Leaves of isoprene-emitting tobacco plants maintain PSII stability at high temperatures

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At high temperatures, isoprene emitting plants display a higher photosynthetic rate and a lower non-photochemical quenching (NPQ) compared to non-emitting plants. The mechanism of this phenomenon, which may be very important under current climate warming, is still elusive. Here, NPQ was dissected into its components, and chlorophyll fluorescence lifetime imaging microscopy (FLIM) was used to correlate the dynamics of chlorophyll relaxation with NPQ and the environment around photosystem II (PSII) in isoprene-emitting and non-emitting plants. The mechanical properties of thylakoid membranes were also measured using atomic force microscopy (AFM) to identify a possible mode of action of isoprene in improving photochemical efficiency and photosynthetic stability. We show that, when compared to non-emitters, isoprene-emitting tobacco plants exposed at rising temperatures display: i) a reduced increase of the NPQ energy-dependent component (qE); ii) stable chlorophyll fluorescence lifetime and amplitudes of the fluorescence decay components; and iii) a

stable thylakoid membrane elasticity. Our study shows for the first time that isoprene maintains PSII stability at high temperatures by preventing the modification of the microenvironment surrounding PSII. Conformational change of PSII and the dissociation of light harvest complex II (LHCII), with the formation of aggregates that are early indicators of PSII damage, do not occur in isoprene-emitting leaves exposed to high temperatures. Isoprene likely optimizes electron transport by creating a more homogeneous distribution of the light-absorbing centers between PSI and PSII. Ultimately, isoprene photoprotects leaves with a mechanism alternative to NPQ, enabling plants to maintain high photosynthetic rate at rising temperatures.

Key words: isoprene, (high) temperature, chlorophyll fluorescence (quenching and lifetime), NPQ, FLIM, AFM, photosynthesis.

Introduction

The photosynthetic apparatus rarely operates under optimal conditions in nature. Rather, photosynthesis usually operates under conditions where environmental factors are either limiting or in excess. Current and future climate warming and associated drought (IPCC 2018) are likely to worsen the situation by increasing photosynthetic constraints and thus leading to lower crop yields (Lesk et al., 2016; Lobell et al., 2013; Zhao et al 2017).

Different ways to improve photosynthesis, especially under limiting conditions, have been proposed (Evans, 2013). The biochemistry of photosynthesis may set the overall limit to the conversion of light to organic matter by photosynthesis, but mechanisms of photoprotection also contribute to maintaining

very high photochemical yields (Zhu et al. 2010). The relaxation speed of photosystem II (PSII) has been considered to play a pivotal role in photoprotection (Murchie and Niyogi, 2011; Kromdijk et al., 2016). This involves rapid activation and modulation of the non-photochemical quenching of chlorophyll fluorescence (NPQ) to dissipate the excess of light energy, mainly as heat (Baker, 2008). Kromdijk et al. (2016) found that increasing the efficiency of NPQ components, especially xanthophyll de-epoxidation, enhanced plant biomass under fluctuating light conditions.

The number and length of warm spells and heat waves are increasing due to climate change. Higher temperatures affect photosynthesis, and heat-stressed plants need to dissipate more excess energy even when exposed to the same light intensity, mainly by NPQ (Baker, 2008) and/or changing the relative sizes of the antennae of the two photosystems by a process called state transition (Allen and Forsberg 2001; Haldrup et al. 2001; Wollman 2001). The ability to rearrange the components forming the thylakoid membrane is one of the photoprotective mechanisms that optimise electron transports. The capacity to quickly cope with the amount of light and energy available seems to be the most important trait for plant fitness and productivity (Kromdijk et al. 2016).

Xanthophylls are formed in the chloroplasts by the methylerythritol phosphate (MEP) pathway, which also produces volatile isoprenoids, namely isoprene and monoterpenes (Loreto and Schnitzler 2010). Isoprene biosynthesis is light and temperature dependent and is highly stimulated by high temperatures (Loreto and Sharkey 1990). Due to its small molecular weight and very low evaporation temperature (34°C), isoprene is largely released into the atmosphere from stomata.

Many plant species may emit isoprene, but substantial emission has only been detected in approximately 20% of the species investigated, and is more typical of deciduous and fast-growing perennial plants in temperate regions (Loreto and Fineschi, 2015). Isoprene protects the photosynthetic apparatus under stressful environmental conditions, especially high temperatures (Singsaas et al. 1997; Velikova et al. 2011). NPQ is lower in isoprene-emitting than in non-emitting plants in stressed conditions (Loreto and Velikova 2001; Behnke et al., 2007; Way et al., 2012). Pollastri et al. (2014) reported a similar effect in unstressed leaves, implying that photosynthesis uses photons more efficiently and needs to non-radiatively dissipate less light energy when isoprene is present.

Various hypotheses have been proposed for how isoprene is able to stabilize photosynthesis, but the mechanism of action remains unclear. Isoprene may exert a positive action and increase synthesis and efficiency of metabolites downstream in the MEP pathway (e.g. xanthophylls); may stabilize and protect photosynthetic membranes from denaturation (Singasaas et al. 1997; Siwko et al. 2007; Velikova et al. 2011); and may operate as an antioxidant in the mesophyll (Loreto and Velikova 2001; Vickers et al. 2009).

Understanding how isoprene-emitting plants can maintain a high rate of photosynthesis at increasing temperatures is thus of great interest. In this paper, we used for the first time different approaches to investigate the effect of high temperatures on the photosynthetic system in transgenic isoprene-emitting and wild-type non-emitting *Nicotiana* tobacco plants (Vickers et al., 2009). After measuring photosynthesis, we dissected NPQ into its components, qE (energy-dependent quenching), qI (photoinhibitory quenching), qT (state transition quenching) (Krause et

Jahns, 2003; Walters and Horton 1991; Johnson et al., 2009). Using fluorescence lifetime imaging microscopy (FLIM), we measured chlorophyll fluorescence lifetime, a kinetic parameter describing the rate of de-activation of the excited state of Chlorophyll (Chl) *a* of PSII. Finally, we characterised the elasticity of the thylakoid membrane and determined chloroplast ultrastructure using atomic force microscope (AFM) and electron microscopy (EM). For the first time, we show that isoprene is able to prevent the modification of the microenvironment surrounding PSII, providing stability to the photosynthetic system at rising temperatures. Our results indicate that isoprene acts as a photoprotection mechanism alternative to NPQ, enabling plants to maintain high photosynthetic rate at rising temperatures.

Materials and methods

Plant materials, and growth and sampling conditions

We used wild-type (WT, isoprene non-emitting) plants, a transgenic line homozygous for isoprene synthase (H, isoprene emitter) and a transgenic line azygous for isoprene synthase (A, non-emitting isoprene) (Vickers et al., 2009) of *Nicotiana tabacum* (cv Samsun). Plants were grown in a growth chamber with the following settings (day/night): 14/10 h photoperiod, light intensity (photosynthetic photon flux density, PPFD) of 500 µmol photons m^{-2} s^{-1}, air temperature of 25/20 °C and relative humidity of 40-60%. Plants were irrigated daily and fertilized weekly with an NPK commercial solution. All measurements were carried out on the first fully expanded leaf of five plants for each genotypewhich were sequentially exposed to increasing temperatures (at +5 °C steps) from 25 °C, as detailed below.

Foliar gas-exchange and chlorophyll fluorescence

Photosynthesis, stomatal conductance and chlorophyll fluorescence were measured using a Licor 6400 XT portable photosynthesis system (LI-COR, Lincoln, USA). Leaves were placed inside the 2-cm^2 leaf cuvette with the following settings: 400 ppm CO_2, 500 µmol m^{-2} s^{-1} of PPFD (10% blue and 90% red light) and sequentially increasing foliar temperatures of 25, 30, 35 and 40 °C. Measurements were recorded when photosynthesis and stomatal conductance of the leaf enclosed in the cuvette were stable.

Non-photochemical quenching NPQ was calculated as:

Equation 1

where Fm and Fm' are the maximal fluorescence, respectively, in dark- and light-adapted leaves (Genty et al., 1989; Maxwell & Johnson, 2000).

NPQ components were measured in plants dark-adapted for 20 min using an Imaging Pam M-series fluorimeter (Heinz Walz GmbH, Germany). Single leaves were placed over a heating element set respectively at 25, 30 and 35°C, and exposed for 10 min at 500 µmol m^{-2} s^{-1} of PPFD, followed by 10 min of darkness.

The NPQ components, qE (energy-dependent quenching) and qI+qT (photoinhibitory quenching and state transition quenching), were calculated using the following equations (Johnson et al., 2009; Krause and Jahns, 2004):

Equation 2

Equation 3

where Fm'' is the maximum level of fluorescence after 10 min of relaxation in the dark.

Isoprene emission

Part of the air flowing from leaves clamped inside the Licor cuvette was diverted at the cuvette outflow into traps containing 30 mg of Tenax and 30 mg of Carboxen (Gerstel GmbH & Co.KG, Mülheim an der Ruhr, Germany) using a pump (Elite 5; A.P. Buck, Orlando, USA) set at 200 ml min^{-1} rate, for 25 min. A gas chromatograph – mass spectrometer (Agilent 7890 GC) with an Agilent HP-INNOWax (30 m × 0.32 mm × 0.15 μm) GC column and a 5975MSD mass selective detector was used for trap analysis. The chromatographic injector was set in splitless mode at 260 °C, and a J&W INNOWax column (50 m, 0.20 mm i.d., 0.4 um df) was used. The oven was maintained at an initial temperature of 40 °C for 1 min, and the temperature was then increased by 10 °C min^{-1} to 130 °C, by 5 °C min^{-1} to 210 °C and by 20 °C min^{-1} to 260 °C, where it was maintained for 3 min. The mass spectrometer was operated with an electron ionization of 70 eV in scan mode in the m/z range of 29-330, at three scans sec^{-1}. The NIST 11.L spectral library database was used to identify isoprene peaks, and the isoprene concentration was calculated using a calibration curve constructed with an isoprene gas standard (99.9%, Sigma-Aldrich, Saint Louis, USA). The Agilent Mass Hunter Workstation software (Agilent 7890A;

Agilent Technologies, Santa Clara, USA) was used to analyze data.

Fluorescence lifetime imaging microscopy (FLIM) and analysis

FLIM analysis was performed on whole leaves using the time correlated single photon counting (TCSPC) module of a Picoquant System (Picoquant GmbH, Berlin, Germany), coupled with a confocal microscope (TCS-SP5 confocal laser-scanning microscope, Leica, Wetzlar, Germania). Excitation light was provided by a pulsed laser at 640 nm with a repetition rate of 40 MHz. We used this wavelength to excite only chlorophyll molecules and to prevent contributions from carotenoids. Detection was set using an imaging bandpass at 650-730 nm. Time-resolved fluorescence emission was collected at 256 × 256 resolution, using a Leica 63x HCX PL APO CS Water objective. FLIM acquisition times ranged from 180 to 240 s and images were collected at approximately 300 counts of photons per pixel to prevent chloroplast movement.

The microscope incubator (Life Imaging Services, Basel, Switzerland) was set at 25 or 30 °C for measuring at the same temperatures at which the plants were exposed. The lifetime data acquired were analyzed using the PicoQuant software package SymPho Time 64.

Fluorescence decay curves were fitted using the following exponential function (N=2):

Equation 4

The average lifetime, τ, was then calculated:

Equation 5

The two components of this double-exponential curve, τ_1 and τ_2, are the short- and

long-lifetime components, respectively, and a_1 and a_2 are their relative amplitude contributions. The model function was first convolved with an experimental instrument-response function (IRF) before fitting the data. We used the instrument-calculated IRF, because its contribution did not affect the decay of the fluorescence curve. The quality of fit was accepted when chi square was approximately 1. Pseudocolor mapping was used to represent the distribution of fluorescence lifetime.

Thylakoid isolation

Thylakoid membranes were isolated as described by Casazza et al. (2001) and Clausen et al. (2014). Twenty grams of the first fully expanded leaves were collected and placed in cold water at 4 °C. The following steps were conducted at 4 °C. The leaves were homogenized in 100 mL of a homogenization buffer containing 0.4 M sorbitol, 5 mM EGTA, 5 mM EDTA, 10 mM NaHCO3, 5 mM MgCl2 and 20 mM tricine at pH 8.4. The homogenate was filtered through three layers of cheesecloth. This solution was centrifuged at 2600 × g for 3 min. The supernatant was discarded, and the pellet was resuspended in 10 mL of a resuspension buffer containing 0.3 M sorbitol, 20 mM HEPES at pH 7.6, 5 mM MgCl2, 2.5 mM EDTA and 10 mM NaHCO3. This step was repeated three times. The pellet was then resuspended for 5 min in 10 mL of a hypotonic buffer containing 20 mM HEPES at pH 7.6, 5 mM MgCl2, 2.5 mM EDTA and 10 mM NaHCO3. The solution was then 0.3 Msorbitol, 20 mM HEPES at pH 7.6, 5 mM MgCl2 and 10 mM NaHCO3. All AFM measurements were performed within 2 h after thylakoid isolation.

AFM measurements

The supernatant was discarded, and the pellet was resuspended in a measuring buffer containing AFM measurements were performed similarly as described in Clausen et al., (2014). Briefly, before experiments, circular glass coverslips were coated with Poly-D-Lysine at 2 mg/ml in MiliQ water for 30 min, then washed 4 times with buffer solution. The thylakoid suspension was added to the coverslip and left for 10 min until thylakoids were settled down, then washed 4 times with buffer solution. Samples were placed on the stage of an optical inverted microscope (TE2000, Nikon, Tokyo, Japan) with a Peltier system to change the temperature of the sample. The actual temperature was measured with a small thermistor submerged in the liquid. For each sample, the stiffness was measured at temperatures rising from 25 ˚C to 45 ˚C with steps of 5 ˚C. To stabilize the temperature in each step the sample was left for 5 min before measurements. Finally, the temperature was returned to 25 ˚C and AFM measurements were carried out again. A custom-built AFM (Alcaraz et al., 2003) was attached to the stage of the inverted microscope with the sample. The measurements were performed on 5 de-enveloped chloroplasts with a Si_3N_4 V-shaped cantilever with a spherical tip (4.5 μm in diameter, nominal spring constant k = 0.01 N/m) glued at its end. The cantilever was 3-D displaced with nanometric resolution by means of piezoactuators coupled to strain gauge sensors (Physik Instrumente, Karlsruhe, Germany) to measure the displacement of the cantilever (z). The deflection of the cantilever (d) was measured with a quadrant photodiode (S4349, Hamamatsu Photonics Inc., Hamamatsu, Japan) using the optical lever method (Alcaraz et al., 2003). The slope of a d–z curve obtained from a bare region of the coverslip was used to calibrate the relationship between the photodiode signal and cantilever deflection. The force (F) on the cantilever was computed as F = kd. At each measurement point,

five force–displacement (F–z) curves were first recorded by vertically oscillating the cantilever with triangular displacement at 1 Hz and peak-to-peak amplitude of 3 μm to reach a maximum indentation of 0.5 μm. Sample indentation (δ) was computed as $\delta = (z-z_c) - (d - d_{off})$, where z_c is the position of the contact point, and d_{off} is the offset of the photodiode. Force–displacement curves were analyzed with the Hertz contact model:

Equation 6

where R is bead radius, ν the Poisson's ratio (assumed to be 0.5) and E the Young's modulus which characterizes the elasticity of the sample. Eq. 6 can be expressed in terms of z and d as:

Equation 7

Non-linear least-squares fit (Matlab, The MathWorks, Natick, MA) was used to estimate E, z_c and d_{off} from the approaching section of the d–z curve for a maximum indentation of 0.3 μm.

Electron microscopy

For transmission electron microscopy (TEM) the thylakoid suspension was first fixed with paraformaldehyde 2% in PBS and then washed with PBS. The sample was post-fixed with osmium tetraoxide 1% in PBS and washed with MilliQ water. The sample was dehydrated with increasing gradients of acetone and included in resin for 72h at 70˚C. Images of 60 nm thick slices were obtained with TEM (JEOL-J1010, JEOL Inc., Peabody, USA) at 80 kV. Samples for scanning electron microscopy (SEM) were processed as for TEM but were dehydrated with gradients of ethanol and dried to the

critical point. Finally, the samples were imaged with JEOL JSM7001F with secondary electron detection.

Statistical analysis

Data are shown as means ± standard errors (SEs). The normality of data distribution was tested using the Shapiro–Wilk Normality Test. Significant differences ($p < 0.05$) between isoprene-emitting and non-emitting lines across the temperature range were analyzed using a one-way analysis of variance (ANOVA) (NPQ components, Flim and EM measurements) and repeated-measure ANOVA (Photosynthesis, isoprene emission, NPQ, Young's modulus) followed by Tukey's test. SigmaPlot was used for the analysis (Systat Software Inc., San Jose, USA).

Results

Photosynthesis was similar in all plants at 25 °C (Fig. 1A). As the temperature raised to 30°C, photosynthesis of WT and A remained stable, but increased in isoprene-emitting H leaves. In all lines photosynthesis was lower at 35 and 40 °C than at 30 °C. However, in the isoprene-emitting H line photosynthesis at 35 and 40 °C was similar to that measured at 25 °C, whereas in WT and A photosynthesis at 35 and 40 °C dropped to rates much lower than that recorded at 25°C. Isoprene emission in H was low at 25 °C but increased significantly with temperature, doubling at 30 °C and increasing further at 35 °C, but decreasing at 40 °C (Fig. 1B).

The major component of NPQ in all lines was the energy-dependent quenching qE, while the sum of qI+qT (photoinhibitory quenching and state transition quenching) was a minor component (Fig. 2). At 25°C, all plants showed similar NPQ,

and similar levels of NPQ components. As the temperature raised to 30°C, NPQ and qE statistically increased in WT and A. However, isoprene-emitting H leaves exposed to 30°C showed stable NPQ and qE values. At 35°C, NPQ and qE raised further in WT and A but were again stable in the H line, while qI+qT increased significantly in all lines.

To provide an indirect measure of the changes in the environment around PSII, we determined the effect of temperature on the relaxation dynamics of excited chlorophyll. To do this we measured chlorophyll-fluorescence lifetime in vivo using Fluorescence lifetime Imaging Microscopy (FLIM) of chloroplasts in the upper layer of mesophyll cells from light-adapted leaves of the three lines. Representative confocal FLIM images of chlorophyll-fluorescence average lifetime (τ) for leaves at 25 and 30 °C are shown in Fig. 3A, and actual values are compared in Fig. 3B. τ was comparable in the three lines ($\tau \approx 1.12$ ns) at 25 °C but was clearly lower at 30 °C than at 35°C only in WT and A ($\tau \approx 0.85$ ns), not in H (Fig. 3b).

Further analysis of the FLIM data identified two quenching processes with long-lifetime ($\tau_1 \approx 1.34$ ns) and short-lifetime ($\tau_2 \approx 0.68$ ns) decay components, which did not vary significantly with temperature in the isoprene-emitting and non-emitting lines (Fig. 3C, D). The amplitude of τ_1 (a_1) was also similar in the three lines at 25 °C (Fig. 3E). However, a_1 was considerably lower ($\approx$ 80%) at 30 °C in the non-emitting WT and A lines but was similar to that measured at 25°C in the isoprene-emitting H line. The amplitude of τ_2 (a_2) was also similar in all three lines at 25 °C (Fig. 3F) but again was lower at 30 °C ($\approx$ -20%, not statistically significant) in the non-emitting WT and A lines. In the isoprene-emitting H line, a_2 was similar at 25 and 30 °C.

The elasticity of isolated thylakoid membranes was measured using AFM at increasing temperatures from 25 to 45 °C and at 25 °C after recovery. The elasticity of the membrane was similar among the genotypes at 25 °C (Fig. 4A) but gradually increased with temperature in the non-emitting lines, becoming significantly higher at 45 °C in WT (Fig. 4B) and at 40 °C in A (Fig. 4C) with respect to the elasticity at 25 ° C. The elasticity of the membrane in the isoprene-emitting H line did not vary across the temperature range (Fig. 4D). The Young's modulus measured after recovery at 25° C was similar to that measured before the heat ramp in all genotypes (Fig. 4B, C, D).

Finally, we used electron microscopy on samples at the growth temperature (25 °C) in order to check the absence of the outer envelope of the membrane and to rule out that some anatomical feature could be responsible of previous results. Specifically, we used scanning electron microscopy (SEM) to investigate the length of the de-enveloped chloroplasts, which were similar in WT and H yet slightly shorter in A (supplementary material 1A, B). We used transmission electron microscopy (TEM) to investigate the height of grana stacks, which was again similar in WT and H but lower in A (supplementary material 1C, D).

Discussion

Isoprene protects photosynthetic membranes against high temperatures and other environmental stresses (e.g. Singsaas et al. 1997, Velikova et al. 2011). Several hypotheses have been made to explain how isoprene protection occurs, but a solid mechanistic interpretation was still needed (Loreto and Schnitzler 2010). Pollastri et al. (2014) first reported a positive effect of isoprene at non-stressful temperatures (supported here). Specifically, these authors found that NPQ increased significantly

less in isoprene-emitting than in non-emitting leaves that were exposed to high temperatures that did not impair photosynthesis. A lower dissipation of energy as heat may thus be needed when isoprene is present, which may in turn indicate that electron flow along the transport chain is more efficient when isoprene is present (Loreto and Schnitzler 2010, Velikova et al. 2011, 2012; Pollastri et al. 2014).

We also found that isoprene-emitting leaves maintained high photosynthesis at temperatures (35 °C) at which photosynthesis of non-emitting leaves began to decrease and that this stability was associated with a reduced temperature-dependent increase in NPQ (Fig. 1). We further analyzed the components of NPQ using FLIM and AFM to determine why isoprene-emitting leaves did not need to increase NPQ when challenged by high temperatures. We found that, compared to non-emitters, isoprene-emitting tobacco leaves exposed to high temperatures had i) a minor increase in the NPQ energy-dependent component (qE) (Fig. 2), ii) a stable average chlorophyll-fluorescence lifetime (Fig. 3 B), iii) a stable amplitude of the components of fluorescence decay (Fig. 3 E, F), and iv) a stable Young's modulus of thylakoid membranes (Fig. 4). We now discuss the meaning of these new findings.

- NPQ does not increase in isoprene emitting leaves exposed to high temperatures because qE remains stable. qE is the main component of NPQ and is associated with non-radiative thermal dissipation. qE tends to increase when leaves are exposed to high temperatures, especially under high levels of light (Kromdijk et al., 2016). The increase in qE is accompanied by the acidification of the chloroplast lumen (Krause et al., 1982), which activates xanthophyll synthesis (Yamamoto et al., 1999; Ruban et al., 2012) and induces protonation of the PsBS protein (Li et al., 2000; 2004), causing

conformational changes in PSII (Horton et al., 2005; Krause et al., 2004; Pascal et al., 2005). qE may not be elicited in isoprene-emitting leaves exposed to high temperatures and consequently the above sequence of events may not occur when isoprene is present (Fig. 2). Velikova et al. (2011) reported that in isoprene-emitting plants exposed to high temperatures: a) the arrays of light-harvesting complex II/photosystem II in the stacked region of the thylakoid grana remained ordered, b) the leakiness of thylakoid membranes to ions did not increase and c) the charge separation state between the redox components on the PSII donor and acceptor sides were not perturbed, maintaining the efficient primary photochemistry of PSII.

- At rising temperatures, the average chlorophyll fluorescence lifetime does not decrease only in isoprene emitting plants. A higher NPQ is generally associated with a shorter fluorescence lifetime, indicating a more chaotic environment where quenching molecules or pathways are activated, (Gilmore et al. 1995, 1998; Briantais at al. 1996; Hartel et al. 1996; Gilmore and Govindjee 1999; Niyogi 1999; Holub et al. 2000, 2007; Sylak-Glassman et al., 2014; Denton *et al*., 2015). Indeed, chlorophyll fluorescence lifetimes are shortened in aggregated LHCII (Tang et al., 2007; Gruszecki et al., 1997; Moya et al., 2001; van Oort et al., 2007; Natali et al., 2016) and in the clustering of pigment-protein complexes (Belgio et al., 2013; Janik et al., 2013, 2015; Natali et al., 2016). The shortening of the excited-state lifetime of detached antennal complexes can become a protective mechanism against photodamage (Unlu et al., 2014). The formation of LHCII aggregates has also been linearly correlated with NPQ *in vivo*, suggesting that LHCII aggregates may represent a protective mechanism to dissipate excess excitation energy in heat-stressed plants

(Tang et al., 2007). Our results therefore indicate that the conformational change of PSII and the dissociation of LHCII from PSII, with the formation of aggregates that are early indicators of PSII damage, do not occur in isoprene-emitting leaves exposed to high temperatures.

- At high temperatures the amplitude of the long-lifetime component of fluorescence decay was conserved in isoprene-emitting leaves. Janik et al. (2017) proposed that the long life-time component of fluorescence decay (1.8 ns) was derived from monomeric or trimeric LHCII antennal complexes, whereas the shorter component (1 ns) was derived from antennal complexes involved in the formation of protein clusters characterized by a pronounced excitation quenching. Matsubara et al. (2011) assigned the longer chlorophyll-fluorescence lifetime (1.5 ns) to unquenched components and the shorter lifetime (0.5 ns) to quenched components associated with photoinhibition, whereas Gilmore et al. (1998) ascribed the shorter component to the effect of zeaxanthin formation. Our data suggest that the number of monomeric or trimeric LHCII antennal complexes do not decrease in isoprene-emitting leaves as temperature increases, leading to a more stable and homogeneous distribution of the light-absorbing centers between PSI and PSII (Janik et al., 2017) compared to non-emitting leaves.

iv) At high temperatures, membrane elasticity does not change in isoprene emitting leaves. The mobility and flexibility of thylakoid membranes are important factors affecting photosynthesis and mechanisms of photoprotection. Thylakoid lipids and proteins are responsible for the dynamic structure of membranes (Ruban et al., 2015) and for enzymatic activity (Loll et al., 2007). Isoprene synthase, which has been

localized in the stroma and in the stromal-facing side of thylakoid membranes (Schnitzler et al., 2005), may alter lipid composition (Velikova et al., 2015) and thylakoid plasticity. The dynamics of thylakoid membranes are important for the migration and diffusion of metabolites, protein complexes such as plastoquinone (PQ), plastocyanin (PC), and LHC and protein supercomplexes necessary for adaptation and repair processes (Moisan et al., 2006;. Chuartzman et al. 2008). AFM is an important tool for studying the mechanical properties and organization of thylakoid membranes (Kaftan et al., 2002; Clausen et al.,2014; Chuartzman et al. 2008; Phuthong et al., 2015). To our knowledge, this investigation is the first to determine the effect of temperature on the mechanical properties of thylakoid membranes. In particular, we measured membrane elasticity (Young's modulus), which is a property linked to membrane stiffness (Kuznetsova et al., 2007). Our data suggest that membrane elasticity in isoprene-emitting leaves does not change as temperature increases whereas the thylakoid membranes of non-emitting leaves loose elasticity and become stiffer as temperature increases. This finding is not related to changes in the chloroplast anatomy, as reported in supplementary materials. The ability to modify the structure and dynamics of lumenal spaces in the light is a very important feature for modulating the diffusion of molecules and the transition state, allowing photosynthetic electron transport (Kirchoff et al., 2011; Amunts et al., 2009). Clausen et al. (2014) reported that membranes become stiffer when exposed to light, since they undergo conformational changes. At rising temperatures, the thylakoid membranes of isoprene-emitting leaves maintain better conditions for molecular diffusion, electron transport rate, dynamic swelling of the lumen and structural and molecular reorganisation, which are fundamental for photosynthesis and

photoprotection.

In conclusion, our study further supports the notion that isoprene maintains PSII stability at high temperatures. Isoprene maintains the dynamic plasticity of the entire photosynthetic machinery thereby preventing conformational changes and the dissociation of antennae or LHCII from PSII. Pollastri et al. (2014) surmised that isoprene exerts its positive effects at high temperatures by cooling chloroplasts. Our data, however, suggest that isoprene acts as a stabilizer of the PSII apparatus and of its environment, in turn sustaining efficient electron transport even in harsh conditions. Compelling evidence is accumulating that isoprene in thylakoid membranes protects the photochemistry of photosynthesis (e.g. Singsaas et al. 1997; Siwko et al. 2007; Velikova et al. 2011, 2012; this study). We can refer to isoprene emission as a photoprotection mechanism and as an alternative to NPQ. A further step could be the measurement of the trimeric/monomeric antennal LCHII complexes in isoprene emitting leaves, at different temperatures in order to verify the results gained with lifetimes analysis. Future studies should also help localize the site of action of isoprene and further identify the molecular mechanisms associated with isoprene-induced PSII stability. Furthermore, in the current and future climate change scenarios, the ability to synthetize isoprene could be considered as a positive trait, enabling plants to be more resilient and productive to thermal stresses induced by global warming or heat waves.

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Conflicts of Interest: None declared.

Author Contributions

SP, FL, JP and MK designed the research. SP and JL performed photosynthesis and chlorophyll fluorescence measurements with the supervision of FL and JP. SP and TH performed the lifetime measurements with the supervision of MK and PH. IJ performed the AFM measurements with the supervision of DN and SP prepared the samples. MM performed isoprene emission measurements. SP performed electron microscopy imaging. SP, IJ and DN made the statistical analysis. SP wrote the first draft of the manuscript, and all authors contributed to the interpretation of the analyses and to the writing and revision of the manuscript.

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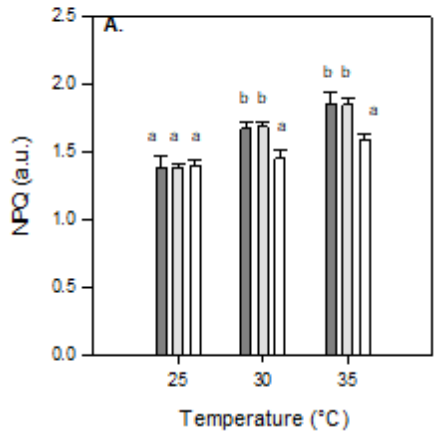
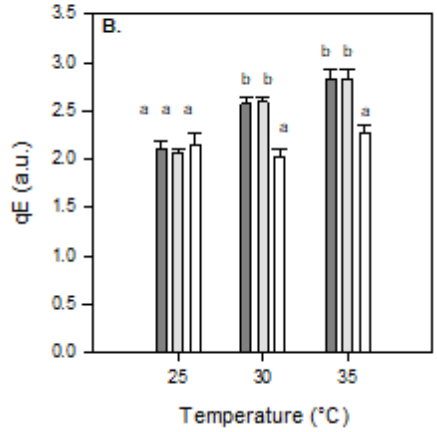
Figure 1. Photosynthesis (A) of wild-type = WT (dark grey circles); azygous line = A (grey circles); and isoprene-emitting line = H (white circles) leaves at 25, 30, 35 and 40°C. In (B) the isoprene emission of H leaves, measured at the same temperatures as in (A) is shown. Means (n = 5) $\pm$ SE are shown in both panels. Repeated-measure one-way ANOVA followed by Tukey's test was performed to define statistical significance ($p < 0.05$) of differences among means. Different letters show statistically significant differences.


A.
NPQ (a.u.)
2.5
2.0
1.5
1.0
0.5
0.0
a a a
b b a
b b a
25
30
35
Temperature (°C)


B.
qE (a.u.)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a a a
b b a
b b a
25
30
35
Temperature (°C)

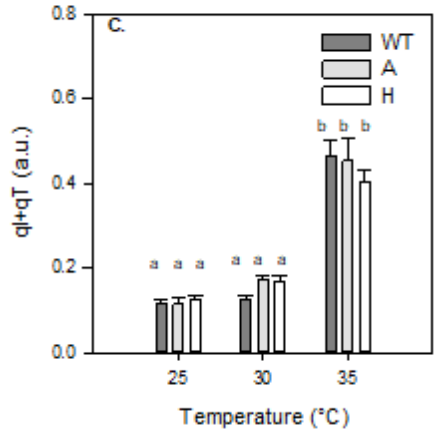
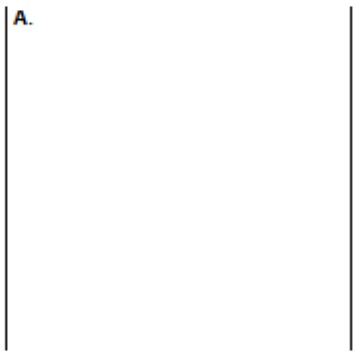
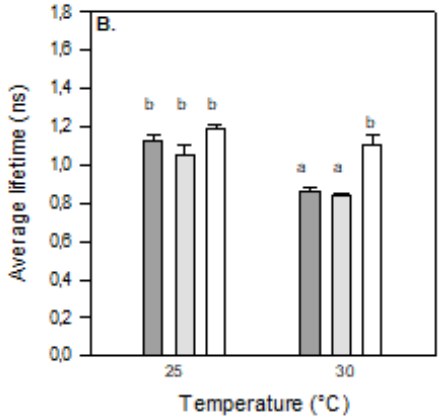
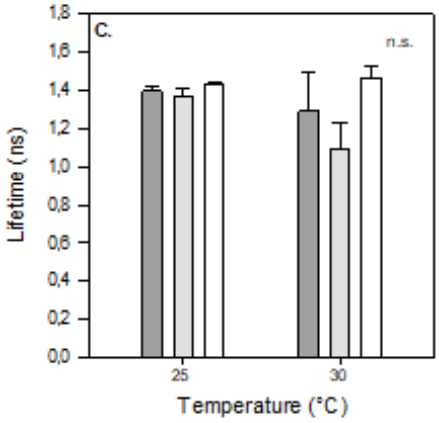

C.
WT
A
H
qI+qT (a.u.)
0.8
0.6
0.4
0.2
0.0
a a a
a a a
b b b
25
30
35
Temperature (°C)

Figure 2. Non-photochemical quenching (NPQ) (A) and NPQ components qE (B) and qI+qT (C) of wild-type = WT (dark grey bars); azygous line = A (grey bars); and isoprene-emitting line = H (white bars) leaves at 25, 30, and 35°C. Means (n = 5) $\pm$ SE are shown. A one-way ANOVA (qE, qI+qT) and repeated measure ANOVA (NPQ) followed by Tukey's test were performed to define statistical significance ($p < 0.05$) of differences among means. Data not sharing the same letters are statistically significant.

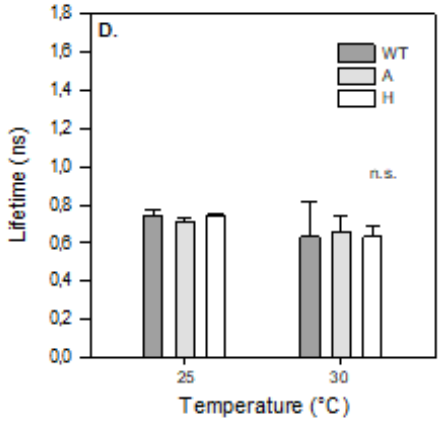


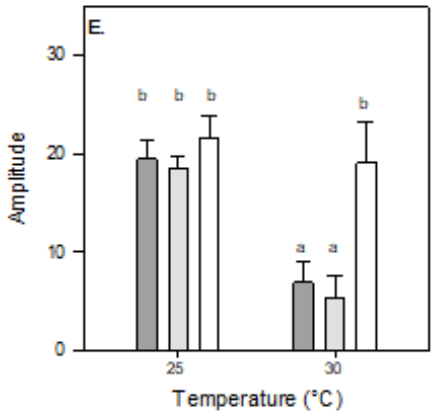


Long lifetime component



Short lifetime component





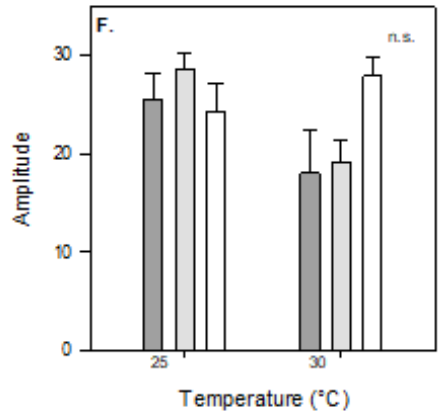


Figure 3. Representative confocal chlorophyll fluorescence lifetime images of chloroplasts located in the upper layer of mesophyll cells of wild-type = WT; azygous line = A; and isoprene-emitting line = H tobacco leaves, measured at 25°C and 30°C (panel A). The color bar below panel A shows the range of fluorescence lifetimes. Scale bar: 10 µm. Average chlorophyll fluorescence lifetimes (B). Fluorescence decay parameters (lifetime and amplitude of long and short lifetime components) (C-F). In all bar panels, bars represent wild-type = WT (dark grey), azygous line = A (grey) and isoprene-emitting line = H (white). Means (n = 5) $\pm$ SE measured at 25 and 30°C are shown. A one-way ANOVA followed by Tukey's test was performed to define statistically significant differences among means ($p < 0.05$). Means not sharing the same letters are statistically significantly different (n.s.: not significant).

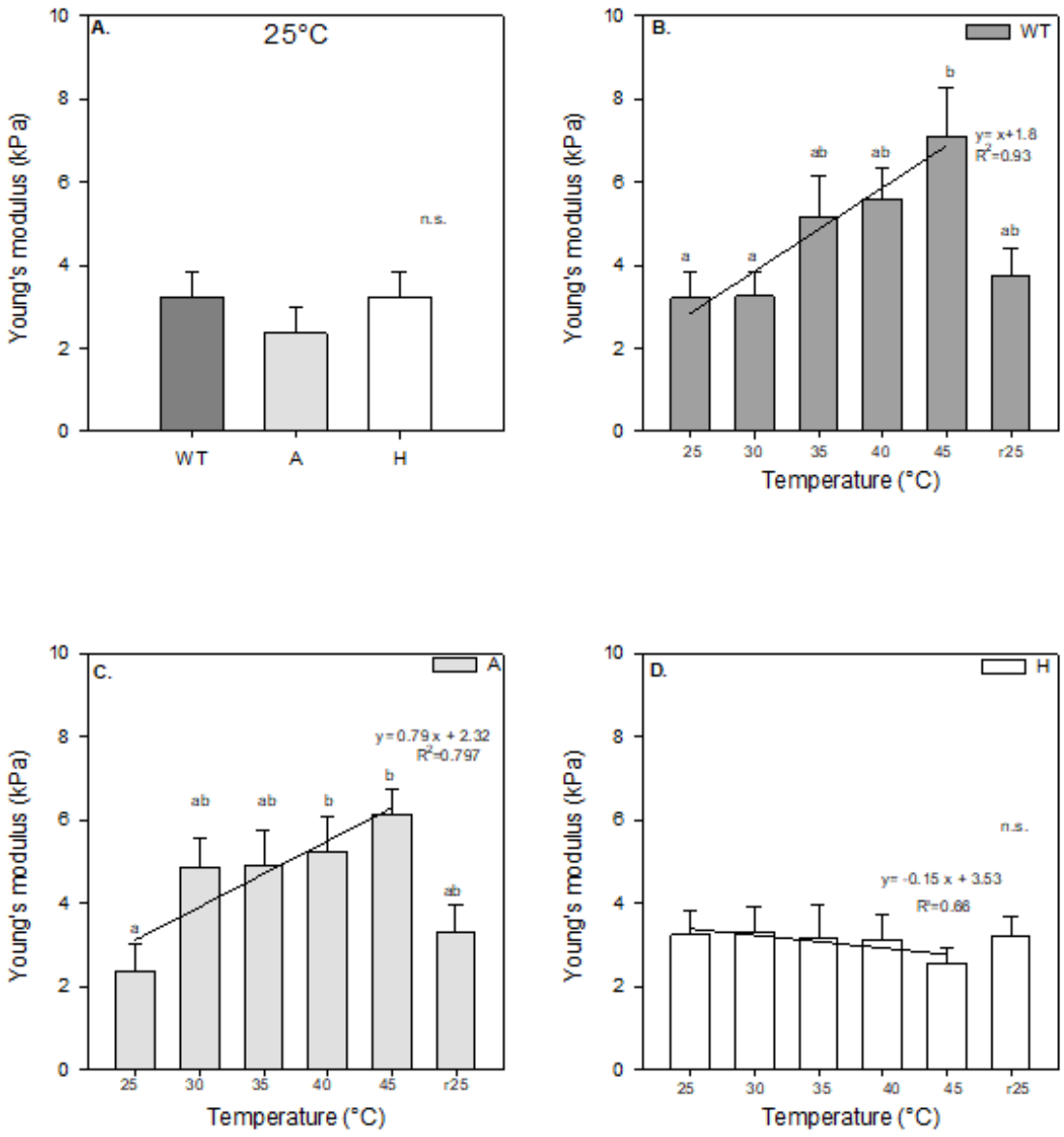
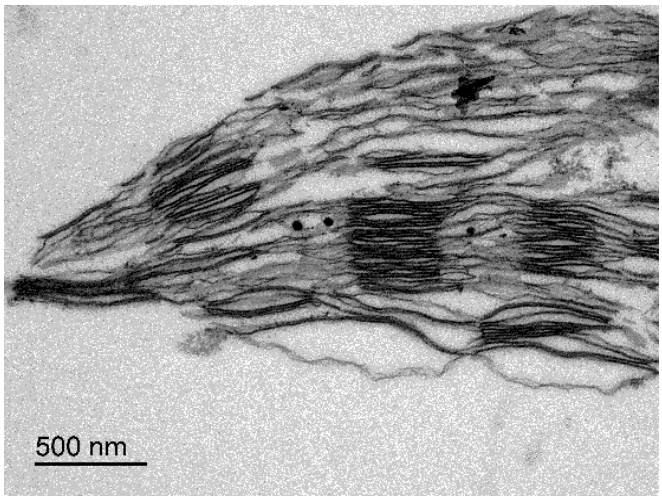
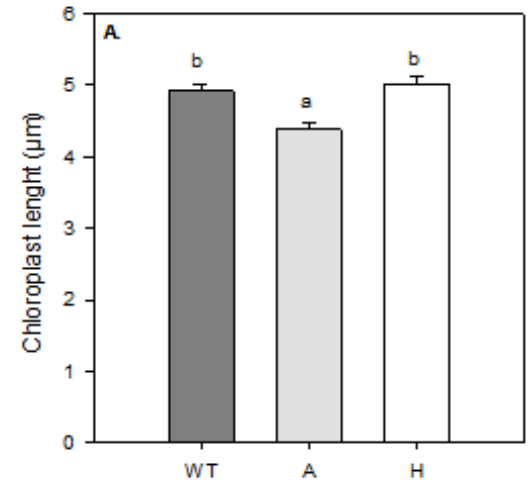
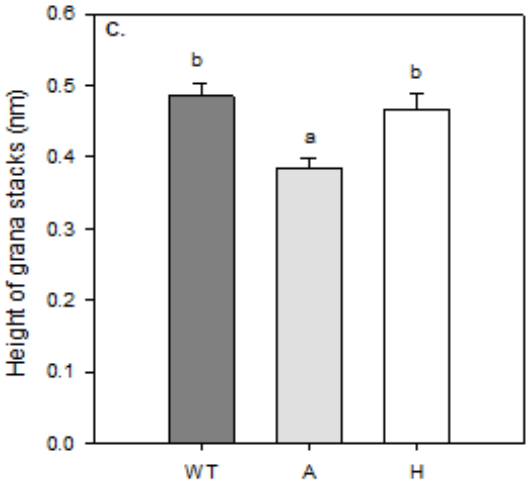


Figure 4. Young's modulus (chloroplast membrane elasticity). In panel (A) wild-type = WT (dark grey bars), azygous line = A (grey bars) and isoprene-emitting line = H (white bars) are compared at 25°C. In panels (B-D) the Young's modulus of WT, A and H, respectively, was measured at rising temperatures (25, 30, 35, 40 and 45°C) and after returning at 25°C (r25). Means (n = 15) ± SE are shown. Repeated-measure one-way ANOVA followed by Tukey's test was performed to define statistically significant differences between means ($p < 0.05$). Data not sharing the same letters are statistically different (n.s.= not significant). Best fits

showing the temperature-dependent (25-45°C) changes of the Young's modulus are also depicted in panels B-D.







Supplementary material, Figure 1. Chloroplast length (A), height of grana stacks (C), and associated de-enveloped chloroplast images by scanning electron microscopy (SEM) B) and transmission electron microscopy (TEM) (D). Wild-type = WT (dark grey bars), azygous line = A (grey bars) and isoprene-emitting line = H (white bars) are compared at 25°C.

Means $\pm$ SE are shown, calculated from at least 30 chloroplasts for each genotype. A one-way ANOVA followed by Tukey's test was performed to define statistically significant differences between means ($p < 0.05$). Data not sharing the same letters are statistically different.