed. What is the regulation pattern for this protein? Under which kind of conditions ISPS is activated? And at which level? Are there different regulatory steps (e.g. transcriptional, translational, and/or in the activity) used by plants to control isoprene biosynthesis? Could isoprene act as a signal molecule for the plant defense system when a stress occurs? Or is its protective role only direct?

The goal of this work is to provide an answer to some of these points not yet clarified. Using different plant species and different

experimental approaches we will try to elucidate the regulation of ISPS function from gene expression to the enzyme activity in the nature, either in unstressed conditions or during and after exposure to environmental stresses driven or exacerbated by climate change (e.g. high temperature, drought or a combination of both); it will be shown that isoprene is able to enhance thermo-tolerance also in plants which naturally do not emit isoprene (like Arabidopsis), and that this protective role is not only directly played, scavenging ROS excess or helping membrane stability, but has also an indirect component, priming the defense system against thermal and oxidative stress. New biophysical evidences supporting the hypothesis that isoprene may also be able to directly interact with chloroplastic membranes increasing their stability during the stress are also presented.

Similarly to isoprene, many questions regarding another class of isoprenoids, the sesquiterpenes, are still open. In order to improve our understanding about the role of sesquiterpenes in plant response to environmental stress conditions, Jonathan Gershenzon group at Max Planck Institute for Chemical Ecology (MPI-CE), Jena, Germany, has transformed *Arabidopsis thaliana* plants, ecotype *col-0*, that emit (*E*)-β-caryophyllene from flowers, whit the *TPS27* gene under the control of the constitutive promoter pro35S, to generate the *TPS27*-overexpressing (pro35S::*TPS27*) *CarS* lines that produce and emit (*E*)-β-caryophyllene in leaf tissues also. Previous results showed that plants overexpressing the *TPS27* gene, as compared to wild-type, were more resistant to stress induced by herbicide

treatment (Gershenzon, personal communication). Genetically engineered model plant species, such as *Arabidopsis thaliana*, overexpressing enzymes that regulate sesquiterpene biosynthesis in plants, may constitute a suitable tool to study the physiological role of sesquiterpenes in plants. Following this line, a molecular, biochemical and physiological characterization of the *CarS* lines will be shown, and how those plants respond to environmental stress, compared to wild-type plants, will be assessed. In collaboration with the MPI-CE we planned to use this tool to demonstrate if sesquiterpenes (like (*E*)-β-caryophyllene) are able to enhance plant thermotolerance, and if this hypothetical protective role is triggered by similar mechanisms to those suggested for isoprene.

IV
On the Regulation of Isoprene Synthase Under Drought Stress

1. Isoprene, photosynthesis and drought

Isoprene biosynthesis is light and temperature dependent and is closely linked with carbon metabolism (Monson and Fall, 1989). By using labelled CO_2 ($^{13}CO_2$), incorporated into the isoprene molecule, it was demonstrated that *ca* 72-91% of the carbon assimilated originates directly form the photosynthetic carbon flux, and that the remaining carbon is derived from alternative sources (Sanadze et al., 1972; Affek and Yakir, 2003; Schnitzler et al., 2004). However, drought stress is known to uncouple isoprene emission from photosynthesis. Short-term experiments on *Quercus virginiana* (Tingey et al., 1981) and *Pueraria montana* (Sharkey and Loreto, 1993) showed that water stress caused substantial reduction in photosynthesis, while isoprene emission was not inhibited or only slightly reduced (Pegoraro et al., 2004). Although it is evident that the ratio between isoprene emission and photosynthesis increases in response to drought, there are still open questions about the causes of the low responsiveness of isoprene emission to drought with respect to photosynthesis, and about the regulation of isoprene biosynthesis under drought stress.

Despite the need to account for the influence of environmental stress factors to reliably estimate the impact of VOC on atmosphere

chemistry, information about the dynamics of isoprene biosynthesis and emission, in response to soil water availability is necessary. Thus, to improve our understanding as on the molecular regulation of isoprene production well on isoprene emission under conditions of decreasing soil water available, a laboratory drought stress treatment was carried out on 1-yr-old *Populus alba* saplings. In this chapter, how isoprene biosynthesis is regulated under drought stress will be investigated, starting from the quantification of mRNA expression levels of *ISPS* gene, following with the ISPS protein concentration, and concluding with ISPS specific activity measurements. The relationship between isoprene emission and photosynthesis when plants are subjected to water stress will be also elucidated.

2. Results

2.2 Isoprene emission and photosynthesis

The drought stress level was quantified by using the well-established technique of the fraction of transpirable soil water (FTSW, Sinclair and Ludlow, 1986). This technique allows precise knowledge to be obtained of water availability and loss by daily weighing, and reveals, if there are, differences in plant response at equivalent levels of soil water deficit. Drought causes an alteration of plant water balance (Sinclair and Ludlow, 1986). When water is fully available from soil, plants absorb it from their roots by the transpiration process. During the first stage of drought stress, when the available soil water content (or fraction of transpirable soil water, FTSW) starts to decrease, plants respond closing their stomata, balancing the transpiration rate

with the lower water absorption from soil (maintaining stable the relative water content, RWC). In the second stage of drought stress, in which there is no availability of soil water, the RWC progressively decreases and plants become dried. The FTSW value at which photosynthesis of our poplar plants began to rapidly decline (corresponding to the second stage of the physiological response of plants to soil water deficit, Sinclair and Ludlow, 1986) was *ca* 30% (FTSW30). At this stage photosynthesis was decreased to *ca* 32% with respect to the control maintained at 100% FTSW). The drying cycle ended at FTSW5, when photosynthesis declined to almost zero (Figure 5A). Following re-watering, the recovery of photosynthesis was vary fast. Two days after re-watering (2 DAR), photosynthesis was 50% of the control unstressed plants, and after seven days (7 DAR) fully recovered the pre-stress values.

The kinetic of isoprene emission in response to water stress was not consistent with the pattern shown by photosynthesis. As drought increased, there was only a slight negative trend of isoprene emission. However, decrease of isoprene emission became significant only during the severe phase of stress. In fact, isoprene emission was significantly reduced (by 23%) at FTSW30, and was at a minimal level (*ca* 49% of the pre-stress values) at the end of treatment (FTSW5, Figure 5B). During the first week of re-watering, isoprene emission completely recovered the pre-stress values. The ratio between isoprene emission and photosynthesis (I/*A* ratio) increased exponentially in response to drought. Consequentially, the percentage of photosynthetic carbon spent as isoprene progressively

increased as FTSW decreased. During the severe phase of the stress this ratio reached *ca* 145% at FTSW10 and 250% at FTSW5 (Figure 5C).

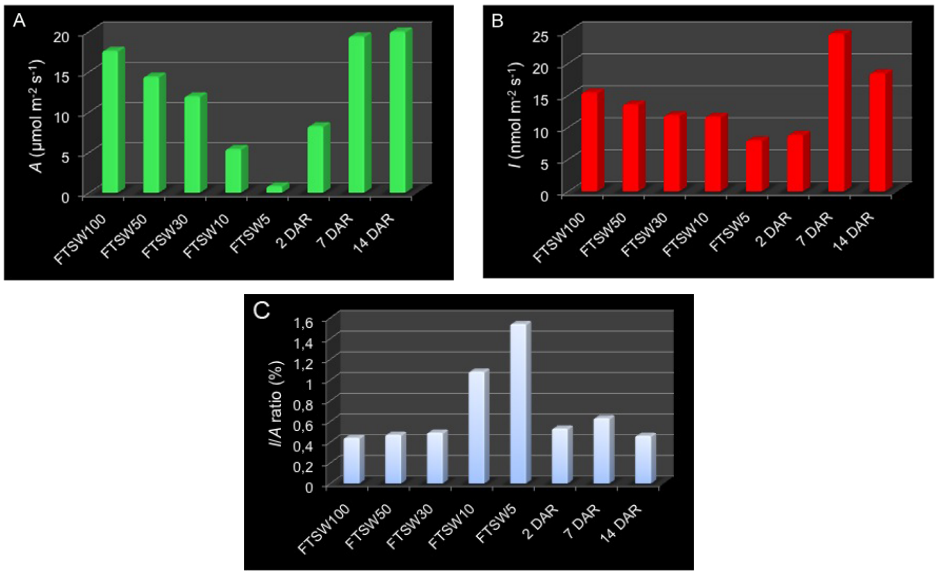

Figure 5. Isoprene Emission and Photosynthesis in Response to Drought Stress.
A. Net photosynthetic rate (*A*) measurements.
B. Isoprene emission rate (*I*).
C. Isoprene emission vs. photosynthesis ratio during and after drought stress.
Note: during the drying cycle, values are shown as the fractions of transpirable soil water (FTSW), at 50% (FTSW50), 30% (FTSW30), 10% (FTSW10), and 5% (FTSW5), respectively. After the drying cycle, values are shown at 2, 7 and 14 days after re-watering (2, 7 and 14 DAR, respectively). Not treated plants, with a FTSW of 100% (FTSW100) were used as a control.

2.3 How isoprene synthase is regulated under drought stress

To follow the regulation pattern of isoprene synthase during drought stress, *ISPS* mRNA expression levels, ISPS protein concentration and ISPS specific activity were measured (Figure 6). The expression of Pa*ISPS* mRNA was significantly reduced only during the severe phase of the stress (FTSW5), remaining high when the stress was

mild (FTSW30 and 10). Pa*ISPS* gene expression rapidly recovered to *ca* 85% of the control value after two days of re-watering (2 DAR), but remained at these levels even after 14 days of recovery (14 DAR). Thus, while isoprene emission completely recovered 14 DAR, Pa*ISPS* gene expression is still down-regulated at this time frame (Figure 6A).

Similarly to Pa*ISPS* gene expression, ISPS protein concentration was significantly reduced only during the severe phase of the water stress (*ca* 58% of the control value at FTSW5). The ISPS protein concentration recovered quickly and completely upon re-watering, reached values similar to those of pre-stressed plants at 2 DAR (Figure 6B).

Isoprene synthase specific activity exponentially decreased during the treatment, arriving to *ca* 16% of the pre-stress values at FTSW5 (Figure 6C). Inhibition of ISPS enzymatic activity occurred at earlier drought stress phase with respect to either the reduction of ISPS concentration or the down-regulation of Pa*ISPS* gene expression. However, ISPS specific activity fully recovered after just two day of re-watering (2 DAR).

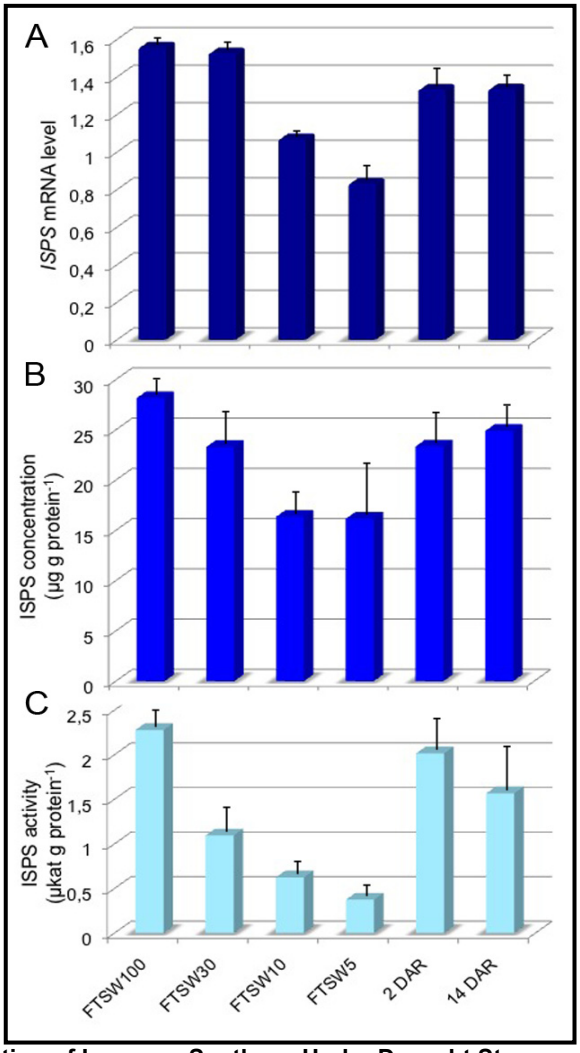

Figure 6. Regulation of Isoprene Synthase Under Drought Stress.

A. Quantitative mRNA expression levels of Pa*ISPS* normalized with respect to the *ACT2* mRNA levels.

B. Measurements of ISPS protein concentration performed by an enzyme-linked immunosorbent assay (ELISA).

C. ISPS specific activity measurements.

Note: during the drying cycle, values are shown as the fractions of transpirable soil water (FTSW), at 30% (FTSW30), 10% (FTSW10), and 5% (FTSW5), respectively. After the drying cycle, values are shown at 2 and 14 days after re-watering (2 and 14 DAR, respectively). Not treated plants, with a FTSW of 100% (FTSW100) were used as a control. Bars represent means ± SE (n = 5, $P < 0.05$).

3. Discussion on the kinetic of isoprene biosynthesis and emission during drought stress

The exposure of *Populus alba* saplings to soil water deficit confirmed previous findings that isoprene emission is less responsive to drought than photosynthesis (Tingey et al., 1981; Sharkey and Loreto, 1993; Pegoraro et al., 2004). The percentage of photosynthetic carbon re-emitted as isoprene was stable during the first, mild phase of the stress, but drastically increased during the severe phase of drought. However, as soil water availability decreased below FTSW50, a progressive steady decline in the rate of isoprene emitted in stress conditions was also observed. These results are consistent with those obtained form studies on the effect of water stress on isoprene (Fang et al., 1996) and monoterpenes emission (Staudt et al., 2002).

The maintenance of high isoprene emission rate when photosynthesis is almost totally inhibited might be due to an increasing contribution of alternative carbon sources. Stored carbon pools, such as xylem-transported glucose and chloroplastic starch, may contribute to isoprene biosynthesis (Affek and Yakir, 2003; Schnitzler et al., 2004). Funk et al. (2004) showed that contribution of recently fixed carbon photosynthates accounted for 84-88% in isoprene in unstressed *P. deltoides* plants, while this contribution decreased to 62% in water-stressed plants. Similarly, experiments conducted by Brilli et al. (2007) with *P. alba*, monitoring the ^{13}C incorporation into isoprene, suggested that carbon recently assimilated accounted for 78 to 90% of the isoprene molecule, in

pre-stress conditions, but only for 16 to 42% of the isoprene molecule during the severe phase of drought stress.

Grote and Niinemets (2008) have recently suggested that the inhibition of isoprene emission under severe water stress conditions is mainly caused by reduced substrate availability rather than by impairment in TPS activity. However, in our experiments, ISPS activity was already significantly inhibited at FTSW30, while isoprene emission was still substantially unaffected by the stress. The inhibition of photosynthesis under drought stress conditions could have indirectly down-regulated ISPS activity, reducing the light-dependent production of DMADP and other photosynthetic intermediates, and their flow through the DOXP/MEP pathway (Lehning et al., 1999). At the beginning of the severe phase of drought stress, ISPS activity down-regulation may then provide a negative feedback, inducing a down-regulation of *ISPS* gene expression, and finally, when the stress reaches the most acute phase, leading the decrease of ISPS protein concentration, by post-transcriptional or translational regulations.

In conclusion, the results of the present study on drought kinetics show that, as long as soil water is available to support transpiration at even limited rates (i.e. between FTSW10 and FTSW5, see Brilli et al., 2007), isoprene emission is not severely inhibited and photosynthesis may not be limited by metabolic factors. It is also confirmed that, when photosynthesis is not fully constrained, freshly fixed photosynthates are the primary substrate for isoprene biosynthesis. On the other hand, because isoprene synthase activity

is sensitive to severe stress, isoprene formation may be sustained by larger contributions from alternative carbon sources, presumably of extra-chloroplastic origin, as soil water availability is increasingly depleted. The maintenance of high levels of isoprene emission, contributed by an increasingly large fraction of the carbon fixed by photosynthesis, is indirect evidence of the importance of isoprene in protecting plants against abiotic stresses such as high temperature and oxidative stress (Sharkey and Singsaas, 1995; Singsaas et al., 1997; Loreto and Velikova, 2001; Sharkey et al., 2001; Velikova et al., 2006), which probably develops under water stress conditions. The findings of this study are likely to be relevant for process-based models that account for stress effects in order to predict the emissions of isoprenoid in globally changing environmental conditions and to scale up the impact of isoprenoid on air chemistry and quality at regional and global levels.

V

When High Temperature and Drought Stress Collide

1. Results

1.1 Photosynthesis and isoprene emission measurements

The severity of drought stress was determined, as well as for drought stress experiment on *P. alba* saplings, as the fraction of transpirable soil water (FTSW). The FTSW decreased faster in plants grown and sampled at 25°C than in plants grown and sampled at 35°C, due to the higher initial stomatal conductance and transpiration rates of plants grown at the lower temperature (Table I). However, at the end of the drought treatment, the residual FTSW was not significantly different at the two temperatures (Table I).

Table I. Fraction of transpirable soil water (FTSW), stomatal conductance (g_s), and transpiration (*Tr*) in *Populus nigra* saplings grown at 25 and 35°C during the drought stress.

Time (days)	Temperature (°C)	FTSW (%)	g_s (mol m^{-2} s^{-1})	*Tr* (mmol m^{-2} s^{-1})
0	25	100 ± 3	0.312 ± 0.085	4.81 ± 0.23
	35	100 ± 5	0.397 ± 0.081	6.56 ± 0.31
14	25	44 ± 8	0.120 ± 0.088	2.23 ± 0.31
	35	77 ± 9	0.186 ± 0.080	4.44 ± 0.24
30	25	27 ± 7	0.060 ± 0.032	1.34 ± 0.12
	35	43 ± 8	0.094 ± 0.037	2.55 ± 0.19
35	25	25 ± 9	0.006 ± 0.003	0.77 ± 0.11
	35	30 ± 6	0.013 ± 0.009	0.98 ± 0.08

Measurements at day = 0 are on pre-stressed leaves. Data represent means ± SD (n = 9).

At the beginning of the experiment (day 0), plants grown at 35°C showed a lower photosynthesis than plants grown at 25°C (Figure 7A). This was expected because of the high photorespiratory rates of C3 plants at high temperatures, which does not indicate the occurrence of stress. Drought caused a reduction of photosynthesis after 14 days and a further strong reduction after 30 days, lowering the rate of photosynthesis to only approximately 5 µmol m^{-2} sec^{-1} in plants grown at both temperatures. Plants were re-watered when photosynthesis was completely inhibited, after 35 days of drought stress at both temperatures. Photosynthesis of re-watered plants grown at 25°C recovered significantly faster than in plants grown at 35°C, and the rates were similar to the pre-stress rates 3 days after re-watering. After re-watering for 15 days (corresponding to day 50 of the experiment) plants grown at both 25 and 35°C had completely recovered to the photosynthetic rates recorded before drought stress.

In contrast to photosynthesis, the isoprene emission rate was higher in plants grown at 35°C than in plants grown at 25°C, before the imposition of drought stress (Figure 7B).

During the development of drought stress, the isoprene emission rate of plants grown at 35°C decreased only slightly, whereas the emission rate of plants grown at 25°C decreased significantly, and reached approximately half of the initial rate after 30 days of drought. When photosynthesis was completely inhibited by a 35-day drought stress, the isoprene emission of plants grown at 25 and 35°C was strongly inhibited but still measurable, averaging 7.5 nmol m^{-2} sec^{-1}.

After a 3-day re-watering phase, the isoprene emission rate recovered in plants grown at both 25 and 35°C. However, isoprene emission did not reach the pre-stress levels even 15 days after re-watering, when photosynthesis had completely recovered. The reduction of isoprene emission after recovering from drought stress was particularly strong in leaves grown at 35°C.

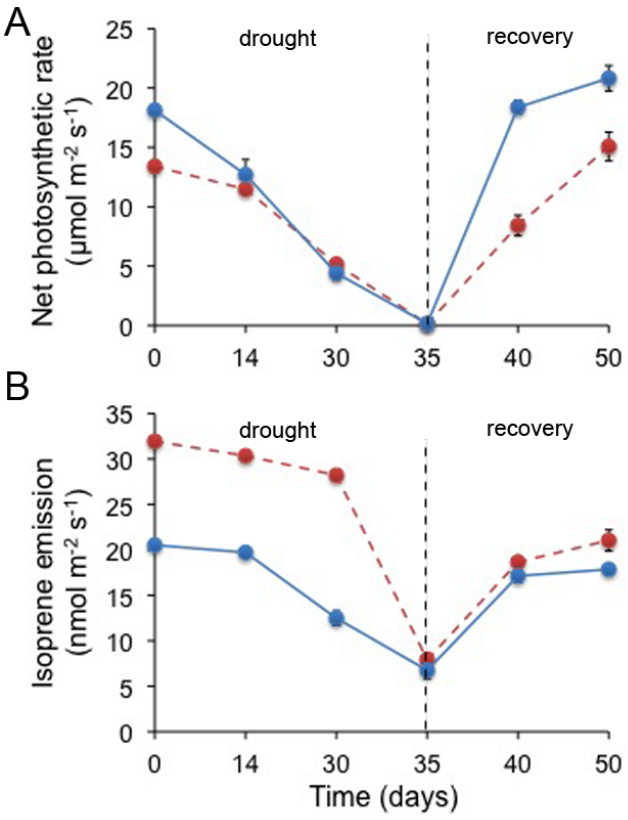

Figure 7. Measurements of Photosynthesis and Isoprene Emission Rates During Drought Stress and Re-watering in Leaves Grown at Different Temperatures.
A. Net photosynthetic rate.
B. Isoprene emission.
Measurements were done on black poplar leaves grown at 25°C (light blue circles) and 35°C (dark red circles) during a 35-day-long drought stress and 15 days of recovery.
All data represent means ± SD (n = 9).

1.2 Phylogenetic analysis of Populus nigra *ISOPRENE SYNTHASE (PnISPS)*

A rooted phylogenetic tree was generated using the putative amino acid sequences of available isoprene synthase (ISPS) genes and terpene synthase b (Tps-b) coding regions that are closely related to known isoprene synthases (Figure 8A). This phylogram was performed with the maximum likelihood analysis and showed that ISPS proteins formed a monophyletic group, closely related to dicotyledonous monoterpene synthases.

Moreover, all putative poplar proteins were closely related to each other, although *P. nigra* showed the lowest homology of sequences with the other poplar species. A BLAST search from the NCBI protein database, using as a query the putative amino acid sequence for ISPS, showed for *P. nigra* ISPS 98% identity with *P. alba* ISPS, 97% identity with *P. tremuloides* and *P. x canescens* ISPS, and 59–73% similarity with terpene synthases (TPS) from other species, including the ISPS from kudzu (Figure 8B).

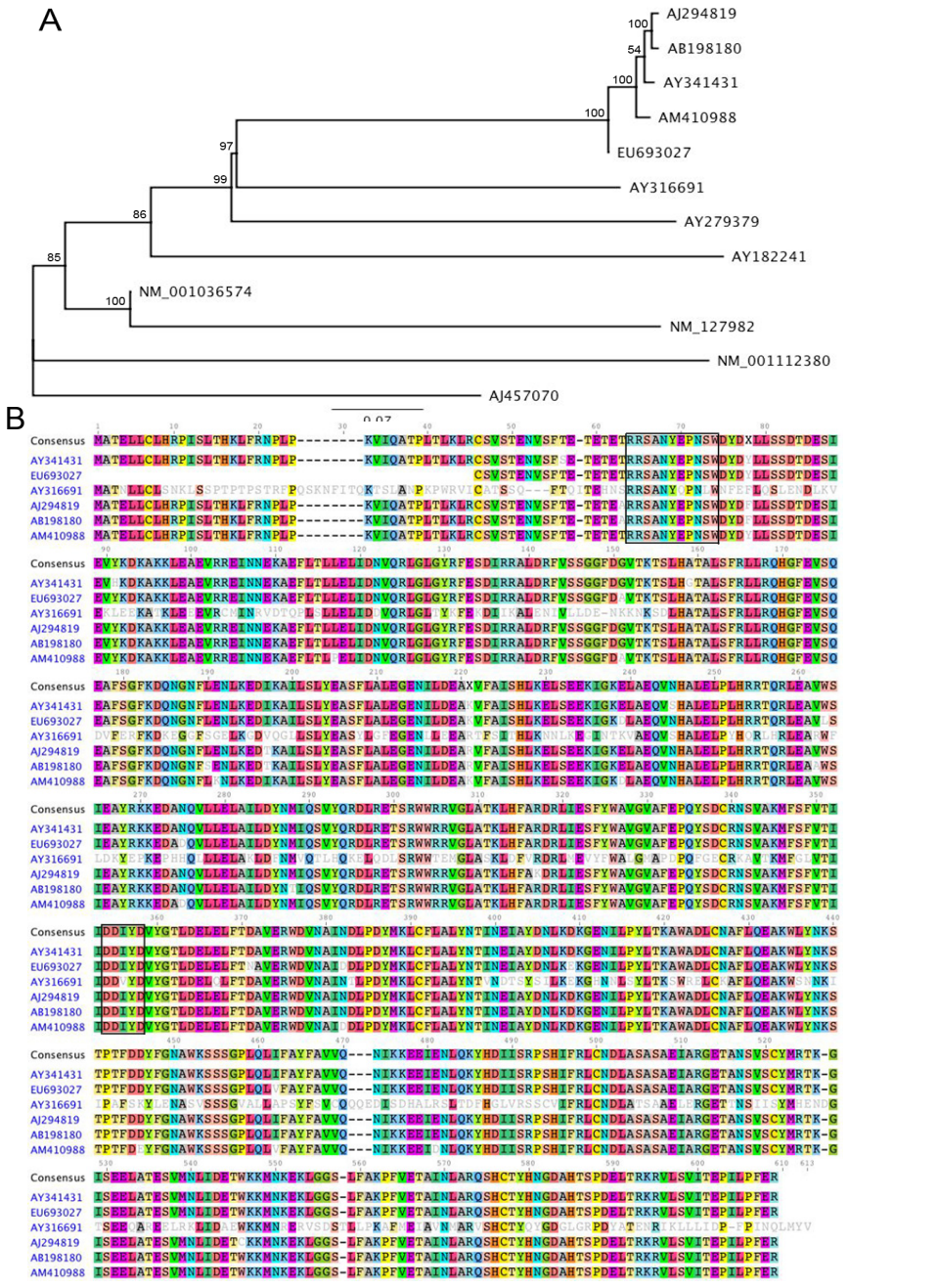

Figure 8. Phylogenetic Analysis of ISPS and Tps-b Proteins.

Figure 8. (Continued)
A. A rooted phylogenetic tree was generated using the ClustalX alignment of the putative amino acid sequences of the note isoprene synthase proteins and the terpene synthase b (*Tps-b*) coding regions, which are more closely related to the isoprene synthases. This phylogram was performed with the method UPGMA and the maximum likelihood analysis. Bold lines show the ISPS proteins.
B. Alignment of the deduced amino acid sequences of the known isoprene synthase proteins. Identical and conserved amino acids residues are denoted by white and gray backgrounds. Consensus amino acid sequence for the ISPS protein is also shown. Dashes indicate gaps inserted for optimal alignment. Frames mark the highly conserved RRX_8W and DDXXD motifs. GeneBank accession numbers: AB198180, *Populus alba* PaISPS; AY341431, *Populus tremuloides* PtISPS; EU693027, *Populus trichocarpa* PtriISPS; AJ294819, *Populus alba* x *tremula syn. X canescens* PcISPS; AM410988, *Populus nigra* PnISPS; AY316691, *Pueraria montana* var. *lobata* PmISPS; AY279379, *Melaleuca alternifolia* putative monoterpene synthase; AY182241, *Malus* x *domestica*, (*E, E*)-alpha-farnesene synthase (MdAFS1); AJ457070, *Cinnamomum tenuipilum* geraniol synthase (CtGERS); NM_127982, *Arabidopsis thaliana* myrcene-(*E*)-beta-ocimene synthase (AtTPS10); NM_001036574, *Arabidopsis thaliana* monoterpene synthase (AtTPS03); NM_001112380, *Zea mays* terpene synthase 10 (ZmTPS10).

1.3 Transcript level, protein concentration and activity of Populus nigra ISOPRENE SYNTHASE (PnISPS)

To investigate the transcriptional regulation of the black poplar isoprene synthase (Pn*ISPS*) gene, quantitative mRNA transcript analysis during and after drought stress at moderately low (25°C) and high (35°C) temperature was performed (Figure 9A). At the beginning of treatment, plants grown at 35°C showed higher Pn*ISPS* mRNA transcript levels than plants grown at 25°C. Drought stress rapidly and strongly affected Pn*ISPS* mRNA transcript levels in leaves grown at 25°C, while transcript levels were unaffected after 14 days of drought in leaves grown at 35°C. When drought stress became severe and photosynthesis was completely inhibited,

Pn*ISPS* mRNA transcript levels also decreased significantly, although they were still measurable, in plants grown at 35°C. After re-watering, Pn*ISPS* mRNA transcript levels did not reach pre-stress values. However, recovery was faster and more complete in plants grown at 35°C than in plants grown at 25°C. Consistent with higher transcript levels, the amount of isoprene synthase protein was also higher in plants grown at 35 than at 25°C, prior to drought stress (Figure 9B). Drought did not induce a complete loss of protein at either temperature. However, when drought stress became severe, after 35 days, the amount of ISPS protein was reduced significantly with respect to pre-stress conditions, and became similar in plants grown at both 25 and 35°C. Re-watering allowed a partial recovery of PnISPS protein, but the amount observed after recovery was lower than the amount observed before stress, and similar at the two temperatures. Prior to the imposition of drought stress, plants grown at 35°C showed much higher PnISPS activities than plants grown at 25°C (Figure 9C). Drought stress negatively influenced PnISPS activity, especially in plants grown at 35°C. In these plants, PnISPS activity started to decrease when drought stress was mild and only partially recovered after re-watering. In plants grown at 25°C, the inhibition of PnISPS activity occurred more slowly and only became evident when the stress was severe. At 25°C, PnISPS activity recovered rapidly and completely upon re-watering. To better identify possible changes in the biochemical control of isoprene emission, the emission rates were plotted against PnISPS activity for data points collected before, during and after drought stress.

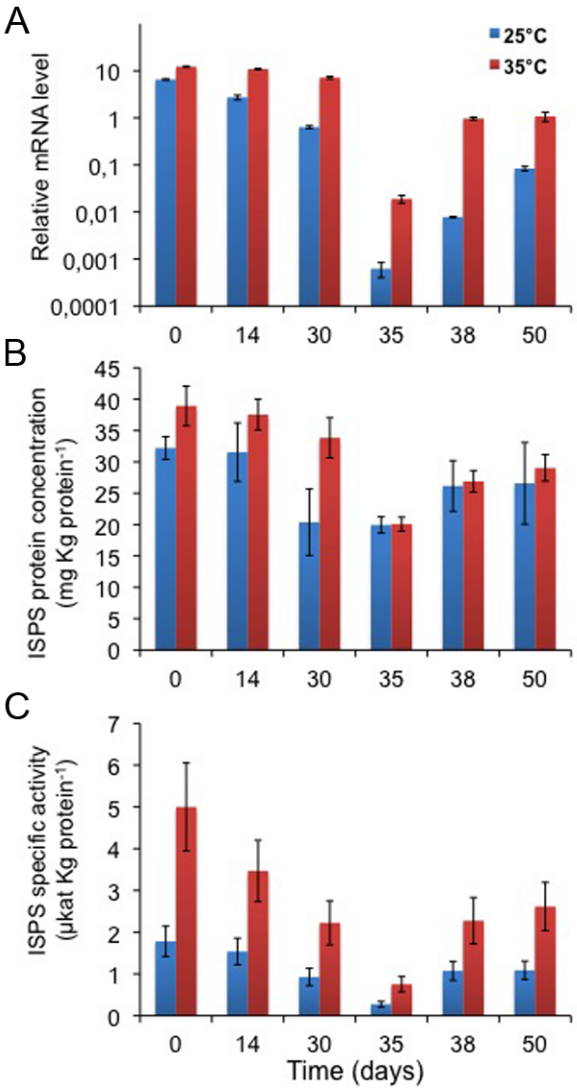

Figure 9. Isoprene Synthase Properties During Drought Stress and Re-watering in Plants Grown at Different Temperatures.
A. Quantitative mRNA transcript level of the Pn*ISPS* gene.
B. Black poplar isoprene synthase (PnISPS) protein concentration.
C. PnISPS specific activity.
Measurements were curried out in leaves of black poplar plants grown at 25°C (blue bars) and 35°C (red bars), during a 35-d-long drought stress and after 15 days of recovery.
Bars represent means ± SD (n = 9).

As the emission is enzymatically controlled, PnISPS activity was generally related to isoprene emission rates along the drought treatment (Figure 10). This relationship also indicates that isoprene emission was not limited by the enzyme activity at both temperatures. A much higher PnISPS activity sustained high rates of

isoprene emission in plants grown at 35°C before drought stress. However, at this high temperature, large reductions of PnISPS activity have no relevant effect on isoprene emission in conditions of mild drought stress. The PnISPS activities of leaves grown at 35°C also do not recover the original rates once drought stress is relieved. Data measured after drought recovery at 35°C lie clearly outside the regression line that correlates data points measured in leaves before and during drought at 35°C.

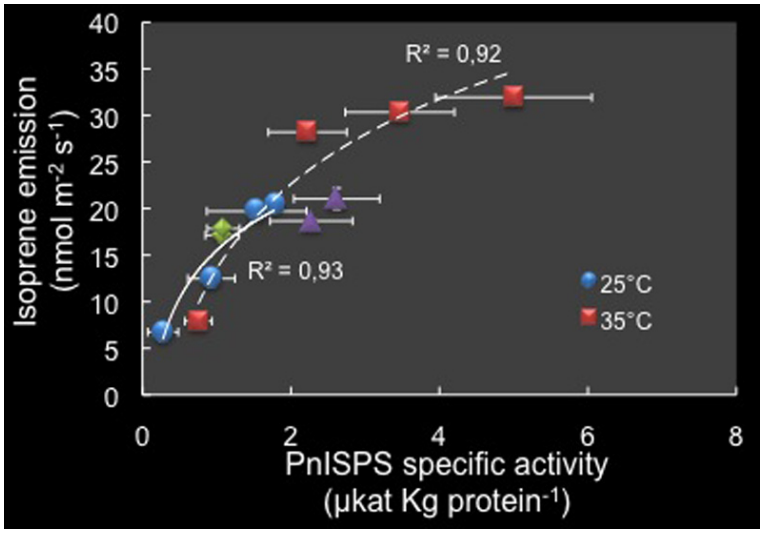

Figure 10. Correlation of Isoprene Emission With Black Poplar Isoprene Synthase (PnISPS) Activity During the Treatments.
Relationship between isoprene emission and PnISPS specific activity in black poplar leaves grown at 25°C (blue circles) and 35°C (red squares) during the 35-d-long drought stress. Data points collected 3 and 15 days after the re-watering are represented for plants grown at 25°C (green rhombus) and 35°C (purple triangles). Symbols represent means ± SD (n = 9). Continuous and dashed lines represent the best fits for relationships at 25 and 35°C, respectively ($P < 0.001$).
Note: Regression lines were generated using the values at pre-stress conditions and during drought stress, excluding values during recovery.

*1.4 Phospho*enol*pyruvate carboxylase activity and leaf dark respiration measurements*

Isoprene is strongly inhibited by elevated CO_2 in *American Populus* species, *Populus tremuloides* (Monson and Fall, 1989) and *Populus deltoides* (Sharkey et al., 1991). The reasons for the reduction of isoprene formation in poplars under elevated CO_2 appeared to be related to the activity of the cytosolic enzyme phospho*enol*pyruvate carboxylase (PEPC), which was proposed to compete with the chloroplastic import of phospho*enol*pyruvate (PEP) for the production of isoprenoid compounds (Rosenstiel et al., 2003; 2004). A negative correlation between isoprene emission rate and PEPC activity was also observed in *P. alba* leaves treated with ozone, high temperature and high light (Loreto et al., 2007). To investigate relationships when drought stress is added to high growth temperature between isoprene and respiration, and if it involves variations in the metabolism of extrachloroplastic pyruvate, dark respiration (R_d) and PEPC activity were measured during the treatment, and were also related to isoprene emission rates. Pre-stress phospho*enol*pyruvate carboxylase (PEPC) activities were twofold higher in plants grown at 25°C then in plants grown at 35°C (Figure 11). The PnPEPC activity decreased by approximately 25% in plants grown at 25°C after a 35-day drought stress, whereas no decrease occurred in plants grown at 35°C. After a 15-day recovery from drought, PnPEPC activity increased in plants grown at 25°C, while no significant change was recorded in plants grown at 35°C.

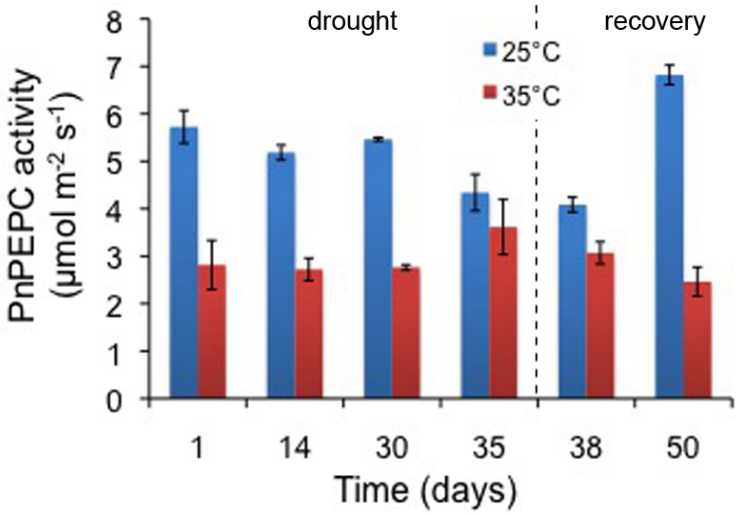

Figure 11. Effect of Drought Stress on PnPEPC Activity.

Phospho*enol*pyruvate carboxylase activity in leaves of black poplar plants grown at 25°C (blue bars) and 35°C (red bars), during a 35-d-long drought stress and 3 and 15 days after re-watering. Bars represent means ± SD (n = 5).

Across the drought stress period and the subsequent recovery, PnPEPC activity was negatively correlated with isoprene emission only in plants grown at 35°C (Figure 12). A positive relationship was instead observed in plants grown at 25°C, but only during the drought stress treatment. During re-watering a similar isoprene emission was not related to contrasting PnPEPC activities. Isoprene emission rates correlated positively with dark respiration rates (R_d) for the whole duration of the drought stress (Figure 13). Before the onset of stress, respiration rates were similar in plants grown at 25 and 35°C, even though their isoprene emission rates were different. Respiration rates decreased to similarly low values at the end of drought stress, in leaves with similar values of isoprene emission.

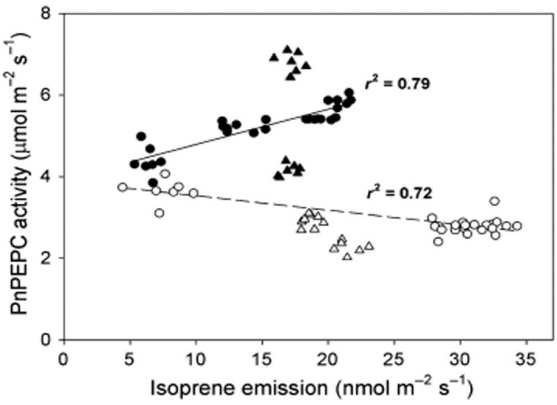

Figure 12. Correlation Between Black Poplar Phospho*enol*pyruvate Carboxylase (PnPEPC) Activity and Isoprene Emission.
Relationship between PnPEPC activity and isoprene emission in leaves of black poplar plants grown at 25°C (black symbols) and 35°C (white symbols). Single data points collected during the 35-day-long drought stress are shown as circles, whereas data points collected 3 and 15 days after re-watering stressed plants are represented with triangles. Regression lines were generated using the values at pre-stress conditions and during drought stress, excluding values during recovery and are shown for the data collected at the two different temperatures ($P < 0.05$).

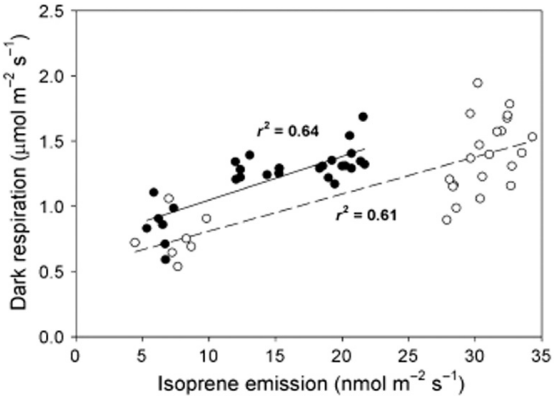

Figure 13. Correlation Between Dark Respiration and Isoprene Emission.
Relationship between dark respiration and isoprene emission in leaves of black poplar plants grown at 25°C (black symbols) and 35°C (white symbols), during the 35-day-long drought stress. Regression lines were generated for the data collected at the two different temperatures ($P < 0.05$).

2. Discussion on temperature-dependent isoprene emission under drought

Previous research conducted in the laboratory suggested that isoprene emission is sustained under drought stress conditions (Sharkey and Loreto, 1993; Tingey et al., 1981) and is not inhibited

by drought-induced stomatal closure (Monson and Fall, 1989). A similar conclusion was reached more recently in field experiments with whole plants exposed to a slowly developing drought stress (Funk et al., 2004; Pegoraro et al., 2004). Biochemical studies (Brüggemann and Schnitzler, 2002) also support this conclusion by showing that ISPS activity in isoprene-emitting oak species is not affected under severe drought stress. However, a recent report on isoprene emission by *P. alba* under soil water limitation indicated that ISPS activity is indeed sensitive to drought, at least in drought-sensitive poplars, and that isoprene emission can be limited at the posttranscriptional level during a drought stress (Brilli et al., 2007). The same report showed that, under severe drought stress conditions, isoprene could be produced from carbon shunted in the pathway from sources other than the chloroplastic, photosynthesis-dependent DOXP/MEP pathway. Drought stress is expected to be exacerbated by the simultaneous occurrence of higher temperatures because of current and future climate change (Centritto et al., 2002; IPCC, 2007). Temperature is the main factor controlling isoprene emission (Loreto and Sharkey, 1990), through transcriptional (Mayrhofer et al., 2005) and biochemical activation of ISPS (Monson et al., 1992). High temperatures, stimulating isoprene emission, might counteract, at least to a certain extent, the inhibition of emission due to concurrent drought stress. Our experiments were planned to investigate the impact of the interaction between rising temperatures and drought stress on isoprene emission. This is likely to be relevant for process-based models that account for stress

effects to predict the effects of global climate change on isoprenoid emissions and, therefore, their future impact on atmospheric pollution (Grote and Niinemets, 2008). As drought developed, inhibition of stomatal conductance and photosynthesis was much more pronounced than that of isoprene emission. This finding confirms that isoprene biosynthesis is even more resistant to drought stress than photosynthesis (Brüggemann and Schnitzler, 2002; Monson and Fall, 1989; Sharkey and Loreto, 1993), and that isoprene emission rates only decrease when photosynthesis is already strongly inhibited by drought (Brilli et al., 2007; Sharkey and Loreto, 1993). More interestingly, our data show that the effects of drought occur independently of growth temperature.

In fact, this study reveals that water limitation overrides the physiological effects of growth at high temperature on isoprene emission during the severe phase of stress and after recovering from drought. This is a surprising result that rules out the hypothesis that increases in growth temperature can alleviate the inhibition of isoprene in drought-stressed leaves.

We searched for possible molecular mechanisms responsible for drought-induced changes in isoprene biosynthesis and emission in plants grown and measured at the two different temperatures. The activity of ISPS, the enzyme catalyzing the conversion of DMADP into isoprene (Schnitzler et al., 2005; Silver and Fall, 1991, 1995), is known to decrease under drought stress in poplar leaves (Brilli et al.,

2007). In unstressed leaves of black poplar, PnISPS activity reflected the dependence of isoprene emission on the plant's growth temperature, as previously reported (Mayrhofer et al., 2005; Monson et al., 1992). To further illustrate this effect, we plotted isoprene emission against PnISPS activity (Figure 10). Isoprene emission correlated with PnISPS activity and data were fitted by a regression curve describing a typical enzyme-controlled relationship. High rates of isoprene emission, such as those recorded in leaves of non-stressed or mildly stressed plants grown at 35°C, were only made possible by coordinately high levels of PnISPS activity. However, in plants grown at high temperatures and characterized by high isoprene emission rates, specific activity of PnISPS decreased during the early stages of drought stress. This decrease might have been caused by one or more, as yet unknown, regulation factors such as the binding of the catalytic site of ISPS protein, or a modulation of post-translational regulation elements.

Irrespective of the actual reason for the inhibition of PnISPS activity in black poplar, this was not reflected by a concurrent inhibition of isoprene emission in the same leaves, indicating that translational limitations to isoprene biosynthesis are unlikely at early stages of drought stress. On the other hand, the absence of negative effects of early drought on isoprene emission was paralleled by maintenance of transcript levels of Pn*ISPS* and the amount of PnISPS protein, indicating that isoprene biosynthesis is controlled at the transcriptional or post-transcriptional level under moderate stress conditions. Interestingly, the decrease in isoprene emission in plants

grown at 25°C, after 30 days of drought stress, was mirrored by a similar reduction of Pn*ISPS* mRNA transcript level and of PnISPS protein concentration. Thus, we hypothesize that during a period of drought isoprene emission is sustained until the stress reduces the capacity to produce the ISPS enzyme, irrespective of leaf temperature. Uncoupling of *ISPS* mRNA and ISPS protein levels is not unique to drought-stressed plants. A similar finding was reported, for instance, in response to diurnal cycles (Mayrhofer et al., 2005; Wilkinson et al., 2006) or to high temperatures (Wiberley et al., 2008).

In our treatments, plants were drought-stressed until photosynthesis was completely inhibited. When drought stress became so severe as to inhibit photosynthesis altogether, isoprene emission was also strongly reduced. Brilli et al. (2007) demonstrated that, under these conditions, the residual emission of isoprene could be attributed to carbon sources entering the DOXP/MEP pathway from sources other than photosynthesis (Lichtenthaler et al., 1997). We show that the photosynthesis-independent isoprene emitted by severely drought-stressed leaves is independent of temperature, since the rates of emission are similar at 25 and 35°C. This result suggests that the alternative source of carbon for isoprene is not affected by temperature. Activity of PnISPS was higher in plants grown at 35°C than in those grown at 25°C, but in both cases was very low after a 35-day drought stress. The low activity of PnISPS might be responsible for the absence of temperature stimulation of isoprene emission under serious drought conditions. However, as

discussed below, PnISPS activity does not explain why the temperature sensitivity of isoprene emission is not restored after re-watering. It is also remarkable that about half of the PnISPS protein was still present after 35 days of stress, when PnISPS activity and gene expression were dramatically inhibited and isoprene emission was four- to six-fold less than under pre-stress conditions, depending on temperature. This result suggests that PnISPS protein has a slow turnover as indicated in Loivamäki et al. (2007b), confirming an earlier indication obtained under different growth conditions (Brilli et al., 2007). The data also imply that the turnover of PnISPS protein might be less affected by growth temperature.

In black poplar plants recovering from a severe drought stress, isoprene emission did not recover to pre-stress levels (Figure 7B). In other cases a complete recovery, and even a stimulation of isoprene emission, was found in plants re-watered after a drought stress (Brilli et al., 2007; Brüggemann and Schnitzler, 2002; Pegoraro et al., 2004; Sharkey and Loreto, 1993). The recovery of isoprene emission was incomplete, particularly in plants grown at 35°C, and emitting constitutively high rates of isoprene before the onset of stress. Since photosynthesis completely recovered from drought stress at both temperatures (Figure 7A), the incomplete recovery of isoprene emission was not caused by a low supply of photosynthetic substrate into the DOXP/MEP pathway. Interestingly, data points for drought recovery do not fit the general relationship between isoprene emission and PnISPS activity identified in Figure 10, indicating that the relationship observed prior to drought stress was no longer

applicable. In plants grown at 35°C and recovering from drought, PnISPS activity was similar to that observed when the drought stress was mild, but the isoprene emission rate was much lower. This is a further indication that severe drought overrides elevated temperature in determining the rate of isoprene emission, and may even remove the positive effect of temperature once the stress is relieved. Moreover, comparative analyses of the properties of PnISPS with actual isoprene emission rates during re-watering demonstrate that plants grown at high temperatures may undergo a coordinated inhibition of PnISPS, which is not rapidly recovered, and may experience other drought induced metabolic limitations that suppress the well-known temperature dependence of isoprene emission. Whether this reflects a simple and transient down-regulation or a true and unrecoverable inhibition of isoprene biosynthesis remains to be determined, possibly using genetic and proteomic tools, and performing longer-term experiments, to understand which are the genes specifically involved in ISPS regulation and in substrate supply.

We found that dark respiration and isoprene emission rates (Figure 13) are also directly related during a prolonged drought stress treatment, independently of growth temperature. This confirms earlier indications obtained under different growth conditions (Loreto et al., 2007) that respiration does not compete with isoprene for phospho*enol*pyruvate (PEP) in the cytosol. The enzyme PEPC was recently identified as an important branch-point of substrate partitioning between isoprenoid biosynthesis and other,

extrachloroplastic competing processes (Rosenstiel et al., 2003; 2004). Pyruvate may also be an important source of extrachloroplastic carbon for isoprene biosynthesis, as shown by intra-molecular labeling studies (Lichtenthaler et al., 1997; Schnitzler et al., 2004). Loreto et al. (2007) also reported that isoprene and PEPC activity are inversely correlated. We found a similar relationship only in plants grown at high temperatures, both during the drought treatment and the recovery. However, isoprene and PnPEPC activity were positively correlated in poplar leaves grown at 25°C, at least during drought stress. Thus, temperature can dramatically change the regulation of extra-chloroplastic carbon feeding into the DOXP/MEP pathway during a drought-stress episode. At high temperature, isoprene synthesis is stimulated and effectively competes with extra-chloroplastic processes. When prolonged drought affects isoprene biosynthesis, however, PnPEPC may more successfully drain PEP to synthesize oxaloacetate in anaplerotic reactions driving respiration in the cytosol. At low temperature, isoprene synthesis is limited by the properties of PnISPS (Monson et al., 1992), and high PnPEPC activity may effectively support both respiration and isoprene biosynthesis along the entire gradient of drought stress.

Interestingly, recovery from drought appears to temporarily unbalance the metabolic control of PnPEPC on isoprene observed at low temperature. In fact, in plants grown at 25°C, low PnPEPC activity persisted after a 3-day recovery, when isoprene emission had already recovered significantly to pre-stress rates.

In summary, we provided biochemical support for the observation that isoprene biosynthesis and emission is insensitive to moderate drought stress. Our data indicate that the stability of transcriptional and post-transcriptional processes leading to ISPS biosynthesis may play a decisive role in determining the response of isoprene to the early stages of drought, in plants grown at both high and low temperatures. Isoprene is related to respiration even under drought stress conditions, but we show evidence that PEPC activity may control isoprene emission during drought only at low temperature. The recovery of isoprene emission after re-watering drought-stressed plants may be subjected to different regulatory mechanisms from those occurring in drought-stressed leaves. Isoprene emission may be restricted by a coordinated down-regulation, or inhibition, of the characteristics of ISPS, from the transcriptional to the posttranslational level, and by the onset of as yet unknown limitations at the substrate level. This is particularly evident in plants grown at high temperatures in which isoprene emission and ISPS protein concentration and specific activity are constitutively high. Drought episodes may therefore reduce the emission of high emitters. Since isoprene is a useful molecule contributing to thermal (Singsaas et al., 1997) and antioxidant protection (Loreto and Velikova, 2001), and probably also to protection against biotic stress factors (Laothawornkitkul et al., 2008; Loivamäki et al., 2008), drought stress may have important and unforeseen consequences in regulating isoprene-mediated plant interactions with the changing global environment.

Drought stress appeared to have a profound influence on the percentage of photosynthetic carbon spent by plants to make isoprene (I/*A* ratio). Previous studies showed that in non-stressed plants *ca.* 1–2% of the photosynthetically fixed carbon is emitted as isoprene (Monson and Fall, 1989; Sharkey et al., 1991; Baldocchi et al., 1995; Harley et al., 1999), whereas under stress conditions I/*A* may exceed 30% (Sharkey and Loreto, 1993; Fang et al., 1996). Pegoraro et al. (2004) found that the proportion of carbon spent as isoprene increased during water-stress periods, with peak values that exceeded 50%. Similarly to the results observed in our studies on drought stress, those peaks were the result of a major reduction in photosynthesis (close to zero), while isoprene emission remained high. However, the percentage of carbon lost as isoprene clearly increased from the beginning through the whole drought treatment. Because of the strong dependence of isoprene emission on temperature, this carbon loss may become even larger in areas with warm climate, where drought-induced stomatal closure may have a large indirect effect on isoprene by increasing leaf temperature. In a climate change scenario with higher temperatures and prolonged droughts, the ratio I/*A* could dramatically increase with significant impact on the global terrestrial carbon balance, especially in regions, such as the tropics, which are estimated to contribute more than 80% of the annual isoprene flux (Jacob and Wofsy, 1988; Zimmerman et al., 1988; Guenther et al., 1995).

VI

ISOPRENE SYNTHASE Overexpression Reveals a Dual Role of Isoprene in the Plant Response to Stress, Priming the Defense System and Helping Membrane Stability

1. Introduction on the *ISPS* overexpression in Arabidopsis plants: a good tool for understanding the protecting roles of isoprene

Isoprene, a volatile compound synthesized by the chloroplastic enzyme ISOPRENE SYNTHASE (ISPS), may improve plant thermotolerance and protect against ozone possibly by "directly" scavenging ROS and quenching ozone products or by intercalating into membrane lipids and changing their properties. We have used Arabidopsis (*Arabidopsis thaliana*) plants engineered to express an *ISPS* gene (with introns) from kudzu (*Pueraria montana* var. *lobata*), generating the *IspS* lines, to test the proposed mechanisms by which isoprene emission protects against high temperature and ROS. It was shown that unstressed *IspS* lines maintain a higher level of H_2O_2, compared to the wild-types, which may prime the defense response, activating a signal cascade started with the activation of specific heat-shock transcription factors (*HSF21*, *HSFA2*, *A3*, and *A4c*) and heat-shock proteins (*HSP18.2*), and was followed by the regulation of genes directly involved in the response to oxidative stress conditions (ascorbate peroxidases, catalases, and superoxide

dismutases). Membrane properties were investigated by low-temperature chlorophyll fluorescence and circular dichroism, which indicated, respectively, that the *ISPS* overexpression induced changes in the energy distribution between the two photosystems and increased the thermal stability of the ordered macro arrays of the pigment-protein complexes in the thylakoid membrane. Both methods indicated a significant effect of isoprene on membrane properties. It is concluded that isoprene plays a dual role: in non-stress conditions it may act indirectly, priming plant response against high temperature and oxidative stresses by the activation of H_2O_2 signalling; then, when an oxidative or temperature stress occurs, isoprene may act directly, both by detoxifying ROS and intercalating into lipids to increase the membrane stability.

2. Results

2.1 Characterization of Arabidopsis ISPS *overexpressing plants*

Sharkey et al. (2005) successfully transformed *Arabidopsis* (ecotype Wassilewskija) with the kudzu ISPS genomic sequence under the control of the Pro35S constitutive promoter, to generate the *Arabidopsis ISPS*-overexpressing *IspS* lines. Using the published DNA sequence for kudzu *ISPS* (GeneBank accession number AY316691), primers in the carboxy-terminal region specific to +949 and +1645 from start codon were designed. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using 1 µg and increasing amounts of total RNA from wild-type and *IspS* plants.

Densitometry indicated the absence of *ISPS* messenger RNA in wild-type plants, whereas in the sense *IspS* plants the amount of mRNA reached saturation point at the lowest amount analyzed (0.25 µg of total RNA, Figure 14A).

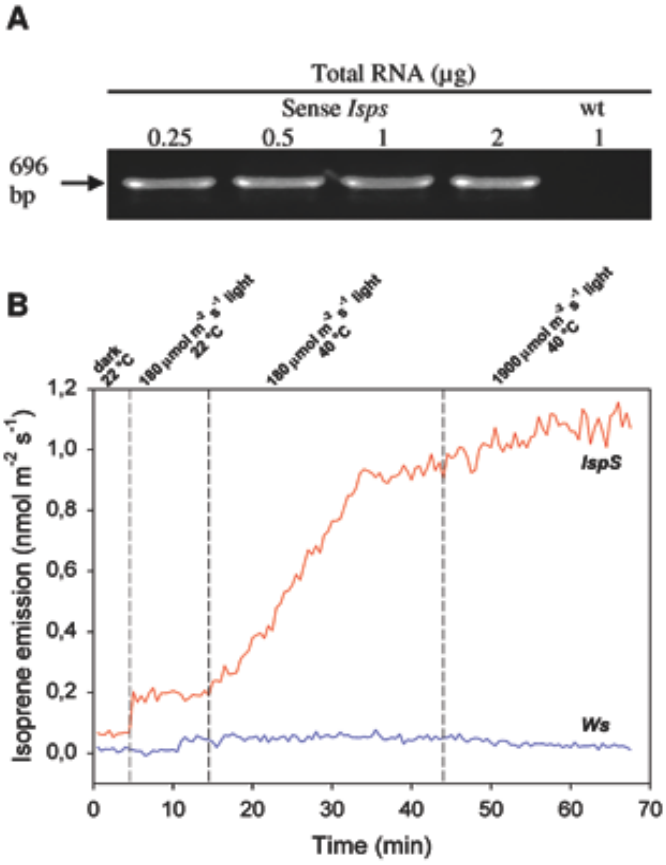

Figure 14. *ISPS* Overexpression and Isoprene Emission in *IspS* Plants.
A. RT-PCR specific for *ISPS* mRNA was carried out with increasing amount of sense *IspS* line total RNA, and with 1 µg of total RNA of wild-type (wt). Products were separated on 1% agarose gel (equal loadings per line).
B. Temperature and light dependence of the isoprene emission measured by PTR-MS on 35-d-old ws and *IspS* plants.

To check if the *ISPS* overexpression triggered isoprene emission, a dynamic time-course of this response before and after increasing temperature and light intensity was followed by proton transfer reaction-mass spectrometry (PTR-MS) measurements (Figure 14B). Isoprene emission was not detected from wild-type plants upon

increasing light intensity and temperature. *IspS* plants displayed a low emission of isoprene in the dark and at 22°C air temperature (less than 0.1 nmol m^{-2} s^{-1}), and an emission around 0.2 nmol m^{-2} s^{-1} under growth conditions (180 µmol m^{-2} s^{-1} light intensity and 22°C air temperature). However, *IspS* plants showed the typical response of isoprene to temperature and light, as isoprene increased to 0.9 nmol m^{-2} s^{-1} after few minutes of exposure to high temperature (40°C air temperature and 180 µmol m^{-2} s^{-1} light intensity) and to 1.1-1.2 nmol m^{-2} s^{-1} when the light intensity was also increased (to 1,900 µmol m^{-2} s^{-1}). Photosynthesis (*A*) measured at growth conditions, was similar in wild-type and *IspS* plants (Table II). Moreover, Arabidopsis *IspS* and wild-type leaves exhibited a similar maximal photochemical efficiency at physiological conditions (Table II).

Table II. Gas Exchange Measurements and Maximum Quantum Yield of PSII in Wild-type (*Ws*) and *IspS* Leaves.

Line	NT	HT	R_{HT}	HT+HL	R_{HT+HL}
			A ($\mu mol\ m^{-2}\ s^{-1}$)		
Ws	8.6 ± 0.6^{a}	4.6 ± 0.3^{b}*	6.6 ± 0.2^{c}*	3.3 ± 0.4^{d}*	4.1 ± 0.1^{e}*
IspS	9.1 ± 0.5^{A}	7.9 ± 0.5^{C}	10.2 ± 0.3^{D}	6.4 ± 0.6^{E}	9.7 ± 0.8^{AD}
			Fv/Fm		
Ws	0.80 ± 0.03^{d}	0.64 ± 0.02^{c}*	0.61 ± 0.02^{c}*	0.24 ± 0.01^{a}*	0.46 ± 0.01^{b}**
IspS	0.81 ± 0.02^{E}	0.73 ± 0.01^{C}	0.76 ± 0.01^{D}	0.36 ± 0.01^{A}	0.62 ± 0.02^{B}

Assimilation rate (*A*) in mature (35-day-old) leaves of *Ws* and *IspS* plants. Measurements of chlorophyll fluorescence (F_v/F_m) were performed on 15 minutes dark-adapted leaves of wild-type *Ws* and *IspS* plants. NT = not-treated plants (23°C and 180 $\mu mol\ m^{-2}\ s^{-1}$ light intensity); HT = high temperature stress; R_{HT} = HT + 24 h of recovery; HT + HL = oxidative stress (high temperature plus high light); R_{HT+HL} = HT + HL followed by 24 h of recovery.
Asterisks (* and **) represent a statistical difference with $P < 0.05$ or 0.01, respectively, between means of different lines at the same treatment (n = 8 ± SD). Superscript letters indicate a statistical difference with $P < 0.05$ between plants of the same line at different treatments.

Wild-type and *IspS* plants were subjected to quantitative *real time* PCR (qPCR) assay to determine the mRNA transcript level of genes involved in isoprene biosynthetic pathway (Figure 15A). There was a marked increase in the amount of mRNA level of all genes up-stream *ISPS* in the sense *IspS* plants with respect to wild-type plants.

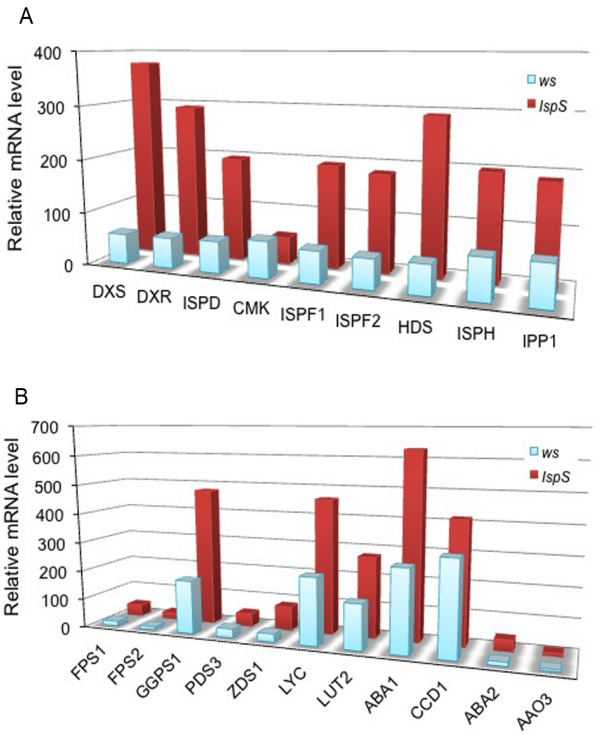

Figure 15. Quantitative Analysis of mRNA Transcript Level of Genes Involved in the Isoprenoids Biosynthetic Pathway.
Expression levels were assayed by qPCR on mature (35-d-old) wild-type (Ws) and transgenic (*IspS*) plants. Relative amounts were normalized with respect to *ACTIN8* expression level (= 100%). Bars represent means ± SD (n = 5-8).
Note: two different scales are used in graphs.
A. qPCR analysis of genes involved in the chloroplastic isoprene biosynthesis.
B. qPCR analysis of genes involved in the carotenoid-abscisic acid biosynthesis.

The only one down-regulated gene was *4-(CYTIDINE-5'-DIPHOSPHO)-2-C-METHYL-D-ERYTHRITOL KINASE* (*CMK*), that converts 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol into 2-phospho-4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol.

The phosphorylation of 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol may therefore be an important step to control the biosynthesis of isoprene and other isoprenoids. Expression profile for genes involved in the carotenoid-abscisic acid biosynthetic pathway was also performed by qPCR analysis (Figure 15B). Sense *IspS* plants showed a high increase (at least twofold) in the mRNA amount of: *GERANYLGERANYL PYROPHOSPHATE SYNTHASE 1*

(*GGPS1*), one of the key enzymes involved in the biosynthesis of diterpenes, carotenoids, tocopherols and phylloquinone; ζ-*CAROTENE DESATURASE* (*ZDS*), involved in the reduction of ζ-carotene to lycopene; *LYCOPENE β-CYCLASE* (*LYC*) and *LYCOPENE ε-CYCLASE* (*LUT2*), by which trans-lycopene is converted respectively to δ- and γ-carotene; and *ZEAXANTHIN EPOXIDASE 1* (*ABA1*), that operates the first step of the abscisic acid biosynthesis. Sense *IspS* plants also showed an increase in the mRNA amount of *CAROTENOID CLEAVAGE DIOXYGENASE 1* (*CCD1*) and short-chain *DEHYDROGENASE REDUCTASE 1* (*ABA2*) genes, codifying for two enzymes involved in the formation of abscisic aldehyde, but no increase was observed in the mRNA amount of *ABSCISIC ALDEHYDE OXIDASE 3* (*AAO3*) gene, that encodes the enzyme catalyzing the conversion of abscisic aldehyde to abscisic acid.

Table III. Leaf Carotenoid/Xanthophyll Profile and Chlorophyll of Wild-type (*Ws*) and *IspS* Lines.

Line	Chl *a*	Chl *a*/*b*	Vx	Ax	Lut	Zx	β-car	Total car.
Ws	1,075.9 ± 86.9	2.6 ± 0.1	15.2 ± 1.1	19.4 ± 1.1	61.7 ± 2.8	3.5 ± 0.8	614.1 ± 30.8	713.9 ± 9.3
IspS	998.4 ± 3.4	2.7 ± 1.1	16.1 ± 1.6	20.7 ± 2.5	69.8 ± 5.8	2.8 ± 0.4	598.3 ± 76.1	707.7 ± 10.1

Values are the means ± SD (n = 8), expressed in μg g Fw^{-1}, of mature (35-d-old) plants grown at 22°C air temperature and 180 μmol m^{-2} s^{-1} light intensity. Vx = violaxanthin; Ax = antheraxanthin; Lut = lutein; Zx = zeaxanthin; β-car = β-carotene.

To investigate if the *ISPS* overexpression-induced up-regulation of the genes involved in carotenoid biosynthesis leads to an increased size of carotenoid pools in the *IspS* plants, carotenoid pigments (xanthophylls and β-carotene) were extracted and analyzed by high performance liquid chromatography (HPLC). Wild-type and *IspS* plants showed the same levels of carotenoid pools (Table III).

To study the role of isoprene in energy transfer inside photosystem II (PSII) the low temperature (77 K) chlorophyll fluorescence emission spectra from isolated thylakoid membranes of wild-type and *IspS* leaves were measured. In this assay, two different isolation buffers (described in Chapter IX, 3.8) were used: Buffer A, containing Hepes, and Buffer B containing Tris. The ratios F735/F685 (an index of energy distribution between the two photosystems) and F695/F685 (an index of energy transfer between chlorophyll-protein complexes in the LHCII-PSII super-complex) were calculated (Table IV). Both wild-types and *IspS* plants do not differ markedly in the energy distribution (Buffer A). However, wild-type reacts to different extents to minor perturbations, such as by washing and suspending the membranes in low concentrations of Tris (Buffer B), which appears to induce a partial dissociation of the oxygen evolving complexes from the PSII supercomplexes. The *IspS* membranes appear to be more resistant to this treatment.

Table IV. Low Temperature (77 K) Fluorescence Measurements in Wild-type (*Ws*) and *IspS* Thylakoid Membranes.

Line	Suspension Buffer			
	Buffer A		Buffer B	
	F735/F685	F695/F685	F735/F685	F695/F685
Ws	1.33 ± 0.08*	0.87 ± 0.03*	1.93 ± 0.24*	1.55 ± 0.09*
IspS	1.60 ± 0.07	0.94 ± 0.03	1.42 ± 0.18	1.11 ± 0.02

Fluorescence measurements are shown as the means ± SD (n = 8) of F735/F685 and F695/685 ratios and were performed in two different suspension buffers (see Chapter IX, 3.8). Asterisks (*) indicate a statistical difference with $P < 0.05$ between the same measurements on different lines.

2.2 ISPS overexpression induces the activation of plant oxidative stress response system

Overexpression of the *ISOPRENE SYNTHASE* gene in *Arabidopsis* plants triggered significant changes in the hydrogen peroxide content when grown in control conditions, as indicated by the higher level of H_2O_2 detected in *IspS* plants as compared to the wild-types (Figure 16A).

Elevated levels of H_2O_2 were associated with higher contents of thiobarbituric acid reactive substances (TBARS) in *IspS* plants (Figure 16B). This indicates a higher membrane lipid turnover in the *IspS* plants. Elevated levels of H_2O_2 were also associated with lower levels of total ascorbic acid (AsA) content in *IspS* plants compared to the wild-types (Figure 16C). Even though *IspS* plants displayed higher H_2O_2 and lower AsA levels in control conditions, these plants

showed no differences in the dehydroascorbate (DhA) content (Figure 16D). *IspS* plants also showed a lower redox state, compared to the wild-types (Figure 16E).

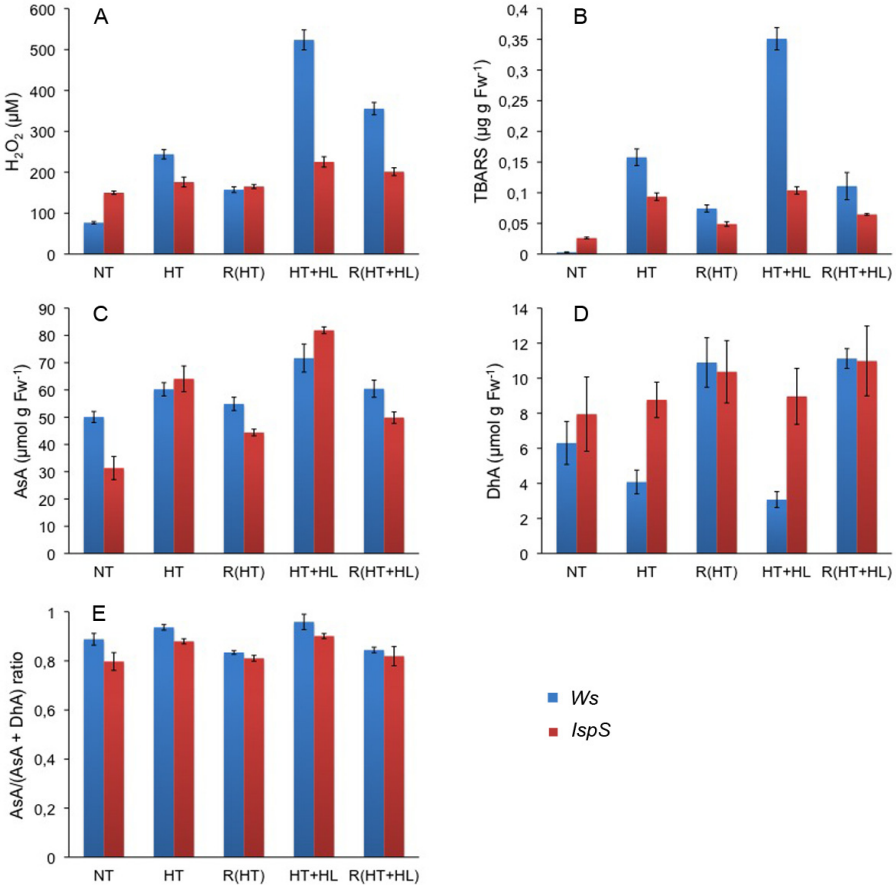

Figure 16. Biochemical Characterization of *IspS* Plants.
All measurements were done in both wild-type (*Ws*) and *IspS* 35-d-old plants before and after high temperature and oxidative stress conditions. Bars represent the means ± SD (n = 4-8). Note: NT = not-treated plants; HT = high temperature treatment (48 h at 40°C); R(HT) = 24 h recovery from HT; HT + HL = combined high temperature and high light treatment (3-d at 40°C and 1,000 µmol m^{-2} s^{-1} of light); R(HT+HL) = 24 h recovery from HT + HL.
A. Hydrogen peroxide (H_2O_2) levels.
B. TBARS content.
C. Total ascorbic acid (AsA).
D. Dehydroascorbate (DhA) levels.
E. Redox state, given as AsA/(AsA + DhA) ratio, of both wild-type and *IspS* plants.

IspS plants showed higher mRNA transcript level of Fe*SUPEROXIDE DISMUTASE 1* (Fe*SOD1*) than wild-type plants. In chloroplasts, superoxide dismutase catalyzes the production of H_2O_2 from singlet oxygen (Asada and Badger, 1984). Moreover, *IspS* plants showed higher mRNA expression levels, compared to wild-type leaves, of *CATALASE 1* and *3* (*CAT1* and *CAT3*), that catalyze the H_2O_2 scavenging in peroxisomes (Figure 17A); chloroplastic *ASCORBATE PEROXIDASE 1* (*APX1*) and *STROMAL ASCORBATE PEROXIDASE* (*sAPX*), that detoxify H_2O_2 excess using ascorbate for reduction (Foyer and Halliwell, 1976). The expression levels of *THYLAKOIDAL ASCORBATE PEROXIDASE* (*tAPX*) mRNA were similar in the two lines (Figure 17B). Evidences have been presented that, in Arabidopsis, heat-shock transcription factors (HSF) play a central role in plant response to heat-shock and high temperature stress by the activation of specific target genes responsible for the defense against temperature changes, e.g. heat-shock proteins (Schöffl et al., 1998a; 1998b; Nover et al., 2001). Quantitative mRNA expression levels of *HSFA4a* (*HSF21*), *HSFA2*, *HSFA3*, and *HSFA4c* (*RHA1*), were measured. Sense *IspS* plants showed an increased expression level of all heat-shock factors mRNA in comparison to wild-type plants (Figure 17C). The main mechanism by which HSF act is the transcriptional activation of specific target genes in response to different stimuli, particularly activating the transcription of heat-shock proteins (HSP). Expression level of *HSP18.2* mRNA was also analyzed by a qPCR analysis. This

protein is activated in response to high temperature or high H_2O_2 levels (Takahashi and Komeda, 1989). *IspS* plants showed higher *HSP18.2* expression levels (about three-fold) compared to wild-type plants (Figure 17C), indicating that HSF up-regulation, induced by *ISPS* overexpression, triggered transcriptional activation of heat-shock proteins specifically involved in the response to heat and oxidative stress.

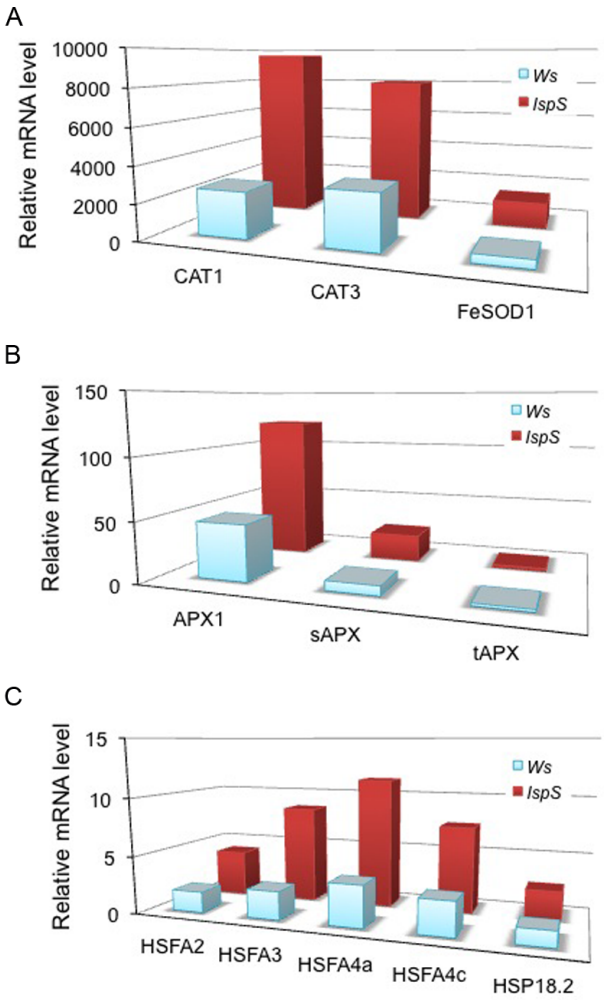

Figure 17. Effect of *ISPS* Overexpression on the Expression Level of Genes Involved in the High Temperature and Oxidative Stress Response.
Expression levels were measured by a qPCR assay and normalized with respect to *ACTIN8* expression level (= 100%). The assay was performed on wild-type (*Ws*) and *IspS* 35-d-old plants in control conditions. Bars represent the means ± SD (n = 4-8).
Note: different scales were used in graphs.
A. Quantitative mRNA expression level for different genes codifying ROS scavenging enzymes.
B. Quantitative mRNA expression level for cytosolic 1, stromal and thylakoidal ascorbate peroxidase genes.
C. Quantitative mRNA expression level for some heat-shock factors and heat-shock protein 18.2 genes.

2.3 ISPS *overexpression-induced thermal protection*

We tested whether *IspS* plants, that emit isoprene, are better protected from high-temperature stress. Five-weeks-old wild-type and *IspS* plants were exposed to 40°C at 180 µmol photons m^{-2} s^{-1} light intensity for 48 hours, and then returned to growth chamber conditions for 24 hours to follow the recovery from stress. At the end of the treatment, leaves of the wild-type plants showed more severe visible foliar injuries, e.g. extensive necrosis of the leaf lamina, than *IspS* plants (Figure 18). The heat-stress-induced H_2O_2 concentration increased in wild-type leaves (about twofold with respect to the pre-stress level). In the *IspS* plants, however, H_2O_2 levels were significantly lower than in wild-type plants (Figure 16A). As expected, wild-type plants also showed considerable membrane damage as indicated by the increment of TBARS. However, TBARS level in *IspS* plants increased after the treatment, but to lower extent, compared to the wild-types (Figure 16B). After a 24-h recovery, both H_2O_2 and TBARS decreased in

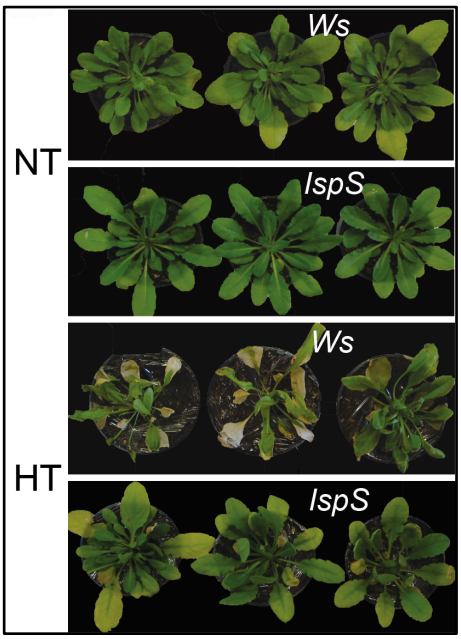

Figure 18. *IspS* Leaves Subjected to High Temperature.
Pictures were taken 1 hour after the end of treatment. Only representative groups of all plants used for the experiments are shown. Note: NT = not-treated plants; HT = high temperature stress: representative photographs of 35-d-old wild-type (*Ws*) and *IspS* plants grown for 48 hours at 40°C.

wild-type plants, but their level remained significantly higher than in pre-stress conditions (Figures 16A and B). High temperature stress resulted in an increase of total AsA content in all plants, which was partially restored after 24 h of recovery (Figure 16C), whereas only wild-type plants showed a decrease of DhA level (Figure 16D). However, *IspS* plants displayed no variation in the DhA content after the treatment, also showing a lower redox state of membranes, compared to wild-types (Figure 16E).

Photosynthesis measured after the treatment declined less and recovered faster (after 24 hours) in *IspS* plants than in the wild-types (Table II). In fact, photosynthesis of *IspS* plants was even stimulated during the recovery from heat-stress with respect to pre-stress measurements (Table II). Stomata remained open during the stress and did not control photosynthesis reduction. The maximal photochemical efficiency in dark-adapted plants was more negatively affected in wild-type than in *IspS* plants (Table II). Interestingly, after 24 h from the treatment the Fv/Fm ratio of wild-type plants did not recover yet (Table II). Clearly, state transition and photochemical quenching of fluorescence were still contributing to reduce the fluorescence yield, especially in *IspS* leaves, after a short dark-adaptation (Walters and Horton, 1991).

To investigate the possible role of isoprene in the stabilization of chloroplastic membranes subjected to high temperatures (Sharkey and Yeh, 2001), measurements of circular dichroism (CD) spectra were performed in wild-type and *IspS* plants after 10 min at 30, 40, 45, 50, 55 and 60°C. Circular dichroism measures the difference in

the extinction of left-handed *versus* right-handed circularly polarized light which arise due to structural asymmetry. Circular dichroism spectroscopy in the visible range is a valuable tool for probing the molecular architecture of the complexes and supercomplexes and their macro-organization in the membrane system (Garab, 1996). In leaves, characteristic CD bands originate from short-range, excitonic interactions, e.g. at around (+)440 and (-)650 nm, originating from PSI and LHCII, respectively, and the so called Psi-type bands (Psi, polymer salt-induced), at around (+)505, (-)675 and (+)694 nm, which originate from chirally organized macrodomains, i.e., the long-range chiral order of the complexes and chromophores. The Psi-type bands usually correlate with the macro-organization of LHCII (Garab and Mustardy, 1999), but ordered arrays of LHCII-PSII supercomplexes also contribute to these CD signals (Kovács et al., 2006). Circular dichroism spectroscopy has been used to determine the thermal stability of the chiral macrodomains and the LHCII trimers (Garab et al., 2002; Dobrikova et al., 2003). It has also been shown that the structural flexibility of LHCII macrodomains depends largely on the lipid environment of the complexes (Simidjiev et al., 1998).

As shown in Figure 19, the amplitudes of the Psi-type CD bands at 48°C were considerably lower in the wild-type than in the *IspS* leaves, while the 440 and 650 nm excitonic bands were hardly affected. The apparent amplitude of this latter band is also influenced by the overlap from the broad Psi-type bands in the red. It was also observed that the transition temperature of the main band, at 694 nm (marked by arrow), was shifted to higher temperature in the *IspS*.

They were found at 39.5 and 47.3°C in the wild-type and *IspS* leaves, respectively (Figure 19, inset). No significant difference could be seen between the *IspS* and wild-type leaves in the trimer-to-monomer transition temperatures of LHCII, between 55 and 60°C, tested with the $CD_{482\text{-}470nm}$ excitonic band pair (Garab et al., 2002). These data show that the *ISPS* overexpression significantly and specifically increased the thermal stability of the chiral macrodomains, i.e., the ordered array of the supercomplexes in the Arabidopsis thylakoid membranes. Higher thermal stability of *IspS* plant membranes supports the hypothesis of a protective role of isoprene against the alteration of membrane stability triggered by high temperature.

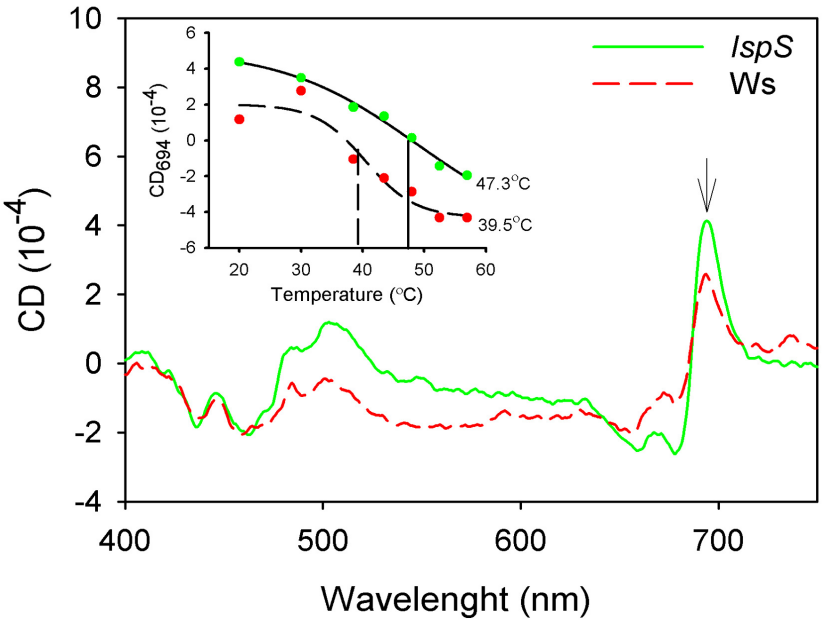

Figure 19. Circular Dichroism (CD) Spectroscopy Measurements.

Circular dichroism spectra at 694 nm, measured at different wavelengths at 48°C, are shown. All CD measurements were performed on thylakoidal membranes extracted from Ws (dashed red line) and *IspS* (continuous green line) mature leaves. Peaks at 694 nm for wild-type (Ws) and *IspS* plants are indicated with a black arrow.

Inset. Intact leaf samples were adapted at 20°C and subjected to increasing temperature (30, 40, 45, 50, 55, and 60°C). For each incubation temperature the CD spectra at 694 nm was measured. All measurements were done on mature 35-d-old wild-type (red dots) and *IspS* (green dots) leaves. The experiment was repeated at least three times, of which only a representative one is shown.

2.4 Effect of oxidative stress on IspS *plants*

The possibility that overexpression of *ISPS* gene might be able to confer protection against a stronger oxidative stress than the high temperature treatment was tested in plants exposed to a combination of high temperature and high light, to mime an oxidative stress. Mature (35-d-old) wild-type and *IspS* plants were exposed to 40°C air

temperature and 1,000 µmol m^{-2} s^{-1} light intensity for 3 days. At the end of treatment, H_2O_2 and TBARS levels were highly increased in wild-type plants but not in *IspS* plants (Figures 16A and B, respectively) indicating that lipids of membranes of wild-type plants were largely damaged by the treatment. After 3 days of exposure to 40°C and 1,000 µmol m^{-2} s^{-1} photons, total ascorbate content of both wild-type and *IspS* plants increased, although to a significantly higher extent in the *IspS* plants (Figure 16C). Previous studies demonstrated that growth under high light might induce the accumulation of ascorbate in leaves (Foyer et al., 1983). *IspS* plants displayed no variation in the DhA content, but in wild-type plants, as expected, DhA decreased after the treatment (Figure 16D). Moreover, *IspS* plants showed a lower redox state, compared to the wild-types, as shown by the AsA/(AsA + DhA) ratio (Figure 16E). These results, like for the high temperature treatment, suggest that *IspS* plants were less affected by oxidative stress conditions with respect to the wild-types. Increased level of total ascorbate in the transgenic plants, during the stress, was not caused by the increased reduction of dehydroascorbate, but, probably, by an increased expression of either *APX* genes or APX activity. Three days of oxidative stress conditions caused a strong decline in the photosynthesis of wild-type plants compared to the pre-stressed values (~58%), while, in the *IspS* plants, this decline was less (~28% of the pre-stress value). In both lines the decline of photosynthesis was not caused by stomatal closure. Photosynthesis and chlorophyll fluorescence were also measured after a 24 h recovery from the

combined high light and high temperature stress in the two lines. *IspS* plants showed a faster recovery compared to wild-type plants (Table II). *IspS* plants also displayed a lower decrease in the maximal efficiency of PSII, compared to wild-type plants, showing a faster and more complete recovery of the Fv/Fm ratio (Table II). At the end of experiment, wild-type plants were clearly damaged, showing large leaf necrosis. On the other hand, *IspS* plants were still alive and showed only marginal leaf necrosis (Figure 20).

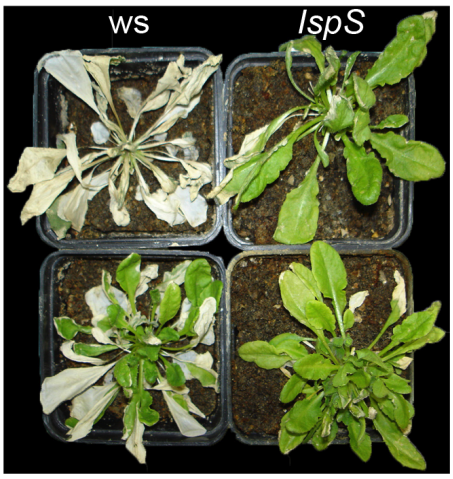

Figure 20. *IspS* Leaves Subjected to Oxidative Stress.
Pictures were taken 1 hour after the end of treatment. Only representative groups of all plants used for the experiments are shown. Representative photographs of 35-d-old wild-type (Ws) and *IspS* plants grown for 3-d at 40°C and 1,000 µmol m^{-2} s^{-1} light intensity are presented.

3. Discussion: what isoprene synthase overexpression reveals about isoprene biological functions?

3.1 Characterization of IspS *plants*

Generation of transgenic *Arabidopsis* plants, transformed with the kudzu *ISOPRENE SYNTHASE* gene (Ku*ISPS*) under the control of the constitutive promoter Pro35S (*IspS* plants) resulted in plants able to synthesize and emit isoprene. These plants constitutively overexpressed the *ISPS* gene, triggering an isoprene emission

regulated by temperature and light (Monson et al., 1992), as shown by the real time, *in vivo* monitoring of isoprene emission (Figure 14B). This result supports the use of Arabidopsis as a model plant to study the impact of isoprene biosynthesis and emission on plant biology and plant interactions with the environment or other organisms. Interestingly, *IspS* plants did not show significant phenological and phenotypical differences compared to wild-type plants in most of the physiological parameters investigated under growth conditions. Wild-type and *IspS* plants showed similar photosynthesis and stomatal conductance, maximal photochemical efficiency of PSII, chlorophyll and carotenoid contents. Other studies showed that Arabidopsis transgenic plants transformed to emit isoprene are characterized by faster growth rate and increased weight of rosette leaves compared to wild-type plants (Loivamäki et al., 2007; Sasaki et al., 2007). The different ISPS gene used for the transformation may cause these contrasting results. In our study the *ISPS* gene from *Pueraria montana* (var. *lobata*) was used, while both Loivamäki et al. (2007) and Sasaki et al. (2007) used Arabidopsis plants that were transformed with the isoprene synthase of poplars: *Populus* x *canescens* and *Populus alba*, respectively. Moreover, while the full-length cDNA sequence of *ISPS* gene was previously cloned into Arabidopsis plants (Loivamäki et al., 2007, Sasaki et al., 2007), we used instead the entire isoprene synthase genomic sequence. Even if all transformations resulted in an overexpression of the gene, and, consequentially, in isoprene emission by plants, in our case, the presence of introns in the *ISPS* gene may cause

different post-transcriptional regulation, with respect to the cDNA sequence.

3.2 ISPS overexpression regulates the isoprenoid pathway genes and the flux of carbon into isoprenoids

Overexpression of the *ISPS* resulted in an up-regulation of the mRNA expression level (at least twofold) of all genes involved in chloroplastic isoprenoid biosynthesis, indicating a positive feedback of enhanced isoprene production for the genes up-stream and down-stream isoprene biosynthesis. The only one gene down-regulated by the isoprene synthase overexpression was *CMK*. In *Arabidopsis*, this kinase plays a key role in the chloroplastic development and function and affects the steady-state mRNA level of different mitochondrial electron transport genes (Hsieh et al., 2008). Considering the strong transcriptional up-regulation of all genes of the isoprene biosynthetic pathway, the phosphorylation of 4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol in 2-phospho-4-(cytidine-5'-diphospho)-2-C-methyl-D-erythritol may be the key regulatory step in the balance of dimethylallyl diphosphate (DMADP) biosynthesis and in the formation of more complex isoprenoids. Our data in fact also show that, despite up-regulation of transcript levels, production of a new isoprenoid into *IspS* leaf cells did not cause changes in the content of photosynthetic pigments formed through the DOXP/MEP pathway and total chlorophyll, suggesting that there is no competition between isoprene and other isoprenoids for carbon sources. The carbon source for isoprene biosynthesis is principally photosynthesis (Monson and Fall,

1989; Lichtenthaler et al., 1997). Cross talk with extrachloroplastic sources of carbon, often reported in the literature (Bick and Lange, 2003; Laule et al., 2002) appear to be very limited in Arabidopsis (Mandel et al., 1996; Budziszewski et al., 2001; Gutierrez-Nava et al., 2004; Guevara-Garcia et al., 2005; Hsieh and Goodman, 2006, 2006; Hsieh et al., 2008). Mature Arabidopsis plants showed a photosynthetic rate of *ca* 8.7 μmol m^{-2} s^{-1} while the maximal isoprene emission rate was *ca* 1,000 times less. Isoprene emission probably does not alter the flux of photosynthetic carbon into the biosynthesis of different isoprenoids and in the photosynthetic metabolism.

3.3 ISPS overexpression regulates the antioxidant system

Higher TBARS content in rosette leaves is generally believed to be a direct consequence of the peroxidation of polyunsaturated fatty acids upon ROS attack on the acyl chains of polyunsaturated fatty acids, highly abundant in thylakoid membranes, producing α,β–unsaturated aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). We were surprised to find a higher TBARS level in *IspS* plants compared to wild-types, indicating a faster membrane lipid turnover. This result is consistent with the higher H_2O_2 levels of *IspS* plants in growth conditions. However, it should be noted that H_2O_2 levels remained in the physiological range in *IspS* plants (e.g. 60 μM-7 mM in Arabidopsis; see Karpinski et al., 1999; Neill et al., 2002). While these levels of H_2O_2 are unlikely to cause oxidative stress, they cause differences in the redox balance between transgenic and wild-type plant cells (see Figure 3E). In chloroplasts, H_2O_2 is mainly

produced by the disproportionation of superoxide ($O_2^{\cdot-}$), a reaction catalyzed by superoxide dismutase during the Mehler reaction. Excess of H_2O_2 in the cell is neutralized by the activity of different ROS-scavenging enzymes, in particular ascorbate peroxidases and catalases (Asada, 1999; for review see Veljović-Jovanović, 1998). The higher H_2O_2 level in *IspS* plants, in control conditions, may be due to a limitation in the APX enzymes activity triggered by the decreased availability of ascorbate. Accumulation of non-toxic H_2O_2 levels is known to induce the activation of various defense-related mechanisms, acting as a signalling molecule for different proteins, such as ascorbate peroxidase (Karpinski et al., 1997; Panchuk et al., 2002; Devletova et al., 2005), glutathione *S* transferase, phenylanine ammonia lyase, and heat-shock factors and proteins (Levine et al., 1994; Desikan et al., 1998; Neill et al., 1999; Grant et al., 2000; Vandenabeele et al., 2003; Volkov et al., 2006).

Indeed, the activation of specific HSF may be the primary mechanism by which H_2O_2 acts as signal molecule. In *Drosophila melanogaster* and human cells increased H_2O_2 levels induced the activation of HSF trimerization (Zhong et al., 1998; Ahn and Thiele, 2003). In Arabidopsis, H_2O_2 regulated the oxidative stress response of APX1 enzyme by the activation of HSF21 (HSFA4a) (Devletova et al., 2005), and also induced an up-regulation of *HSP17.6* and *HSP18.2* mRNA expression level (Volkov et al., 2006). Moreover, overexpression of *HSF3* resulted in the up-regulation of *APX1* and *sAPX* genes (Panchuk et al., 2002). *IspS* plants showed higher mRNA expression level, compared to the wild-types, of four different

heat-shock factors (*HSFA2*, *A3*, *A4a*, and *A4c*) involved in the plant response to high temperature and oxidative stress conditions (Nover et al., 2001; Zimmermann et al., 2004; Devletova et al., 2005; Fortunati et al., 2008b). Equally up-regulated were the genes of those enzymes activated by HSF, namely APX1 and sAPX, as well CAT1, 3, and FeSOD1. These results support the hypothesis that higher H_2O_2 levels induced by isoprene biosynthesis in *IspS* plants activated a signal cascade that primes plant defense mechanisms against heat- and oxidative-stress damages.

3.4 IspS *plants have enhanced resistance to thermal and oxidative stress*

Davison et al. (2002) reported that overexpression of β-carotene hydroxylase gene in Arabidopsis was able to increase the content of xanthophylls and total carotenoids, enhancing plant resistance to oxidative stress (1,000 μmol photons m^{-2} s^{-1} and 40°C air temperature). Under similar experimental conditions, overexpression of the *ISPS* gene also enhanced high light and high temperature stress resistance, but without a concurrent increase of carotenoids. These results indicate a specific protective role of isoprene against environmental stress conditions, uncoupled from the classical antioxidant functions of non-volatile carotenoids synthesized in the chloroplasts (Niogy, 1999). The hypothesis that overexpression of *ISPS* gene leads to enhanced tolerance to high temperature and/or oxidative stress conditions via the regulation of genes involved in the plant response to stress and via changes in the redox balance of the

plant cells, was therefore investigated. As also observed by other authors (Behnke et al., 2007; Sasaki et al., 2007), *IspS* plants showed a higher resistance to the high temperature stress compared to wild-types. More interestingly, we show that this protective effect is even stronger when plants are subjected to a high light intensity stress that exacerbates the oxidative pressure. At the end of these treatments, *IspS* plants were clearly less damaged than wild-types as evidenced by physiological (photosynthesis and chlorophyll fluorescence) and biochemical (H_2O_2, TBARS and AsA content), and as also clearly indicated by visual inspection of leaf necroses (Figure 5). *IspS* plants not only clearly displayed a high ROS scavenging efficiency under stress conditions, as previously observed with either oxidative (Loreto and Velikova, 2001) or thermal stress conditions (Velikova and Loreto, 2005). These plants also recovered photosynthetic rates and fluorescence values faster than wild-type plants, indicating an overall low damage of photosynthetic structures under stress conditions (Walters and Horton, 1991). As briefly highlighted above, we surmise that the alteration of H_2O_2 content observed in *IspS* plants under control conditions can play a role in priming plant protection against stresses. Isoprene-emitting leaves of *IspS* plants, exhibiting genetically regulated higher levels of H_2O_2, have constitutively enhanced capacity to generate antioxidants upon stress occurrence. In general, changes in the redox balance of leaves were observed comparing transgenic and wild-type plants in control conditions. In fact, *IspS* plants displayed a lower AsA/(AsA + DhA) ratio, compared to wild-types (Figure 16E).

Another proposed mechanism of action of isoprene is based on the capacity of this molecule to directly stabilize the thylakoid membranes (Sharkey and Yeh, 2001). The localization of ISPS protein in the stromal side of thylakoidal membranes (Wildermuth and Fall, 1998; Schnitzler et al., 2005), and the hydrophobic nature of isoprene are expected to assist with its partition into photosynthetic membranes (Siwko et al., 2007). Thylakoid membranes become leaky to protons at high temperature (Pastens and Horton, 1996; Bukhov et al., 1999; Schrader et al., 2004) and lipophylic isoprene partitioned into membranes can prevent the formation of water channels responsible for the membrane leakiness at high temperature (Singsaas et al., 1997; Sharkey et al., 2001). Isoprene could also enhance hydrophobic interactions within thylakoids and thereby stabilize interactions between lipids and/or membrane proteins during episodes of heat-shock or high temperature stress conditions (Sharkey and Yeh, 2001). We bring two further lines of evidence in support of this theory. First, results obtained with the low temperature fluorescence measurements suggested that in the absence of isoprene the oxygen evolving complex is unpaired. Preservation of the structure of thylakoid membranes in *IspS* plants may be due to enhanced monomer to oligomer forms of light harvesting complex (LHC) (Apostolova et al., 2006). Second, the experiment using the CD spectroscopy, a new biophysical approach for studying isoprene, showed a higher thermal stability for *IspS* plants thylakoid membranes compared to wild-types. In fact, the transition temperature of the disassembly of the chiral

macrodomains in *IspS* membranes was shifted *ca* 8°C to higher temperatures compared to the wild-type, clearly indicating the higher stability of *IspS* membranes at high temperatures.

In conclusion, in this study we aimed to characterize the *IspS* plants that are able to emit isoprene. Our results suggest that isoprene emission promotes a signal transduction mediated by H_2O_2, which results in the activation, in non-stress conditions, of the antioxidant system that protects against high temperature and oxidative stress. We also confirm with new biophysical data the possible direct role of isoprene in membrane stabilization, possibly by intercalating into lipid tails of thylakoidal membranes (Siwko et al., 2007). Previous studies attributed the protective role of isoprene to its scavenging properties when reacting with ozone and ROS (Loreto and Velikova, 2001; Loreto and Fares, 2007) during different environmental stress conditions. It can be hypothesized that, in the *IspS* plants, isoprene plays a dual role: in non-stress conditions it may act indirectly, priming plant response against high temperature and oxidative stresses by the activation of H_2O_2 signalling; then, when an oxidative or temperature stress occurs, isoprene may act directly both by detoxifying ROS and intercalating into lipids to increase the membrane stability (Figure 21). Genetic engineering (applied to isoprene biosynthesis) is a valuable tool to study mechanisms by which isoprene helps plants cope with stress, and represents a potentially powerful way to generate plants better adapted to harsh environments.

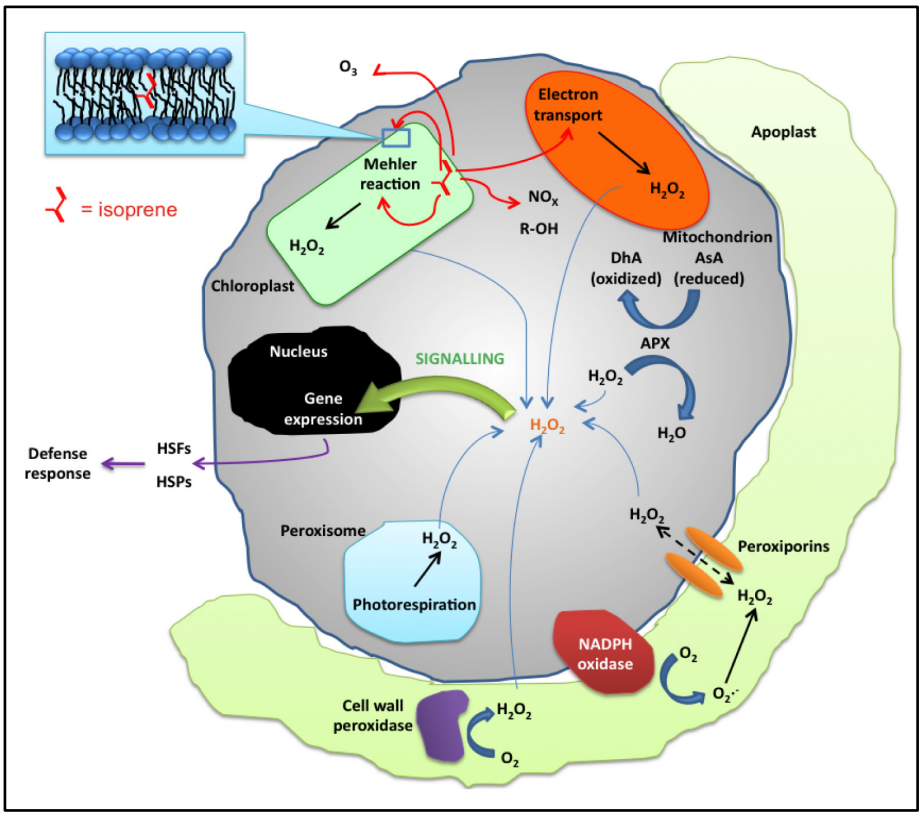

Figure 21. Function and Possible Interactions of Isoprene in the Plant Cell.
Isoprene is produced by DMADP as substrate in a reaction catalyzed by the ISPS enzyme in the chloroplast. Different may be the roles played by isoprene in the plant defense against oxidative damages (red arrows), helping the membrane stability by intercalating in the lipid tails; detoxifying ROS and nitric oxide excess; or interacting with potentially dangerous molecules such as ozone. Overexpression of *ISPS* gene and, consequentially, the constitutive isoprene biosynthesis, changes the redox balance, increasing chloroplastic H_2O_2 content by an alteration of the Mehler reaction, photorespiration, and the mitochondrial electron transport. Hydrogen peroxide levels are also determined by the activity of APX via the ascorbate-glutathione cycle and peroxisomal CAT, NADPH oxidase, and cell wall peroxidases (blue arrows). Hydrogen peroxide may reach the cytosol from the apoplast diffusing across membrane lipids or via membrane channels, such as peroxiporins. Hydrogen peroxide increased levels in the *IspS* plants, acting as a signal molecule (green arrow), activate the plant response against oxidative stress conditions via the up-regulation of specific HSF and HSP (purple arrows).

VII

TPS27 Overexpression in Arabidopsis as a Suitable Tool for Studying the Physiological and Molecular Role of (*E*)-β-caryophyllene

1. Introduction and experimental set-up on *TPS27* overexpressing plants

The main aim of this study was to assess whether (*E*)-β-caryophyllene emission has a protective role in leaves against environmental stress conditions, and, in particular, against short and long term heat stress. The physiological, biochemical and molecular responses of *Arabidopsis thaliana* plants overexpressing the *(E)-β-CARYOPHYLLENE SYNTHASE* (*CARS*) gene in leaves (Gershenzon et al., unpublished), during and after a long heat stress exposure, were investigated. We wanted in particular to assess the ability of transformed plants to tolerate environmental stresses and the extent to which those stresses have damaged the photosynthetic apparatus that is able to produce sesquiterpenes. Heat stress-induced damage is mediated by ROS, such as superoxide and H_2O_2. Variations in the quantity of this flavonoid are involved in the protection against ROS. The size of the anthocyanin pool was measured, as well as the quantity of chlorophyll *a* and *b* and carotenoids. We followed the stress-induced membrane lipid peroxidation, by quantifying the thiobarbituric acid reactive substances (TBARS) accumulation, during the different phases of

stress. Finally, the transcriptional expression of some genes involved in the chloroplastic and cytosolic isoprenoid biosynthetic pathways was followed.

2. Results

2.1 Stress-induced membrane damages are delayed in the CarS *plants*

To study the biological functions of caryophyllene overproduction in plants, transformed Arabidopsis *CarS* lines were subjected to heat stress conditions (48 hours at 37°C), and the first 48 hours of recovery were also followed. No significant differences in the total carotenoid content were observed between wild-type and transformed *CarS* plants in pre-stress conditions. Heat stress induced a slight decline in the wild-type total carotenoid content after 48 hours exposure, while *CarS* plants did not showed significant variation overall the treatment (Figure 22A). Stress-induced anthocyanin accumulation in the wild-type was already evident after 24 hours of heat stress exposure, continuously increasing during the stress, but declining to constant levels when plants were allowed to recover at growth temperatures (Figure 22B). On the other hand in the *CarS* plants, stress induced significant anthocyanin accumulation only in the late stages of exposure (after 48 hours), decreasing progressively during the recovery.

Accumulation in leaf tissues of malonyl dialdehyde (MDA), a secondary product of membrane fatty acid peroxidation, has been considered to be a reliable marker of stress-induced membrane

damages. We found that heat stress induced a significant increase of the MDA accumulation in the wild-type plants (an increase of about 2.5 times after a 48 hour exposure), only partially recovered when plants were transferred back to the growth chamber (Figure 22C). Otherwise, no significant alterations in the membrane status were found in the *CarS* plants, suggesting that cellular membranes of the *CarS* plants were more protected against heat stress-induced lipid peroxidation with respect to the wild-type membranes.

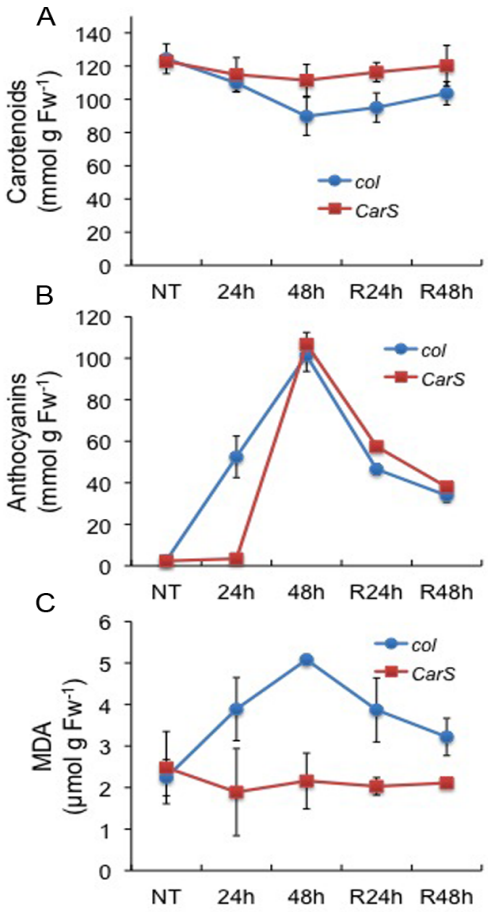

Figure 22. Stress-Induced Membrane Damage of *CarS* Plants.
Quantification of stress-related compounds during and after 48 hours of heat stress (37°C) in the wild-type (*col*) and transgenic *CarS* 35-d-old plants.
A. Total carotenoids content.
B. Anthocyanins quantification.
C. Malonyl dialdehyde (MDA) quantification.
Note: NT = not treated control plants; 24h and 48h represent plants after 24 and 48 hours of heat stress, respectively; R24h and R48h represent plants recovered in growth chamber conditions for 24 and 48 hours, respectively.

Both chlorophyll (chl) *a* and chlorophyll *b* contents were significantly higher in the *CarS* plants, in pre-stress conditions, than in the wild-types. Though total chlorophyll concentration was higher in the *CarS* plants,

these differences did not seem to significantly affect the chl *a*/*b* ratio. Chl *a* and *b* content of both wild-type and *CarS* plants decreased after 24 hours stress exposure, remaining constant overall the treatment, and showing only a partial recovery after the stress (Figure 23A). The ratio between chlorophyll *a* and chlorophyll *b* contents it is an important tool to know if the photosystems are damaged, and, in physiological conditions, this ratio should be around 2.5 for a correct functioning of the photosystems. Although the total amount of chlorophylls (*a* + *b*) was higher in the *CarS* plants, the ratio was the same than in the wild-type. This ratio strongly differed only after 24 hours of recovery, when it decreased in the *CarS* plants, in comparison to the pre-stress values, reflecting a decrease of chl *a* and a concurrent increase of chl *b*.

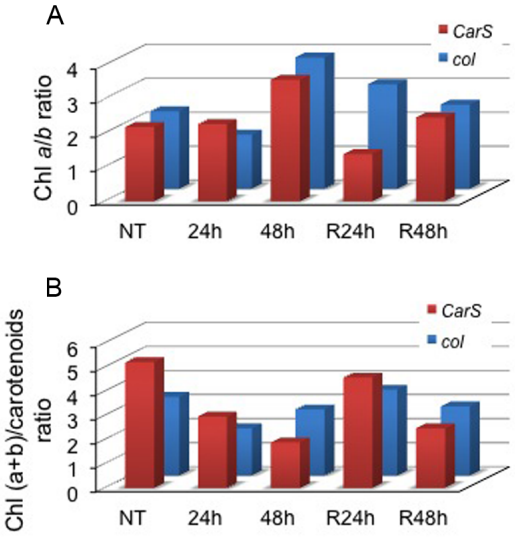

Figure 23. Effect of Heat Stress on Total Chlorophyll and Carotenoid Content in *CarS* Plants.
A. Chlorophyll (Chl) *a*/*b* ratio.
B. Chl (*a* + *b*)/carotenoids ratio.
Note: NT = not treated control plants; 24h and 48h represent plants after 24 and 48 hours of heat stress, respectively; R24h and R48h represent plants recovered in growth chamber conditions for 24 and 48 hours, respectively.

The Chl (*a* + *b*)/Carotenoids ratio, in pre-stress conditions, was about 60% higher in the *CarS* plants compared to the wild-types, but was similar in both lines during the stress (Figure 23B). This result

suggests that less carotenoids were available, per unit of chlorophylls, in the *CarS* plants with respect to the wild-type once, for both light harvesting and dissipation of non-photochemical excess energy. On the other hand, this ratio due the disproportional decrease of total chlorophyll and carotenoids content gradually decreased in both *CarS* and wild-type plants.

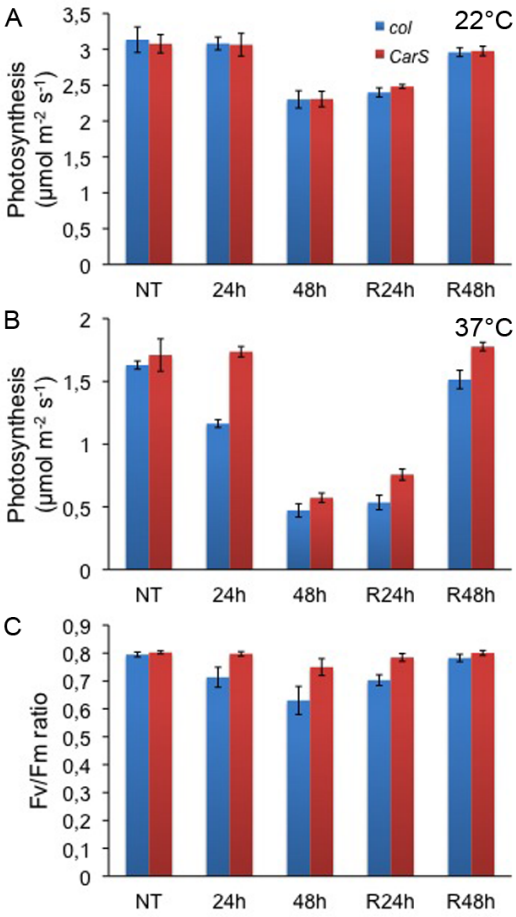

Figure 24. Photosynthesis of *CarS* Plants is Resistant to Heat Stress.

A. Photosynthesis measured at 22°C.

B. Photosynthesis measured at 37°C.

C. Chlorophyll fluorescence measured as Fv/Fm ratio.

Note: NT = not treated control plants; 24h and 48h represent plants after 24 and 48 hours of heat stress, respectively; R24h and R48h represent plants recovered in growth chamber conditions for 24 and 48 hours, respectively.

A significant reduction of photosynthetic carbon fixation rate, measured at 22°C, was detected only after 48 hours of heat stress, when photosynthesis was *ca* 40% of the pre-stress values), but without differences between the wild-type and *CarS* plants (Figure 24A). Carbon fixation rate measured at 37°C

progressively decreased in the wild-type plants overall the treatment, while in the *CarS* plants a significant decrease of carbon fixation rate was observed only after 48 hours, i.e. delayed with respect to the changes observed in the wild-type (Figure 24B). This trend was consistent with the progressive decrease of chlorophyll fluorescence (Fv/Fm) measured in the dark, which was much more evident in the wild-type than in the *CarS* plants (Figure 24C). This result reflects the photosystem II (PSII) impairment especially in the wild-type leaves, and to a lesser extent in the CarS leaves.

2.2 Molecular characterization of CarS *plants*

To characterize the *CarS* transgenic plants, four different transformed lines (L1, L6, L9 and L10) were grown for 35 days in growth chamber conditions. Phenological parameters were measured, such as primary root elongation and diameter, rosette leaf length and surface area, germination time. None of these parameters showed significant differences when comparing *CarS* and wild-type plants (data not shown).

Sesquiterpenes, such as (*E*)-β-caryophyllene, are synthesized via the cytosolic mevalonate-dependent isoprenoid biosynthetic pathway, by the condensation of isopentenyl diphosphate (IDP) and geranyl diphosphate (GDP). One of the key metabolite in this metabolic pathway is synthesized by a large family of farnesyl diphosphate synthase (FPS), which are present in Arabidopsis in two different isoforms: FPS1 and FPS2. FPS catalyzes the biosynthesis of farnesyl diphosphate (FDP) from isopentenyl diphosphate and

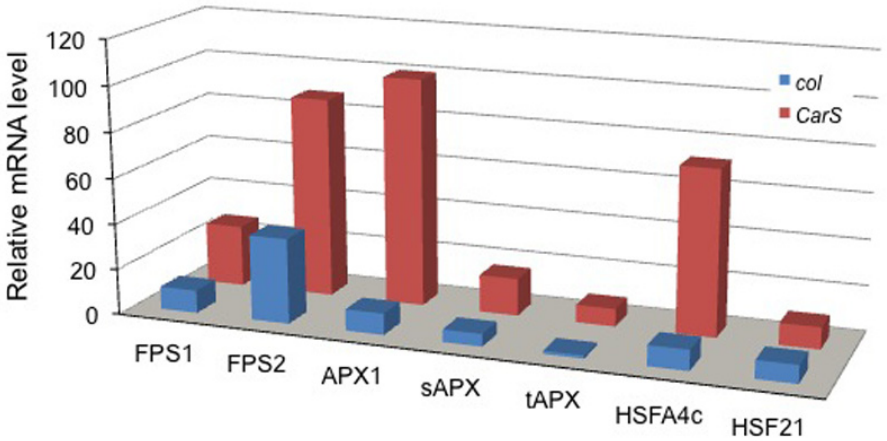

Figure 25. Expression Profile of Genes Related to Oxidative and Heat Stress Response.

Quantitative mRNA expression analysis of farnesyl diphosphate synthases (FPS1 and FPS2), ascorbate peroxidases (APX1, sAPX and tAPX), and the heat shock factors HSFA4c and HSF21. This analysis was curried out on 35-d-old wild-type (col) and transgenic CarS plants in control conditions.

Note: expression levels were measured by a qPCR assay and normalized with respect to *ACTIN8* expression level (= 100%).

geranyl diphosphate (GDP). This reaction may be considered as the rate-limiting step in the cytosolic isoprenoid biosynthesis (Cunillera et al., 1996). Overexpression of *CarS* gene in transgenic plants does not seem to be limited, at transcriptional level, by FPS; mRNA expression levels of *FPS1* and *FPS2* measured in control conditions were significantly higher in the *CarS* plants than in wild-type once (Figure 25).

If *CarS* plants are more resistant or tolerant to high temperature stress conditions, an improved production or an activation of the enzymes involved directly or indirectly in plant response to stress may occur. To investigate this, quantitative mRNA expression levels of ascorbate peroxidase genes (*APX1*, *sAPX* and *tAPX*; ascorbate

peroxidase cytosolic, stromal and thylakoidal, respectively), and of two different heat-shock transcription factors (*HSF21* and *HSFA4c*), involved in plant response to heat and oxidative stress, were measured (Figure 25). Ascorbate peroxidase detoxifies the excess of peroxides, such as hydrogen peroxide, using ascorbate as a substrate. This enzyme catalyzes the electron transfer from ascorbate to peroxide, producing dehydroascorbate and water as products. tAPX and sAPX are chloroplastic isozymes, bound respectively at the thylakoidal and stromal side of the thylakoid membranes, while APX1 is located in the cytosol. Stromal and thylakoidal APX expression can be involved in the response to oxidative stress. These genes were much more expressed in *CarS* than in wild-type plants, being the difference particularly evident for the stromal APX. mRNA transcript level of sAPX was approximately 4 times higher in *CarS* than in wild-type plants (Figure 25). It has been demonstrated that in the absence of APX1 the entire chloroplastic H_2O_2-scavenging system of *Arabidopsis thaliana* could collapse, followed by enormous H_2O_2 increase, and, finally, by protein oxidation (Devletova et al., 2005). Our results show that *CarS* transgenic plants have constitutively higher mRNA expression level of APX1, if compared to the wild-type levels. They also indicate that in Arabidopsis neither heat nor oxidative stress-induction of ascorbate peroxidase genes should be attributed to heat shock elements (HSE) sequences, the binding sites for HSF that are present in the promoter region of the APX genes (Storozhenko et al., 1998). To verify if a higher HSF mRNA expression level caused the

transcriptional up-regulation of APX1, tAPX and sAPX genes, a quantitative qPCR analysis of *HSF21* and *HSFA4c* was performed. *HSFA4c*, directly involved in the plant response to heat shock and heat stress conditions, was strongly up-regulated, with an expression approximately 5-fold higher in the *CarS* than in wild-type plants. No significant differences in the *HSF21* gene expression, activated by oxidative stress conditions, were found (Figure 25).

To investigate the heat stress-dependant expression of genes involved in the sesquiterpenes biosynthesis and in the response to stress, quantitative mRNA expression levels of *FPS2* and *APX1* genes, during and after a high temperature treatment, was measured. In particular, mature *CarS* and wild-type plants were incubated in a growth chamber at 37°C for 2 days, and the gene expression of *FPS2* and *APX1* was measured at different times (30 minutes, 1, 24, and 48 hours) of the heat stress treatment. Our results show that mRNA level of the wild-type *FPS2* gene rapidly decreased after 30 minutes at 37°C, maintaining the same expression levels along the entire treatment. *CarS* plants displayed a much higher *FPS2* gene expression level, in pre-stress conditions, with respect to the wild-type plants, but this expression drastically dropped just after 30 min of treatment, reaching levels similar to those of wild-type leaves (Figure 26A).

Cytosolic ascorbate peroxidase showed two different expression patterns in wild-type and *CarS* plants. In the wild-types, mRNA transcript level of *APX1* rapidly raised just after 30 minutes, reaching the maximal value after 24 hours of treatment, when the level was

20-fold higher than pre-stress levels. After 48 hours of heat stress the expression level of APX1 declined, again reaching physiological levels observed in pre-stressed leaves (Figure 26B). On the other hand, *CarS* plants showed a strong induction of mRNA expression level of the *APX1* gene already in pre-stress conditions (approximately 10-fold higher compared to wild-type values). However, during the treatment, *APX1* mRNA transcript level remained at the same levels, and decreased during the recovery period (Figure 26B).

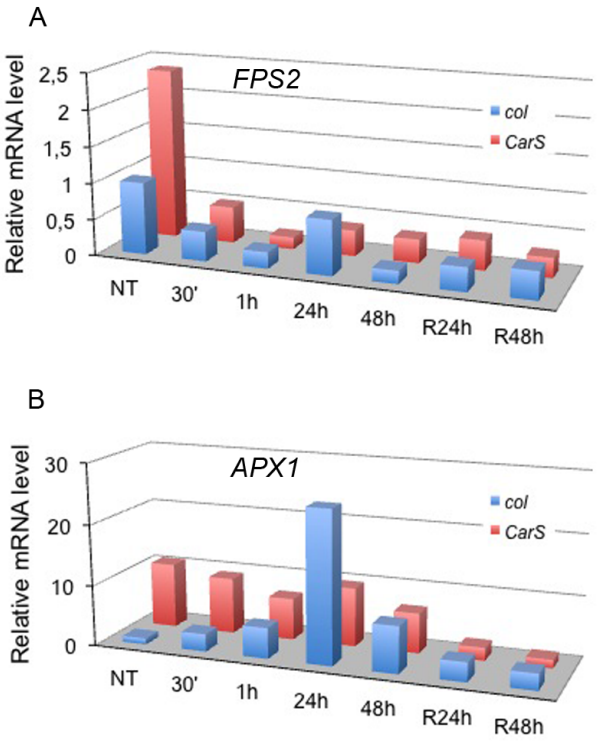

Figure 26. Expression Profile of FPS2 and APX1 During and After the Heat Stress.
Quantitative mRNA expression analysis of *FPS2* and *APX1* genes was curried out on mature 35-d-old wild-type *col* and *CarS* plants.
A. Relative mRNA level of FPS2.
B. Relative mRNA level of APX1.
Note: NT = control not-treated plants; 30', 1h, 24h, and 48h represent the incubation time of plants at 37°C; R24h and R48h represent the recovery period of 24 and 48 hours, respectively. Expression levels were measured by a qPCR assay and normalized with respect to *ACTIN8* expression level (= 100%).

3. Discussion on the thermal protection conferred by *TPS27* overexpression in Arabidopsis

Heat stress has a complex impact on cell function, suggesting that several processes are involved in the cellular response to high temperature and in acquiring thermotolerance. The ability to withstand heat and to acclimate to supra-optimal temperatures may be important as well in the prevention of heat damages as in the efficiency of the repair system of the more heat-sensitive cellular components. Prevention of stress-induced damage actively involves a large number of molecular and biochemical responses, initiated by the up-regulation of specific genes, to ensure timely and adequate antioxidant defense. These responses include enzymatic antioxidant activity, to neutralize stress-generated reactive oxygen species (ROS), as superoxide radicals or hydrogen peroxide (Panchuk et al., 2002), and the transcriptional activation of specific heat shock factors, specifically involved in the heat stress response (Nover et al., 2001).

Our results showed that photosynthetic carbon fixation rates assayed at elevated temperatures (37°C) were significantly lower than the values assessed at 22°C in both wt and *CarS* leaves. This reflects the well-known stimulation of photorespiration by high temperature due to the difference in the solubility of the two competing substrates of RuBisCo (O_2 and CO_2), in turn favoring RuBisCo oxygenation over carboxylation (Lea and Leegold, 1999) and enhancing cyclic electron transport processes. These processes are considered to be mechanisms protecting photosynthesis against

stress-induced damages (Ort and Baker, 2002). However, when assayed at 37°C net photosynthetic rates rapidly declined in the wild-type plants, already after 24 hours of stress exposure, whereas at the same period photosynthesis remained constant and similar to pre-stress levels in *CarS* plants. Thus, sustained photosynthesis of *CarS* plants under heat might either partially be attributed to either a different contribution of photorespiratory processes to photosynthesis or to enhanced thermotolerance ability. To investigate whether *CarS* plants were more tolerant to heat stress than the wild-types, the stress-induced membrane degradation status of these two plants was compared.

High temperature affects membrane-linked processes due to the alteration of membrane fluidity and permeability (Alfonso et al., 2001). Membrane damages may frequently occur because heat stress generation of ROS exceeds the capacity of the antioxidant network to cope with it, which may promote lipid peroxidation cascades (Girotti, 2001; Vellosillo et al., 2007). Lipid peroxidation results in the accumulation of malonyl dialdehyde (MDA), a secondary product of membrane unsaturated fatty acid peroxidation (Hodges et al., 1999, Girotti, 2001). High levels of MDA in plant tissues are correlated with high levels of oxidative damages to lipid membranes (Heath and Packer, 1968). Wild-type plants accumulated after 48 hours of stress exposure 2.5 times higher quantities of MDA as compared to pre-stress conditions. On the other hand, *CarS* plants showed an enhanced thermotolerance, maintaining membrane integrity overall the heat stress treatment, as shown by

the MDA levels of *CarS* leaves that displayed no significant variations overall the treatment.

Ascorbate peroxidases (APX) have been demonstrated to play a central role in the detoxification of ROS in plant cells (Asada, 1999; Mittler et al., 2004; Murgia et al., 2004; Devletova et al., 2005). Thus it is conceivable that the increased expression/activity of this enzyme is functionally linked to an increase in hydrogen peroxide concentration in the cytosol, especially during heat stress conditions. In our experiments it was shown that gene expression of all APX isoforms (cytosol and chloroplast localized) was enhanced in the *CarS* plants with respect to WT plants already in pre-stress conditions. Clearly this renders *CarS* plants more capable to cope with ROS, even when the ROS level is not that high. It may be also hypothesized that this activation of APX enzymes may have a dual function: beside acting as a protective agent, against hydrogen peroxide rise under stress conditions, it may regulate the hydrogen peroxide-dependent signaling pathway, and the consequent generation of heat shock response, especially under non stressful conditions (Panchuk et al., 2002). The heat shock response is primarily activated by heat shock transcription factors (HSF), which recognize specific DNA sequences found in the promoter regions of many *APX* genes, the heat shock elements (HSE). The transcriptional activation of target genes by HSF enables an increased expression of specific genes involved, directly or indirectly, in the response to heat stress conditions. Compared to wild-type, unstressed *CarS* plants show a higher mRNA transcript level of

HSFA4c, induced by high temperature, but not of *HSF21*, which is involved in the response to oxidative stress. The increased expression of these *HSF and APX* genes could confer the *CarS* plants a more sensitive protection against high temperature. However, the sequences of events and receptors by which the HSF-dependent expression of ascorbate peroxidase genes is activated remain prevalently unknown and need further investigations.

A good parameter to check the physiological status of plants during heat stress is the efficiency of photosystem II, which was shown to be particularly sensitive to stress caused by elevated temperatures (Berry and Bjorkman, 1980; Yordanov, 1986). The maximum efficiency of PSII is well described by the ratio of variable to the maximum chlorophyll fluorescence, Fv/Fm (Genty et al., 1989). In pre-stress conditions both wild-type and *CarS* plants showed a optimum Fv/Fm value, around 0.8, which allows us to infer that the lines were similar in their intrinsic photochemical efficiency. On the other hand, progressing heat stress induced a gradual decline in the Fv/Fm ratio only in the wild type. The decline was about 20% after 48 hours of exposure at 37°C (Figure 24C). The Fv/Fm ratio of *CarS* plants remained constantly high for the whole duration of heat treatment. A change in Fv/Fm is generally due to a change in the efficiency of non-photochemical quenching (Maxwell and Johnson, 2000), and values lower than the optimum (0.83, Björkman and Demmig, 1987) indicate impairement of photochemistry of photosynthesis due to photoinhibition. Photoinhibition was also shown to be a secondary effect of heat stress induced damages

(Berry and Bjorkman, 1980). These results suggest that not only general membrane stability is enforced in *CarS* plants, but also that thylakoids of transgenic, sesquiterpene emitting plants are less sensitive to heat stress-induced damages.

Total chlorophyll concentration in plants has a dominant control over the amount of solar radiation that a leaf absorbs; therefore its foliar concentration controls the photosynthetic potential and its photochemical reactions. Chlorophylls generally decrease under stress or during senescence and the ratio of Chl *a*/*b* changes with abiotic factors such as light (Fang et al., 1998). In particular, the concentration of Chl *a* is considered to be the limiting factor in the utilization of light for photosynthesis. Chlorophyll *a* was shown to be more intensely degraded than Chl *b* under stress exposure (Wolf, 1956). Thus, the reduction of Chl *a*/*b* ratio under stress conditions generally reflects an increased stress-induced bleaching of Chl *a* (Lichtenthaler et al., 1981, Lichtenthaler and Wellburn, 1983). Chlorophyll *a*/*b* ratios observed in both wild-type and *CarS* plants were relatively low, around 2.1 (Figure 23). These values are characteristic for plants grown in low light, like our Arabidopsis plants (see Chapter X, 4.1). Chlorophyll *a*/*b* ratio declined already after 24 hours of stress in the wild-type plants, while remained constant in the *CarS* plants. These results also support the observation that the photochemical apparatus of *CarS* plants is resistant to heat. The results are in fact consistent with other studies showing that in many plant species heat stress tolerance is associated with higher Chl *a*/*b* values, while sensitivity is generally accompanied by the decrement

of Chl *a* values, and thus of the Chl *a*/*b* ratios (Heckathorn et al., 2002). Absolute concentrations of both Chl *a* and *b* decreased also in *CarS* and wild-type plants, after 24 hours of heat exposure. However, we did not attribute this reduction to a stress effect, but rather to a fast adaptation of the plants that were transferred from growth chamber light intensity of 50 μmol m^{-2} s^{-1}, to the slightly higher 125 μmol m^{-2} s^{-1} light intensity of the treatment conditions.

The ratio between chlorophyll (*a* + *b*) and carotenoids is believed to be another sensitive marker of plant physiological status in many stress situations (Lichtenthaler et al, 2007). Our results show that in pre-stress conditions and in the initial phases of stress the chlorophyll/carotenoid ratio was higher in the *CarS* plants (Figure 23). This suggests that less carotenoid amount is available for either excess energy dissipation, photosynthetic functions or stress induced ROS neutralization, per unit of chlorophyll. Higher chlorophyll content may allow on one hand efficient light use of the transformed plants, but on the other hand also increases the possibility of excited chlorophyll triplet formation under saturating light conditions. Chlorophyll triplet formation may favor energy transfer reactions to molecular oxygen, leading to the formation of damaging reactive singlet oxygen molecules (Telfer and Barber, 1989; Vass et al., 1992; Macpherson et al., 1993; Telfer et al., 1994; Hideg et al., 2002).

The PS II maximal efficiency, carbon fixation rates and the lack of lipid peroxidation in the early phases of stress imply that *CarS* plants are protected through a compensatory yet unknown mechanism, independent of carotenoid protection (there was no difference in the

carotenoid pool of wild-type and *CarS* leaves before and during the treatment, see Figure 22A) and attributable to the overexpression of beta caryophyllene synthase. Whether this mechanism is mediated directly by the antioxidant action of (*E*)-β-caryophyllene, similar to that elucidated in the case of isoprene (see previous chapters) or is rather due to an indirect effect, e.g. resulting in the up-regulation of endogenous antioxidant system of transformed plants that allows plants to face oxidative stresses with a higher antioxidant-mediated protection (as indicated by the increased transcriptional level of *APX* genes in the *CarS* plants in pre-stress conditions) remains to be further studied.

It should be further remarked that a 24-h exposure to heat induced the accumulation of anthocyanins in wild type plants but not in *CarS* plants. Anthocyanins are water-soluble flavonoids, and represent the third major group of plant pigments in leaves. Anthocyanins can modify the light environment within the leaf and have the potential to regulate photosynthesis and limit the occurrence of photoinhibition and photo-bleaching (Barker et al., 1997), thereby acting as photoprotectants (Steyn et al., 2002; Close and Beadle, 2003), or antioxidants (Gould et al., 2002). *CarS* plants accumulated anthocyanins only in a late stress phase, after 48 hours, reaching values of anthocyanins similar to the wild-type one. The arly induction of anthocyanin is therefore assumed to be a further compensatory mechanism that is not needed in heat resistant *CarS* plants.

Farnesyl diphosphate synthases (FPS) and geranylgeranyl diphosphate synthases (GGPS) are two enzymes active at the branch point of the cytosolic and chloroplastic terpenoid metabolism, respectively, playing a key regulatory role in controlling IDP flux into different families of terpenoids (Tholl et al., 2004). The level of *FPS2* expression in transformed plant is initially higher than in wild-types, suggesting up-regulation of the enzyme by overexpression of *TPS27* gene. However, decay of *FPS2* gene expression during the stress suggests a reorientation of the IDP in other linked pathways.

4. Conclusions

The plant cells and tissues are often exposed to environmental conditions that cause acute and chronic stress. Consequently, plant survival requires efficient and constantly updated stress response networks, in order to detect, monitor and react to environmental changes. Elucidating the large family of stress response mechanisms and their role in acquired stress tolerance is of basic importance.

Heat stress can rapidly damage proteins leading to a dramatic decrease of activity of essential enzymes, such as of light harvesting complezes of photosystems and RuBisCo, and inducing oxidative symptoms that are caused by inefficient light use for carbon fixation.

We have shown that the transformation of *Arabidopsis thaliana* plants to constitutively overexpress the (*E*)-β-caryophyllene synthase (*TPS27*) gene in leaves (Gershenzon et al., unpublished) enhances transcriptional activity of many genes involved in oxidative stress defense of plants, already in pre-stress conditions. It was also shown

that photosynthetic carbon fixation, photosystem operations and membrane properties are less damaged in the *CarS* plants than in the wild type exposed to long-term heat stress.

Finally, all together these results reveal a pattern that could be assimilated to that also obtained with the Arabidopsis plants transformed to overexpress the isoprene synthase gene (see chapter VI). Can we surmise that all volatile isoprenoids exert a similar protective action increasing plant resistance to high temperatures?

We will try to postulate such a function in the next chapter.

VIII

The “merging hypothesis” to respond to a still actual question: Why plants emit isoprenoids?

In the 1995, Thomas Sharkey and Eric Singsaas, in a letter to *Nature*, first went beyond the simplistic ‘waste of photosynthetic carbon’ hypothesis to explain plant emission of isoprene and other volatile organic compounds (Jones and Firn, 1991). They introduced a pioneer ‘biological’ hypothesis, driven by the observation that thermo-tolerance is enhanced in isoprene-emitting plants. From there, during the last decade an ever-increasing number of researchers and laboratories are constantly adding something new to support that original idea, even if, until now, a conclusive study on the biological function of isoprene is not yet been done (Sharkey et al., 1996; Rosenstiel et al., 2003; Velikova and Loreto, 2005; Siwko et al., 2007; for review see Sharkey and Yeh, 2001; Liu et al., 2005; Sharkey, 2005).

Although isoprene is the most abundant volatile organic compound (VOC) released from vegetation, it is only one component of the very large family of molecules produced by the plant secondary metabolism, family of which isoprenoids are the most representative group. Isoprenoids, like isoprene, have been largely investigated, reaching to interesting results, which point out a possible function for some isoprenoids as repellants of herbivores or attractants of predators (Arimura, 2000; Pennisi, 2005; Schnee et al., 2006). Otherwise, it was also been hypothesized an ‘opportunistic’,

finely regulated, biosynthesis and emission of isoprenoids (Owen and Peñuelas, 2005). However, the debate about the evolutionary and biological significance of the biosynthesis and emission of the volatile organic compounds by plants is still opened. Richard Firn and Clive Jones (2006) asked if "…we need new a hypothesis to explain plant VOC emissions", questioning the opportunistic hypothesis for isoprenoid emission. Susan Owen and Josep Peñuelas (2006) and later Pichersky et al. (2006), responded addressing the reminder that "…volatile isoprenoids would not have evolved with a specific role because they are secondary metabolites, and that any role they have now is fortuitous…" and "…organisms have to generate substantial chemical diversity for a few compounds to have any likelihood of possessing biomolecular activity". Owen and Peñuelas (2006), with the amazing opportunistic hypothesis, claimed that isoprenoids might be side products synthesized and emitted as a consequence of the primary metabolism carbon flux. The comment of Eran Pichersky, Thomas Sharkey and Jonathan Gershenzon (Pichersky et al., 2006) pointed out "…secondary metabolites were historically defined as 'secondary' because we did not know why plants made them. Often, for lack of a better explanation, they were hypothesized to be 'waste products'…". As more compounds have been investigated more functions have been found. This is the case of isoprenoids, for which several biological functions have been demonstrated. Pichersky et al. (2006) conclude their letter with the suggestion to follow the 'biological way' to explain the isoprenoid biosynthesis and emission. "…To propose that most

secondary compounds have no current function and are produced by forward-looking plants for future use or as side products of the formation of other metabolites that are required is not only contrary to our understanding of how evolution works but also ignores a multitude of recent papers on the varied and fascinating functions of secondary compounds…".

This debate between scientists that support neither a biological role nor a waste of carbon for the volatile organic compounds is doomed to go on for the next years, at least until we will be able to completely demonstrate that each isoprenoid produced by plants has a specific biological function. Looking beck the results obtained with isoprene and (*E*)-β-caryophyllene, looking on the recent variety of works on these and other isoprenoids, and also on the evolutionary history of isoprenoid biosynthesis (for an overview see Trapp and Croteau, 2001), it is possible that plants emit volatile compounds, as a part of the secondary metabolism, combining them to release a bland of molecules with specific functions, independently from the plant species. Plants have been developed evolving their capacity to regulate more and more finely the biosynthesis of even more specific family of isoprenoids to better respond to specific environmental conditions (herbivore attach, pathogen infection, high temperatures, photoinhibition and oxidative stress, drought, or wounding). Therefore, filogenetically distant compounds, like isoprene (an hemiterpene) and (*E*)-β-caryophyllene (a sesquiterpene), synthesized and emitted by the same plant, like Arabidopsis, produce similar effect, showing the same biological function of

thermal protection. This ‘merging’ hypothesis brings beck the explanations, not only the thermo-tolerance, for the isoprenoid emission to a unique multiple biological function: protection of plants against the environmental (biotic and abiotic) stress conditions, and enhancement of the plant fitness (for example improving their attraction of pollinator insects). The ‘merging’ hypothesis overlap isoprenoids coming from different plant species suggesting that the biosynthesis and utilization or not of a specific volatile compound in two different species should not be attributable to the fact that this compound may be or not ‘waste of carbon’, but because that plants have evolved in a different way, adapting their emission pattern to different environments, and, consequently, selecting different isoprenoids to respond to similar phenomenon.

IX
Methods Used to Study Isoprenoids

1. Methods for drought stress experiment

1.1 Water stress experiment

Populus alba L. saplings were propagated from physiologically mature trees growing in a clonal provenance trial in Italy. When the cuttings had rooted they were potted in 5-dm^3 pots, filled with commercial soil, and grown in a growth chamber (Sanyo Gallenkamp, Loughborough, UK) under controlled conditions. The relative humidity was maintained between 50 and 60%, the light intensity was 800 µmol m^{-2} s^{-1} (daylight period 12 h), and the temperature was 30/25°C day/night. The plants were regularly watered to pot water capacity and fertilized with Hoagland solution once a week in order to supply mineral nutrients at free access rates. The dehydration treatment was initiated 2 months after the appearance of the first fully expanded leaf. On the afternoon preceding the initiation of the experiment, plants were fully irrigated and excess water was allowed to drain overnight. After draining, the pots were weighed on a digital balance (model QS32A; Sartorius Instrumentation, Goettingen, Germany) to determine the initial hydrated weight of the pot (i.e. pot water capacity). Each pot was then enclosed in a plastic bag that was tied around the stem to prevent soil evaporation. Eight plants were then water-stressed by withholding water, while another five saplings continued to be well watered to pot capacity (control). Thereafter, every morning the

plastic bags were unwrapped to weigh the water stressed saplings (daily pot weight) and to water the control plants. Then, the fraction of transpirable soil water (FTSW) was calculated as:

$$FTSW = \frac{[daily\ pot\ weight] - [final\ pot\ weight]}{[initial\ pot\ weight] - [final\ pot\ weight]}.$$

The physiological lower limit of available soil water (FTSW endpoint) was defined as the FTSW at which stomatal conductance approached zero (i.e. soil water decreased to a level where there was no longer water available to support transpiration) (Sinclair and Ludlow, 1986). The water-stressed pots were then weighed to determine the final pot weight. The severity of water stress was calculated as the fraction of the available water at pot water capacity. The time until plants reached FTSW endpoint varied between 30 and 35-d, reflecting differences in water loss among plants. At FTSW endpoint the water-stressed saplings were re-watered daily to pot capacity over a 15-d recovery period.

1.2 Gas exchange measurements on Populus alba *leaves*

Photosynthesis (A), stomatal conductance (g_s) and internal CO_2 concentration (C_i) were measured daily using a portable gas exchange system (LI-6400; Li-Cor, Lincoln, NE, USA) between 10:00 and 17:00 h. Gas exchange measurements were carried out under photosynthetic photon flux density (PPFD)-saturated conditions (1,000 μmol m^{-2} s^{-1}). Leaf temperature was set at 30°C, and the relative humidity in the leaf cuvette ranged between 45 and 50%. Isoprene emission from leaves was detected simultaneously with

CO_2 and H_2O gas exchange measurements by connecting the outflow of the Li-Cor 6400 cuvette to a gas chromatograph with a photoionization detector (Synthec Spectras BTX Analyzer GC 855; Synthec, Groeningen, the Netherlands). Calibration using an isoprene gaseous standard (60 nL L^{-1}) was performed daily before measurements and sensitivity was > 0.1 ppb.

1.3 Measurement of PaISPS activity and PaISPS protein concentration

Leaf samples were homogenized in liquid nitrogen in a chilled (4°C) mortar. Two hundred mg fresh weight of this material was suspended in 4 mL of plant extraction buffer (PEB: 100 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 100 mM $CaCl_2$, 1.5% (weight/volume; w/v) polyethylene glycol (PEG) 1500, 5% (v/v) glycerol and 0.1% (v/v) Tween 80). Polyvinylpolypyrrolidone (PVPP) (250 mg) and dithiothreitol (DTT) (20 mg) were added before use and the mixture was stirred on ice for 15 min. The homogenate was centrifuged at 16,000*g* for 20 min. In the meantime, PD-10® (Amersham Pharmacia, Piscataway, NJ, USA) columns were equilibrated with 25 mL of isoprene synthase buffer (ISB: 50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$ and 5% (v/v) glycerol) and 2 mM DTT. After centrifugation, 2.5 mL of the clear supernatant of the homogenized leaf sample was added to the columns. Finally, the ISB (3.5 mL) was added the column. The protein extract (3.5 mL) was collected and stored at -80°C (Schnitzler et al., 2005). The total protein concentration was measured by the Bradford assay using bovine serum albumin (BSA) as a standard.

ISPS activity was then assayed using the procedure described by Lehning et al. (1999). The ISPS protein concentration was determined using an enzyme-linked immunosorbent assay (ELISA) with polyclonal antibodies generated against His-tagged recombinant poplar ISPS protein, as described by Schnitzler et al. (2005). Ninety-six well microtiter plates (Greiner, Frickenhausen, Germany) were precoated with anti-ISPS-IgG diluted 1:500 in phosphate-buffered saline (PBS: 50 mM NaPi, pH 7.2, and 140 mM NaCl) overnight at 4°C. The wells were then washed four times with PBS plus Tween 0.1% (v/v) for 1 min. This procedure was repeated between all subsequent steps. (1) Residual binding sites were blocked with PBS plus BSA 0.2% (w/v) for 1 h. (2) To bind the ISPS protein, 2.5 µg of protein extract was diluted in PBS and then incubated for 2 h at room temperature. (3) The plates were incubated for 1 h with the second anti-ISPS-IgG antibody, which was conjugated with horseradish peroxidase (HRP; BioGenes, Berlin, Germany). (4) Each well was filled with 200 µL of a staining solution containing the HRP substrate tetra methylbenzidine (TMB), and the plate was incubated for 45 min at room temperature. Following the addition of 10 µL 2 N H_2SO_4 to increase the staining intensity, plates were analyzed using a plate reader (Tecan Spectra Image; SLT, Crailsheim, Germany) at 450 nm. Each sample/standard was performed in triplicate.

1.4 Relative mRNA transcript levels of PaISPS *gene*

Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of ISPS Total RNA (2.5 µg), extracted from 200 mg fresh mass of leaves at 0-4°C using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), was reverse-transcribed (RT) with SuperScript II RNaseH reverse transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was performed using Platinum Taq DNA polymerase (Invitrogen) with RT product as a template. The PCR reaction was carried out using *P. alba* isoprene synthase gene (PaISPS)-specific internal primers (sense primer PaISPS-Fw2: 5′-GTCGTTTGGAGCATTGAAGCATACCG-3′ and antisense primer PaISPS-Bw2: 5′-ATGTTTTCCCCCTTGTCCTTCAGATTGTGTC-3′) of *P. alba*. PaISPS gene expression was normalized using the ACTIN2 gene, with two specific primers (sense primer PaACT-Fw1: 5 -GCAGGCATCCACGAAACCACATACAACTC- 3′ and antisense primer PaACT-Bw1: 5′-CACCTTGATTTTCATGCTGCTTGGGGC-3′).

2. Method for combined drought and high temperature stress

2.1 Populus nigra *growth conditions and experimental design*

One year old saplings of black poplar (*Populus nigra* L.) were transplanted during spring into 6 L plastic pots containing a mixture of 50% commercial potting soil and 50% sand, and placed in two growth chambers Fitotron® (Sanyo-Gallenkampf, http://www.sanyo-biomedical.co.uk/), under the following environmental conditions: photosynthetic photon flux density (PPFD) 900 µmol photons m^{-2} s^{-1} of light intensity at the canopy level, day length of 12 hours, and

relative humidity level of ~ 50%. Air temperature during the light hours was set at 25°C and 35°C in the two phytotrons since transplantation. When light was switched off, air temperature of both phytotrons was 3-4°C lower than during the day. The drought stress was initiated one month after transferring the saplings into the phytotrons.

Nine saplings were randomly chosen in each phytotron as control plants and regularly well watered to full soil capacity for the entire experimental period. Nine more plants were instead subjected to the drought stress treatment, at the two growth temperatures by withholding water. The drought severity was parameterized monitoring the fraction of transpirable soil water (FTSW) as shown in Sinclair et al. (1998). The end-point of drought stress (i.e. the point at which photosynthesis and stomatal conductance of all plants declined to zero) was reached after 35 days of drought, with a FTSW of about 30% at both 25 and 35°C. Drought-stressed plants were then re-watered to field capacity and thereafter were watered daily over the following 15-day long recovery period.

2.2 Gas-exchange and isoprene emission measurements on P. nigra *leaves*

Isoprene emission, photosynthesis and respiration rates, and leaf water exchange indicators (stomatal conductance and transpiration) were measured during drought stress and re-watering. All gas-exchange experiments were done on fully expanded leaves attached to the plants, choosing the 4-6 leaves from the apex, during the

central part of the day (h. 11.00-13.00). A 6 cm^2 area of a single leaf was clamped in the gas-exchange cuvette of the LI-6400 infrared gas analyzer (Li-Cor, http://www.licor.com/). Measurements were carried out in conditions identical to those experienced during growth. Leaves were exposed to a flux of synthetic air, free of contaminants and pollutants, composed by N_2, O_2 and CO_2 in atmospheric concentrations (80%, 20% and 370 ppmv, respectively). The incident light intensity was maintained at a PPFD of 900 µmol photons m^{-2} s^{-1} and a relative humidity of 40-50%. When measuring dark respiration, leaves were darkened for 30 min prior to measurements. The leaf temperature during the assay was set at 25 or 35°C, in the two batches of plants grown at the same temperature. The leaf temperature was measured with an array of three thermocouples near to the center and at two margins of the abaxial leaf side. Experiments were carried out when physiological parameters (photosynthesis, transpiration, respiration and isoprene emission) were at steady state, about 15-20 min after inserting the leaf in the cuvette. Photosynthesis, respiration and transpiration were measured with the CO_2/H_2O differential infrared-gas analyzer LI-6400.

Isoprene emission was measured on-line with a Proton Transfer Reaction-Mass Spectrometer (PTR-MS, Ionicon; http://www.ptrms.com/) by diverting a small flux of the air flowing out of the cuvette to the instrument. The PTR-MS was calibrated daily by injecting an isoprene standard (70 ppbv), and measurements were validated with gas-chromatography measurements (Synthec

Spectras BTX Analyzer® GC 855; Synthec; http://www.syntech.nl/). Further details about the gas-exchange set-up and physiological measurements are reported by Loreto et al. (1996) and Brilli et al. (2007). Details on the theory and practice of the PTR-MS technique are reported by Lindinger et al. (1998). At the end of each measurement the leaf area used for gas-exchange measurements was rapidly frozen in liquid nitrogen and used for the biochemical assays.

2.3 Cloning of the Populus nigra isoprene synthase *(Pn*ISPS*) cDNA and phylogenetic analysis of the sequence*

Total RNA of black poplar leaf samples was extracted by grinding 0.2 g of frozen tissue to fine powder in liquid nitrogen and then adding the tissue to a 15 mL round-bottom tube containing 1 mL RLT buffer (Qiagen; http://www.qiagen.com/) and 0.01 g PVP-40. After shaking the tubes for 1 min by vortexing, the tissue was homogenized with polytron for 30 sec. After homogenization 450 µL 5 M potassium acetate, pH 6.5, were added to 0.4 v/v, and mixed with the tissue by inversion 4-6 times, and incubated on ice for 15 min. The samples were transferred into 2 1.5 mL micro centrifuge tubes, centrifuged at 12000 rpm for 15 min at 4°C, and then the supernatant was transferred to two 1.5 mL tubes. One half volume of 100% ethanol was added and mixed by pipetting and the samples were transferred into RNeasy Plant Mini Kit (Qiagen) spin columns. RNA was extracted following the Qiagen's handbook instructions for isolation of total RNA from plant cells and tissues. First-strand cDNA was

synthesized from 1 µg total RNA extracted, using the SuperScript III reverse transcriptase (Invitrogen; http://www.invitrogen.com/). The single stranded cDNA mixture was used as template for the polymerase chain reaction (PCR) to amplify the full-length *PnISPS* cDNA. For the PCR reaction was used the *Platinum* Taq DNA Polymerase, *High Fidelity* (Invitrogen), with a primer's pair specific for the white poplar isoprene synthase (PaISPS-Fw3: 5'-ATGGCAACTGAATTATTGTGCTTGC-3' and PaISPS-Bw3: 5'-TTATCTCTCAAAGGGTAGAATAGGCTCTG-3'). The PCR product of 1,788 bp was sub-cloned into pGEM T-Easy vector (Promega). The exact sequence of cloned fragments was analyzed by sequencing (Bio-Fab Research; http://www.biofabresearch.it/) and deposited in the gene bank (EMBL accession number: AM410988). ClustalX (Thompson *et al.*, 1997) was used for multiple alignment of ISPS and other TPSs. Phylogenetic tree of the putative ISPS and TPS amino acid sequences was constructed using the MEGA2 software by the maximum likelihood analysis with 1,000 repeats (Kumar et al., 2001), applying the UPGMA algorithm.

*2.4 Quantitative mRNA expression analysis of Pn*ISPS *gene*

First-strand cDNA mixture from black poplar leaves was prepared as described previously. For each point of the experiment 3 leaves harvested from 3 different saplings were used. Quantitative polymerase chain reactions (qPCRs) were performed in an Mx3000*P*® (Stratagene; http://www.stratagene.com/) system. qPCR reactions were assembled in a total volume of 20 µL as described by

Pellissier et al. (2006) with some modifications: 2 µL of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 0.4 µL of 2 mM dNTPs, 0.8 µL of 50 mM $MgCl_2$, 6 µL of 2 M D-(+)-trehalose dehydrate (Fluka; http://www.sigmaaldrich.com/), 0.5 µL formamide, 0.1 µL of a 1/100 SYBR Green I dilution in dimethylsulfoxide (Molecular Probes/Invitrogen), 0.2 µL (1 U) of *Taq* DNA Polymerase, recombinant (Invitrogen), the specific primers for Pn*ISPS* gene (sense primer PnISPS-Fw2: 5'-GTCGTTTGGAGCATTGAAGCATACCG-3' and anti sense primer PnISPS-Bw2: 5'-ATGTTTTCCCCCTTGTCCTTCAGATTGTC-3') (300 nM each, final concentration), 10 ng cDNA, and MQ water to 20 µL. qPCR reactions were always carried out in triplicate wells, using the following conditions: 2.30 min at 95°C, followed by a total of 40 three-temperature cycles (30 sec at 95°C, 1 min at 58°C, and 1.5 min at 72°C). The couple of primers used amplified a fragment of 461 bp. Pn*ISPS* gene expression was normalized to *ACTIN* gene of black poplar (Pn*ACT*) (EMBL Acc. Num.: AM777847), using two specific primers (sense primer PnACT-Fw: 5'-GCAGGCATCCACGAAACCACATACAACTC-3' and anti-sense primer PnACT-Bw: 5'-CACCTTGATTTTCATGCTGCTTGGGGC-3'). To quantify the qPCR amplification results cDNA plasmid standards, specific for Pn*ISPS* and Pn*ACT* genes, were constructed. To construct the cDNA standards, total RNA from black poplar leaves was extracted, and both isoprene synthase and actin cDNA fragments were synthesized by RT-PCR, using the same primers previously described. Each of these fragments was purified with

QIAquick PCR Purification Kit (Qiagen), cloned into pGEM-T Easy Vector System (Promega; http://www.promega.com/), and transformed into DH5α competent cells. Plasmid DNA was purified from transformed cells using QIAGEN Plasmid Mini Kit (Qiagen). The cDNA plasmid concentration was measured by NDR ND1000 Nanodrop (Nanodrop Inc.; http://www.celbio.it/). The corresponding copy number was calculated using the following equation:

$$1\ \mu g \text{ of } 1{,}000 \text{ bp DNA} = 9.1 \times 10^{11} \text{ molecules.}$$

In each qPCR amplification serial dilution (10 folds) of plasmid cDNA, ranging from 10^{-1} to 10^{-7} input cDNA copies was used as a standard curve (each standard curve was performed as triplicate). All qPCR data were elaborated by the MxPro v3.2 software (Stratagene). To verify if the primer pair used for the qPCR analysis of the Pn*ISPS* gene produced a specific amplification, a RT-PCR experiment was carried out using the same primers and the product was analyzed in a 1% agarose gel electrophoresis. The DNA fragment was excised from the agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) following the handbook instructions. The purified fragment was then cloned into pGEM-T Easy vector (Promega) and sequenced.

2.5 PnISPS protein extraction

Poplar leaves were rapidly killed and homogenized in liquid N_2 using mortar and pestle. All further steps were performed at 4°C. Two hundred milligram fresh weight were suspended in 4 mL plant extraction buffer (PEB: 100 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$, 100

mM $CaCl_2$, 1.5% (w/v) PEG 1500, 5% (v/v) glycerol, 0.1% (v/v) Tween 80), with 200 mg PVPP and 20 mM DTT added directly prior to use and stirred on ice for 15 min. The homogenate was centrifuged at 18,000*g* for 20 min, and in the meantime PD-10 (Amersham Pharmacia/GE Healthcare; http://www.1.gelifesciences.com/) columns containing Sephadex G-25 M were equilibrated with approximately 25 mL of isoprene synthase buffer (ISB: 50 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$, 5% (v/v) glycerol) adding 2 mM DTT directly prior to use. When the equilibration buffer was run into the columns, 2.5 mL of clear supernatant, collected after centrifugation of homogenate, were added to the columns and the eluent was discarded. Finally, 3.5 mL ISB were added and protein extract was collected and stored at -80°C.

2.6 Measurement of PnISPS specific activity

Protein concentration of all samples was measured by the Bradford assay using BSA as a standard. PnISPS specific activity was assayed using a modified procedure of Lehning et al. (1999): 20 mM $MgCl_2$, 15 µL protein solution, 0.1 mM DMADP (in ISB), and ISB to a final volume of 100 µL were added to 2 mL gas-tight vials, and the mixture was incubated for 1 h at 30°C. The enzyme reaction was determined by removing the reaction mixture from the closed vials with a syringe, and washing the vials with 150 µL distilled water. Samples of the headspace were analyzed by PTR-MS system as described in Loivamäki et al. (2007a). Calibration of the system was

performed using 2 mL vials filled with a calibration standard (10.9 ppmv isoprene in N_2, Messer Griesheim, http://www.airliquide/loesungen/produkte/gase/). Tests without protein extracts were carried out in parallel to correct for the non-enzymatic background formation of isoprene. All measurements of PnISPS specific activity were normalized with the background isoprene formation.

2.7 Quantification of PnISPS protein

Isoprene synthase protein concentration was determined by an ELISA assay (Schnitzler et al., 2005), which was set up according to the QIAexpress® and Assay Handbook (Qiagen). 96-well microtiter plates (Greiner; http://www.chemie.de/) with high protein binding capacity were pre-coated with anti-ISPS-IgG diluted 1:500 in 200 µL of phosphate buffer saline (PBS: 50 mM NaP_i pH 7.2, 140 mM NaCl) over night at 4°C. Before the next step, as well as in between all following steps, the wells were washed four times with 200 µL PBS-Tween (0.1% (v/v) Tween 80 in PBS) for 1 min each. In the second step, residual protein binding sites were blocked with PBS-BSA (0.2% (w/v) BSA in PBS) for 1 h. For binding of PnISPS protein, 2.5 µg protein extracts in 200 µL PBS per well were incubated at room temperature (RT) shaking carefully for 2 h. Thereafter, the second anti-ISPS-IgG antibody (diluted in PBS-BSA), which was conjugated with horseradish peroxidase (HRP), was added to all wells, and the plates were incubated for 1 h at RT. After the final washing, each well was filled with 200 µL of HRP substrate, a ready-to-use tetra

methylbenzidine (TMB) staining solution® (Sigma). The plates were incubated at RT for 45 min. After that time the staining intensity was enhanced by addition of 10 µL 2 N H_2SO_4. The resulting yellow colored complex was analyzed with a plate reader (Tecan Spectra Image, SLT; http://www.tecan.com/) at 450 nm. For calibration purified 6xHis-tagged ISPS protein was used in a range of 0.01-0.3 µg protein. Each sample/standard was performed as triplicate.

*2.8 Phospho*enol*pyruvate carboxylase (PEPC) activity measurements*

Two cm^2 black poplar leaf disks free of midrib were used for PnPEPC activity assay. Leaf material was extracted in a pre-chilled mortar with 1.5 mL extraction buffer (100 mM HEPES-KOH pH 7.2, 10 mM DTT, 0.3% (w/v) Triton X-100, PVP-40, 5 mM $MgCl_2$), centrifuged for 5 minutes at 11,000*g* at 4°C. The supernatant fraction was immediately assayed for PnPEPC activity, in an assay buffer containing 25 mM Tricine-KOH pH 8.1, 5 mM $MgSO_4$, 5 mM $NaHCO_3$, 5 mM DTT, 0.2 mM NADH, and 5 U malic dehydrogenase (from porcine heart). The reaction was initiated by the addition of 2 mM phospho*enol*pyruvate at room temperature. NADH absorbance changes were registered for 5 minutes at A_{340}, with a sampling frequency of every 0.2 min. (Rosenstiel et al., 2004). Total protein contents of samples were determined according to Bradford (1976). For calibration BSA standard was used.

2.9 Statistical analysis

Measurements from *P. alba* and *P. nigra* leaves were carried out on at least 5 replicates (different leaves of different plants). When describing the relationship between PEPC activity and dark respiration with isoprene emission, single data points are shown and relationships obtained at the different temperatures were best fitted by first order regression lines. In all other cases, data are shown as means ± standard deviations. Means were statistically separated by Tukey's test. Different letters indicate statistically different means between groups (different days of treatment at the same temperature), and asterisks (*) indicate statistically different means within groups (same day of treatment at two different temperatures). Differences that were not statistically significant are not shown. All statistical analyses were conducted using SigmaStat® (SPSS; http://www.spss.com/). Statistical tests were performed on *IspS* and *CarS* transgenic lines using Stat32 (SigmaStat, 2004 Systat Software). Differences intra-groups were detected by *t*-test. One-way ANOVA test was used for inter-group tests.

3. Methods used for the characterization of *IspS* plants

3.1 Growth conditions of IspS *plants*

Arabidopsis (*Arabidopsis thaliana*) seeds, ecotype Wassilewskjia (Ws), and transgenic *IspS* plants (for transformation and selection of *IspS* lines see Sharkey et al., 2005), were sterilized in 50% commercial bleach and 0.01% SDS for 10 min, and then with 4-5 distilled water washes. Treated seeds were planted in Petri dishes on

growth medium (1.5% agar, 1% sucrose, and 0.5X MS basal medium, enriched with Gamborg's vitamins, adjusted to pH 5.7 with NaOH). *IspS* transformed plants were selected by growing them on growth medium with kanamycin 50 mg L^{-1}. Dishes were incubated for 48 h at 4°C to synchronize the germination, and grown in an Arabidopsis growth chamber (Percival Scientific, USA) for two weeks. Finally, plants were transferred to pots (7 cm) on a sterile medium made up of 40% sand, 35% turf and 25% soil. Conditions in the growth chamber were: 8 h light/16 h dark, photoperiod; 180 µmol m^{-2} s^{-1} light intensity; 23°C (day) and 19°C (night) air temperature; 65% relative humidity. Plants were irrigated three times per week with distilled water.

3.2 High temperature and oxidative stress experimental design

Five-week-old plants were used in this study. For the high temperature treatment, *IspS* and wild-type plants were incubated at 40°C air temperature, 180 µmol m^{-2} s^{-1} light intensity, at *ca* 65% relative humidity for 48 hours. For oxidative stress experiment, both wild-type and transgenic lines were incubated at 40°C air temperature, 1,000 µmol m^{-2} s^{-1} light intensity, at *ca* 65% relative humidity for three days. After the treatments, some plants were transferred to the growth chamber for 24 hours, to follow the recovery. At the end of treatment, and after the recovery period, physiological measurements were made and leaf samples, for further molecular and biochemical analysis, were collected and stored at -80°C. At the end of all treatments pictures of stressed and control

plants were taken with a digital camera. Wild-type and transgenic *IspS* plants, with the same age of stressed plants, left in the growth chamber were used as control in both experiments.

3.3 Physiological parameters of IspS *plants*

Two entire plants of the same line (wild-type or *IspS*) were removed (as shown above) from the incubator and placed in a 1.7 L *Arabidopsis* glass cuvette for gas-exchange and isoprene emission measurements (Tholl et al., 2006). Plants were exposed to synthetic air made by mixing O_2, N_2, and CO_2 from cylinders scrubbed of contaminants. The concentration of the three gases (20%, 80%, 370 μL L^{-1}, respectively) was set with mass flow controllers. The airflow was set at 2 L min^{-1}. During gas exchange measurements leaf temperature, light intensity, and relative humidity were maintained at constant levels close to growth conditions (23°C, 180 μmol m^{-2} s^{-1}, and 65%, respectively). The CO_2 and H_2O exchange between leaves and air were measured with a LI-6262 infrared gas analyzer (Li-Cor, Lincoln, Neb, USA). Measurements of isoprene emission from *Arabidopsis* leaves were conducted with a Proton Transfer Reaction-Mass Spectrometer (PTR-MS, Ionicon, Innsbruck, Austria). Chlorophyll fluorescence was measured at pre-stressed conditions, at the end of high temperature treatment and after a 24-h recovery, with a portable chlorophyll fluorometer Mini-Pam (Walz, Effeltrich, Germany). The rate between variable and maximal fluorescence (Fv/Fm), which indicates the maximal quantum yield of PSII, was measured in leaves dark-adapted for 15 minutes.

3.4 RT-PCR and quantitative qPCR analysis

Total RNA was extracted from 50 mg of each leaf sample using the TRIZOL reagent (Invitrogen, CA, USA) following the manufacturer's instructions. Two µg of total RNA was reverse transcribed using the Super Script III (Invitrogen) reverse transcriptase to synthesize the first-strand cDNA mixture, which was used for the polymerase chain reaction (PCR). PCR reactions were curried out with *Platinum* Taq DNA Polymerase (Invitrogen) using kudzu *ISPS* gene specific internal primers (see Table V); the final products were stained with ethidium bromide, run in a 1% agarose gel electrophoresis and displayed by a UV transilluminator.

First-strand cDNA mixture from *Arabidopsis* leaves was prepared as described previously. For each point of the experiments, three leaves harvested from three different plants were used for both wild-type and *IspS* plants. Quantitative *real time* polymerase chain reactions (qPCRs) were performed in an Mx3000*P*® (Stratagene, CA, USA) system using the protocol described in Fortunati et al. (2008a). For the complete list of primers used in the RT-PCR and qPCR reactions see Table V.

Table V. List of RT-PCR and qPCR Primers Used for the mRNA Expression Analysis of *IspS* plants

Locus Tag	Name	Primer sequence
AY316691	KuIspS[a]	5'-ATGGAAGTGTATTTTTGGGCGTTGGG-3' 5'-GCTCTCGGTTCATCTTCTTCCACTCTGC-3'
At4g15560	DXS	5'-CTCTTAATGGGTTCGGCTTCG-3' 5'-TATGTTTGTTCCACAGCATCCCAC-3'
At5g62790	DXR	5'-GTTCAATCCAATCCCTAAACTCTCAGG-3' 5'-CGGGCAACCTCAATCACTCCT-3'
At2g02500	ISPD	5'-GTATCCACGACTCTGCCCGACCAT-3' 5'-CATTTTTCATGCATACCCTTGACACG-3'
At2g26930	CMK	5'-GCAAATGCTACTACCTCATCCGCCG-3' 5'-ATTCAGGTGCTGGTGTTATCGTGG-3'
At1g63970.1	ISPF1	5'-GGCTGCGAAGCTCACTCCGATGGCG-3' 5'-ATCAAAAGTATGTTTTTCTTAAAAGATGAG-3'
At1g63970.2	ISPF2	5'-TGAGACTCATGGACGAGGCAGGG-3' 5'-GCAGTCCAAGACACAGTTTCCAAT-3'
At5g60600	HDS	5'-CGAGCAGGTCTTCACTCCTTTGG-3' 5'-GCCTATGCTTTTCTTCAAACGGTGC-3'
At4g34350	ISPH	5'-ACCAAAATCGTCCATTGAAGTGAAC-3' 5'-GGTCGGGTTATGAATGATTTCGTTAG-3'
At5g16440	IPP1	5'-CGTAATCCAAAATGTCTACTGCTTCACTA-3' 5'-GGCATTTCTTACACCAAGCACATTC-3'
At5g47770	FPS1	5'-GGGAGAGAAAGTCAGTCCGATTGTG-3' 5'-ACCACGGCGAGTGACAGAGTTATCC-3'
At4g17190	FPS2	5'-ATGGCTTGAACGGATGCTTGACTAC-3' 5'-CGCAAGCAACAGGAAGATAAAATGAG-3'
At4g36810	GGPS1	5'-TCCACGAAGCGATGCGTTACTC-3' 5'-TCAATCTCATCATCACTTCCTCCACC-3'
At4g14210	PDS3	5'-TGGAGGCACTTTCATCTGGAGGTTG-3' 5'-GGGTGCTGGTAGGACATCTGGG-3'
At3g04870	ZDS1	5'-CGATTGCTCTTATCTCAGTTGATTGGC-3' 5'-GTCTCTCATTGCTCCATCGGGGTCG-3'
At3g10230	LYC	5'-GTTGATTTGGCTATTGTTGGTGGTG-3' 5'-GGGAAAACCCAGTGGCATCAAG-3'
At5g57030	LUT2	5'-GGTGATGGTGCTTTGGATCTAGTGG-3' 5'-ACCTCAACCTCCACGCCGTATGC-3'
At5g67030	ABA1	5'-GGAGTTTTTGCTCTTCTCGTCGGTG-3' 5'-GTGACGCCGCAGGAGTGAAAGTATTC-3'
At3g63520	CCD1	5'-GCAGCAGCATCATCTCAGTCCATC-3' 5'-TTGGGTGAGCAGTGAAGGAGTGG-3'

At1g52340	ABA2	5'-TCACTGGAGGAGCCACAGGGATAGG-3' 5'-GCGTAAGGCGAAACACAGTTCACAC-3'
At2g27150	AAO3	5'-ATCCGATTCATAAGCGGTTCGCGG-3' 5'-GTAACCTGTACCCGTGTTGCCTAC-3'
At1g20630	CAT1	5'-CTGCTCTGGAAATCGTGCAGAAGTTGC-3' 5'-AGAAACCAAACCGTAAGAGGAGCATA-3'
At1g20620	CAT3	5'-CACTCAGAGACATCGCCTTGGACCG-3' 5'-CGTGGGTGAGACGTGGCTCCGATAG-3'
At4g25100	FeSOD1	5'-TCCAGAACCGAAGACCAGATTACAT-3' 5'-CTTGACACACACAAAACGCACACAC-3'
At1g07890	APX1	5'-GTCCATTCGGAACAATGAGGTTTGAC-3' 5'-GTGGGCACCAGATAAAGCGACAAT-3'
At4g08390	sAPX	5'-TGCTAATGCTGGTCTTGTGAATGCTT-3' 5'-CCACTACGTTCTGGCCTAGATCTTCC-3'
At1g77490	tAPX	5'-CAGAATGGGACTTGATGACAAGGAAA-3' 5'-ATGCAGCCACATCTTCAGCATACTTC-3'
At2g26150	HSFA2	5'-AACAATGTTTGGAGGCTATGAATG-3' 5'-ACACAAATGTACAACAAACCACACTCA-3'
At5g03720	HSFA3	5'-ACAATACCGAGTTTCAGCAACACCG-3' 5'-AGAAAGGAACAATCAAACTCATCAAT-3'
At4g18880	HSFA4A	5'-CTCAGAGAATCCTGGCTCAACCG-3' 5'-GCTTATCATTACCATTGAAGTCTGC-3'
At5g45710	HSFA4C	5'-TGAACTCAGAGCCGCCCGTTACCGT-3' 5'-TGTCACTCCTGCCTCAAACAATAGA-3'
At5g59720	HSP18.2	5'-AGAGAGGAGCAAGGAGAACGAAGAG-3' 5'-CATAACACAACAAGCCAAGAAAAAAAC-3'
At1g49240	ACT8	5'-ATGAAGATTAAGGTCGTGGCA-3' 5'-TCCGAGTTTGAAGAGGCTAC-3'

[a]The primers pair KuIspS-Fw and KuIspS-Bw was used for the RT-PCR analysis of isoprene synthase gene (KuIspS) in the transgenic *IspS* plants

3.5 Photosynthetic pigments and chlorophyll content analysis

For leaf pigments extraction, each sample was homogenized in liquid nitrogen with mortar and pestle, and 50 mg of fine powder were incubated in the dark at room temperature (25°C) for 10 min in 1 mL pure acetone. Then, the suspension was centrifuged for 10 min at 15,000*g* and 4°C. The pellet, containing pigments, was re-suspended in 500 µL of acetone, centrifuged again, and, finally, the supernatants

were combined. Each sample was injected and analyzed for the pigments content in a System Gold HPLC (Beckman, Fullerton, CA, USA). System calibration was performed using chlorophylls (Chl *a* and *b*), β-carotene, and violaxanthin pure standards.

3.6 Hydrogen peroxide content and lipid peroxidation assay

Hydrogen peroxide levels were determined as described by Velikova et al. (2000). Frozen leaf material (0.1 g) was homogenized in an ice bath with 1.2 mL 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000*g* for 30 min and 0.4 mL of the supernatant was added to 0.4 mL 10 mM potassium phosphate buffer (pH 7.0) and 0.8 mL 1 M potassium iodide (KI). The absorbance of supernatant was read at 360 nm. The coloured reaction product of H_2O_2 with KI develops within 25 min and is stable for at least 2 h. The content of H_2O_2 was given on a standard curve.

Lipid peroxidation was determined in terms of thiobarbituric acid reactive substances (TBARS**)** according to the method of Heath and Parker (1968) based on the interaction of malondialdehyde (MDA), a product of membrane lipids breakdown, with thiobarbituric acid (TBA). Leaf material (0.1 g) was homogenized in 1.2 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000*g* for 30 min and 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) TBA in 20% TCA. The mixture was incubated in a boiling water bath for 30 min, and then quickly cooled in an ice bath. Absorption of the solution was recorded at 532 nm. The value for non-specific absorption at 600 nm was subtracted. For calculation of the content

of MDA-TBA complex the molar extinction coefficient of 155,000 cm^{-1} was used.

3.7 Ascorbic acid quantification

The ascorbate redox state and total ascorbate (AsA) content of leaf extracts was determined according to Law et al. (1983). The spectrophotometric assay is based on the reduction of Fe^{3+} to Fe^{2+} by ascorbate in an acidic solution and on the formation of a 525 nm absorbing pink complex upon its reaction with 2,2-bipyridyl. Total ascorbate (ascorbate + dehydroascorbate) was determined through the reduction of the dehydroascorbate present in the extracts to ascorbate by dithiothreitol (DTT). 0.15 g leaf material free of midrib was extracted in 1 mL ice-cold 6% (w/v) trichloroacetic acid (TCA), centrifuged for 5 min at 15,600*g* at 4°C and 0.2 mL of the supernatant assayed for ascorbate content in an assay mix containing 0.6 mL 0.2 mM sodium-phosphate buffer (pH 7.4), 1 mL 10% (w/v) TCA, 0.8 mL 42% (w/w) phosphoric acid and 0.8 mL 2,2'-dipyridyl. A_{525} was determined after incubating the assay-mix at 42°C for 40 minutes in a water bath in the presence of 0.4 ml 3 % (w/v) $FeCl_3$. Total ascorbate content was assayed in the same assay mix, containing in addition 0.2 mL 0.5% (w/v) N-ethyl-maleimide (NEM) and 0.2 mL 10 mM DTT. Dehydroascorbate (DhA) contents were calculated from the difference between total ascorbate and ascorbate. Calculations were based on a standard curve by assaying commercially available ascorbate and dehydroascorbate standards.

3.8 Thylakoid Membranes Isolation and Low Temperature (77 K) Fluorescence Measurements

Thylakoid membranes form both wild-type and transgenic *Arabidopsis* leaves were isolated as previously described in Harrison and Melis (1992), and then suspended in 350 mM sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, and 40 mM Hepes, pH 7.8 (Buffer A), or 350 mM sorbitol, 5 mM $MgCl_2$, 10 mM NaCl, and 50 mM Tris, pH 8 (Buffer B). The chlorophyll content of all samples, estimated by using the method described in Lichtenthaler (1997), was adjusted to 20 μg mL^{-1}. Low temperature (77 K) chlorophyll fluorescence spectra from the isolated thylakoid membranes were recorded from 660 to 780 nm with a Jobin Yvon JY3 spectrofluorimeter (Jobin Yvon ISA, Longjumeau, France) supplied with a low temperature device. Excitation wavelength was 436 nm with 4 nm of excitation and emission slits, respectively. Thylakoid membrane emission spectra were normalized by adding 1 μM fluorescein sodium salt to the medium as an internal standard (Krause et al., 1983).

3.9 Circular Dichroism (CD) Spectroscopy and Temperature Dependence

Circular dichroism (CD) was measured in a Jobin Yvon CD6 dichrograph (Jobin Yvon ISA, Longjumeau, France) equipped with a thermostated sample holder. Circular dichroism was measured in absorbance units, the spectra, between 400 and 750 nm, were recorded in 1 nm steps with an integration time of 0.3 sec and a band-pass of 2 nm. For the measurements of thermal stability, intact

leaves of Arabidopsis were placed in the sample holder and was dark-adapted for 10 min at 20°C. Then the temperature was gradually increased to 30, 40, 45, 50, 55 and 60°C. The leaves were incubated for 10 min before the measurements at the same temperature. The transition temperatures in the wild-type and *IspS* leaves for the main Psi-type CD band, at (+)694 nm, were calculated from inflection point of the fitted curve of the temperature dependence of the CD amplitude.

4. Methods for studying *CarS* plants

4.1 Growing up CarS *plants and high temperature experimental setup*

In this study, *Arabidopsis thaliana* plants ecotype *col-0*, transformed with the (*E*)-β-caryophyllene synthase gene (*CarS* lines) under the control of a constitutive promoter to over-express the (*E*)-β-caryophyllene synthase and to emit (*E*)-β-caryophyllene from leaves (Gershenzon et al., unpublished) and wild-type *col-0*, were used as plant material. Seeds of both lines were sterilized using 50% commercial bleach added with 0.01% SDS, and washed several times with distilled water. After sterilization, seeds were grown on Petri dishes containing a solid medium (1.5% plant agar, 8% sucrose, 0.5X MS basal medium added with Gamborg's vitamins, at pH 5.7). One week after germination, plantlets were transferred from in vitro culture to soil. Growth chamber conditions were as follow: 50 µmol m^{-2} s^{-1} photons light intensity, 22/18°C day/night air temperature, 65% relative humidity, 8/16 h photoperiod). All

Arabidopsis thaliana lines (*col-0* and *CarS*) were given by Jonathan Gershenzon of the Max Planck Institute for Chemical Ecology, Jena, Germany.

Samples of 2-weeks-old seedlings and fully expanded rosette 5-weeks-old plants were collected and stored at -80°C for molecular and biochemical analysis. Heat stress treatment was performed on both *col-0* and *CarS* 2- and 5-weeks-old plants, switching them from growth chamber to an incubator at 37°C and continuous 125 µmol m^{-2} s^{-1} photons light intensity for two days. Samples of both lines were collected at the beginning of the experiment and after 30 minutes, 1, 24 and 48 h of treatment. Another batch of plants was allowed to recover at growth temperature in the growth chamber after 24 hours of treatment. All samples during the treatment and the following recovery were subjected to physiological measurements and then collected for further analysis. All experiments were performed in duplicate.

4.2 Physiological Measurements on CarS *plants*

Photosynthesis measurements were performed either on whole intact rosettes or on single leaves. In whole plant measurements, whole Arabidopsis rosettes were inserted in a temperature controlled whole plant cuvette, hosting the intact plant in its growth vessel. To avoid soil moisture evaporation and soil respiration, the soil was isolated and covered with a Teflon layer. The cuvette system was flushed with a flux of synthetic air reflecting the ambient air composition (80% N_2, 20% O_2 and 370 ppm CO_2) deprived of

contaminants. The cuvette, during photosynthesis measurements, was maintained at either 22°C (growth temperature) or treatment temperature, at 37°C, exposed to growth light intensity of 170 µmol m^{-2} s^{-1} photons provided by an Osram-Power Star 1000 HQT source (Osram, Munich, Germany), and to 50% relative humidity. H_2O and CO_2 gas exchange measurement was performed by LI-6262 infrared gas analyzer (Li-Cor, Lincoln, NE, USA), and steady state photosynthesis was recorded. In an other set of experiments gas exchange measurements were performed with a LICOR 6400 portable gas exchange system on single leaves, in the temperature and light controlled cuvette system of the LICOR 6400 instrument. A 2 cm² leaf area was enclosed in the cuvette flushed with synthetic air (80% N_2, 20% O_2 and 370 ppm CO_2). Chlorophyll fluorescence was assessed on the leaf surface enclosed in the cuvette. The ratio between variable and maximal fluorescence (Fv/Fm) was measured in dark-adapted (20 min) leaves at the beginning of each measurement. Leaves were then adapted to 170 µmol photons m^{-2} s^{-1} light intensity in the cuvette, given by the LICOR 6400 inbuilt light source giving 90% red and 10% blue light. Steady state gas exchange was assessed at 22°C.

4.3 Anthocyanin determination

25 mg of frozen leaf material was sampled and homogenised in 1 mL of acidified (1% HCl) methanol and centrifuged at cold 15 min at 10,000*g*. Supernatant was saved and pellet was resuspended in 0.5 mL of acidified methanol and centrifuged 5 min at 10,000*g*. The

collected supernatants were passed through a 0.20 µm syringe filter (Ministart RC4, Sartorius). The optical density of the cleared supernatant was scanned between 400 and 700 nm and anthocyanins were measured as described by Mancinelli (1984), using the function $OD_{530} – 0.25\ OD_{657}$ to account for the contribution by chlorophylls.

4.4 Chlorophyll and carotenoid quantification

25 mg frozen leaf material was sampled and homogenised in 1 mL of a solution acetone/Tris buffer (80/20 volume; pH = 7.8, 50 mM), and centrifuged at cold for 5 min at 5,000*g*. Supernatant was saved and conserved at dark; pellet was resuspended one time in 1 mL and another time in 0.5 mL of acetone 80% and centrifuged at cold for 5 min at 5,000*g*. OD was measured at 663, 647, 537 and 470 nm and pigments concentration was determined taking the presence of anthocyanin into account as described by Sims and Gamon (2002).

4.5 Lipid peroxidation assay on CarS *plants*

For the measurements of the extent of lipid peroxidation in leaves, the thiobarbituric acid reactive substances (TBARS) test was used. The occurrence of malondialdehyde (MDA), a secondary end product of polyunsaturated fatty acid oxidation is a useful index of general lipid peroxidation. 40 mg frozen plant leaf material was homogenized in 80% ethanol extracting medium in the dark. The extract was centrifuged at 8,000*g* for 5 minutes in the dark, at 4°C. The pellet was separated into two equal aliquots. One aliquot was assayed in

the presence of 0.65% thiobarbituric acid (TBA), the other in the absence of TBA to correct for interferences in absorption due to the presence of anthocyanins in the extract, in an assay mix containing 20% trichloroacetic acid (TCA) and 0.01% butylate hydroxytoluene (BHT). All samples were incubated for 30 minutes in a block heater at 95°C. Absorbance was read at 440, 532 and 600 nm. Malonyl dialdehyde equivalents were calculated according to Hodges et al. (1999), to correct for aspecific absorbance interference of anthocyanins and total soluble sugars present in the samples.

4.6 *Real time qPCR analysis*

Plant material was homogenized in liquid nitrogen and total RNA was isolated using the TRIzol® Reagent (Invitrogen, CA, USA) according to the Invitrogen™ handbook, and then treated with RQ1 RNase-free DNase (Promega). First strand cDNA was synthesized from 1 µg of total RNA using the RT-IMPROM-II reverse transcriptase (Promega). The concentration of nucleic acids (RNA and cDNA) was measured using NDR ND1000 (Nanodrop Inc., USA). Semi-quantitative polymerase chain reaction (PCR) analysis was carried out with Platinum Taq DNA Polymerase (Invitrogen, CA, USA), using specific primers for (*E*)-β-caryophyllene synthase (*TPS27*) gene. mRNA expression level of *ACTIN8* (*ACT8*) gene was used to normalize the expression level of the other genes (for the sequence of primers used, see Table VI).

Table VI. List of PCR Primers Used During the Experiments Performed With the CarS Plants.

Protein Name	mRNA GeneBank Acc. No.	Primer Sequence	Length (bp)
APX1	X59600	5'-GTCCATTCGGAACAATGAGGTTTGAC-3' 5'-GTGGGCACCAGATAAAGCGACAAT-3'	326
sAPX	X98925	5'-TGCTAATGCTGGTCTTGTGAATGCTT-3' 5'-CCACTACGTTCTGGCCTAGATCTTCC-3'	417
tAPX	X98926	5'-CAGAATGGGACTTGATGACAAGGAAA-3' 5'-ATGCAGCCACATCTTCAGCATACTTC-3'	299
HSF21	AT4G18880	5'-TGCTAAAAGGGTTCTAGATTCGTTG-3' 5'-TTGGTGAGGAAAGGTGGAAGTGAGC-3'	449
RHA1	AT5G45710	5'-TAATGGAGGTTCAAGCTCACTTCC-3' 5'-TCCCAAAACGTTAGCGAAGATTC-3'	679
TPS27	AT5G23960	5'-CTCGCATCAAAAGAGGGAAAGGAA-3' 5'-GATGAGTGGCTTCCCGTTGTTCCTG-3'	574
FPS1	AT5G47770	5'-GGGAGAGAAAGTCAGTCCGATTGTG-3' 5'-ACCACGGCGAGTGACAGAGTTATCC-3'	537
FPS2	AT4G17190	5'-ATGGCTTGAACGGATGCTTGACTAC-3' 5'-CGCAAGCAACAGGAAGATAAAATGAG-3'	588
ACT8	AT1G49240	5'-ATGAAGATTAAGGTGGCA-3' 5'-TCCGAGTTTGAAGAGGCTAC-3'	458

The real time qPCR assay was performed in 12.5 µL of reaction mixture composed by 10 ng of cDNA and Brilliant SYBR Green QPCR master mix (Stratagene, USA) (final concentrations: 1.25 units of SureStart® Taq DNA polymerase, 1.25 mM $MgCl_2$, 0.8 mM dNTP mix, and 300 nM gene-specific primers) using an Mx3000P (Stratagene, USA) instrument. Amplification of PCR products was monitored via detection of fluorescence released by the intercalated SYBR Green I dye (Stratagene; 1:20 dilution of 1:3,000 stock solution). The following program was applied: initial polymerase

activation: 95°C, 10 min, then 40 cycles at: 95°C, 30 sec; 57°C, 45 sec; 72°C, 1 min; and finally one cycle at 72°C for 3 min. PCR conditions were optimized for amplification efficiency > 95% for all primer pairs used. Efficiency was determined by comparison of experimentally determined and theoretically expected threshold values in dilution series of the same cDNA using 500, 100, 10, or 1 ng per reaction.

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