Running title: Isoprene emission and plant metabolism under drought stress

Isoprene production in transgenic tobacco alters isoprenoids, non-structural carbohydrates and phenylpropanoids metabolism, and protects photosynthesis from drought stress

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ABSTRACT

Isoprene strengthens thylakoid membranes and scavenges stress-induced oxidative species. The idea that isoprene production might also influence isoprenoid and phenylpropanoid pathways under stress conditions was tested. We used transgenic tobacco to compare physiological and biochemical traits of isoprene emitting (IE) and non-emitting (NE) plants exposed to severe drought, and subsequent re-watering. Photosynthesis was less affected by drought in IE than in NE plants, and higher rates were also observed in IE than in NE plants recovering from drought. Isoprene emission was stimulated by mild drought. Under severe drought, isoprene emission declined, and levels of non-volatile isoprenoids, specifically de-epoxidated xanthophylls and ABA, were higher in IE than in NE plants. Soluble sugars and phenylpropanoids were also higher in IE plants. After re-watering, IE plants maintained higher levels of metabolites but isoprene emission was again higher than in unstressed plants. We suggest that isoprene production in transgenic tobacco triggered different responses, depending on drought severity. Under drought, the observed trade-off between isoprene and non-volatile isoprenoids suggests that in IE plants isoprene acts as a short-term protectant, whereas non-volatile isoprenoids protect against severe, long-term damage. After drought, it is suggested that the capacity to emit isoprene might up-regulate production of non-volatile isoprenoids and phenylpropanoids, which may further protect IE leaves.

Key words: abscisic acid (ABA), carotenoids, drought stress, flavonols, hexoses signaling, isoprene, MEP pathway, phenylpropanoids, xanthophylls

Summary statement:

Isoprene exerts important functions in plant defense. In the present study we show that photosynthesis of transgenic tobacco plants in which isoprene biosynthesis was made possible, is less affected by drought, and that isoprene production is associated to increased biosynthesis of secondary metabolites during and after drought.

INTRODUCTION

Isoprene (2-methyl-1,3-butadiene), as many other secondary metabolites, provides general protection against oxidative stress under wide range of environmental conditions (Vickers *et al*. 2009a). Isoprene may act as thermoprotectant of the photosynthetic apparatus by enhancing the integrity of thylakoid membranes (Singsaas *et al*. 1997; Velikova *et al*. 2011), as well as by scavenging stress-induced reactive oxygen and nitrogen species (ROS/RNS) (Loreto *et al*. 2001; Velikova *et al*. 2005; Vickers *et al*. 2009b). These functions are of crucial significance in countering the deleterious effects of stress-induced excess excitation energy in the photosynthetic machinery, playing a role against multiple stresses, including drought.

Drought stress inhibits photosynthesis. The over-reduction of photosynthetic electron transport, along with stimulation of photorespiration and a network of other responses result in increased production of ROS (Hernández *et al*. 2012). Isoprene production is normally linked with photosynthesis, which provides around 80% of carbon used for isoprene biosynthesis (Brilli *et al*. 2007). However, isoprene biosynthesis still occurs when drought-induced stomatal and mesophyll limitations greatly depress and even totally inhibit photosynthesis. This indicates that plants use multiple sources of carbon to maintain isoprene production (Funk, Mak & Lerdau 2004; Brilli *et al*. 2007), and further suggests that isoprene could also play a role in ameliorating plant performance under drought stress (Brüggemann & Schnitzler 2002; Pegoraro *et al*. 2004).

Isoprene may indeed serve as a short-term thermo-protective agent under drought stress, e.g. in *Quercus* spp. (Brüggemann & Schnitzler 2002). Recently, Beckett *et al*. (2012) showed that isoprene production by the resurrection plant *Xerophyta humilis* increased under mild drought stress (when relative water content, RWC, was at 80%), presumably strengthening membranes and providing protection for the photosynthetic apparatus. However, isoprene production decreased under extreme desiccation (when RWC was below 5%), and levels of zeaxanthin and lutein increased. The latter two non-volatile isoprenoids presumably operated photosynthetic membrane protection more efficiently than isoprene under extreme desiccation (Beckett *et al*. 2012). This suggests tight regulation and co-operative functions of non-volatile and volatile isoprenoid metabolism during drought stress.

Isoprene and non-volatile isoprenoids (e.g. carotenoids) are formed through the same metabolic pathway (methylerythritol phosphate, MEP pathway) that also produces abscisic acid (ABA), a pivotal molecule in drought stress protection. ABA induces stomatal closure and many other biochemical and physiological adjustments that allow plants to tolerate drought stress (Wilkinson & Davies 2010). Isoprene emission may

correlate with foliar ABA concentrations, as inhibition of isoprene biosynthesis through inhibition of the MEP pathway resulted in a sharp reduction in leaf ABA concentration in *Populus alba* (Barta & Loreto 2006). Furthermore, isoprene emission is linearly correlated with water use efficiency (WUE) in poplar species naturally emitting different rates of isoprene (Guidolotti, Calfapietra & Loreto 2011). Since WUE is co-regulated by stomatal closure, isoprene emission may again proxy ABA levels.

Vickers *et al*. (2009b) have shown that isoprene may induce tolerance to generic oxidative stress from ozone fumigation. Similarly, Behnke *et al*. (2010) showed that transgenic grey poplars with suppressed isoprene biosynthesis suffered from greater oxidative stress as compared with wild-type plants under high temperature. Since suppression of isoprene biosynthesis in transgenic poplars down-regulated genes for polyphenol biosynthesis (Behnke *et al.* 2010), it may suggested that tolerance to oxidative stress might be enhanced through a stimulation of both isoprenoids and polyphenols biosynthesis when isoprene is produced. However, the mechanism(s) underlying the inter-relation between different secondary metabolic pathways is (are) far from being elucidated. New insights might come from recent molecular works. The introduction of Anthocyanin Pigments1 (PAP1) transcription factor in rose plants greatly enhanced the biosynthesis of volatile isoprenoids (Zvi *et al*. 2012).

Besides having a direct role on drought-induced stomatal closure, ABA has also long been reported to participate in stress-induced metabolic readjustment (for a review, see Fujita *et al*. 2011). In particular, by increasing the transcripts of several myb genes (Denekamp & Smeekens 2003; Luo *et al*. 2012), ABA may up-regulate the biosynthesis of phenylpropanoids, particularly flavonols and anthocyanins (Tossi, Lamattina & Cassia 2009; Perrone *et al*. 2012; Tossi *et al*. 2012). R2R3-MYB transcription factors indeed coordinately regulated flavonoid and isoprenoid biosynthesis in conifer trees (Bedon *et al*. 2010). It is therefore possible that, in drought-stressed plants, isoprene biosynthesis influences ABA production, indirectly regulating the biosynthesis of flavonoids.

Recently, a novel isoprene-emitting transgenic tobacco model was used to examine the response of isoprene and photosynthesis to mild drought stress (Ryan *et al*. 2014). This study demonstrated that isoprene-emitting plants under drought stress accumulate less ROS than non-emitting plants, though the effect was statistically significant only in one line. We have used the same transgenic tobacco tool but our experiment consisted of a prolonged and severe drought stress, followed by re-watering. It is known that, in nature, isoprene emission is resistant to stress, being sustained by photosynthetic carbon until photosynthesis is minimally active, and by other carbon sources when photosynthesis is totally suppressed (Brilli et al. 2007). We aimed at investigating i) the protective effect of isoprene against a severe stress in a transgenic plant that naturally does not produce isoprene; ii) the consequences of artificial isoprene biosynthesis on plant metabolism. In addition to photosynthesis, we examined the effect of isoprene emission on accumulation of (1) non-volatile isoprenoids (including ABA and carotenoids); (2)

phenylpropanoids, which have been supposed to be involved in ABA-mediated drought responses (Perrone *et al*. 2012); (3) non-structural carbohydrates, as sugar signaling is thought to be involved in regulating accumulation of high-order isoprenoids and phenylpropanoids (Flores-Perez *et al*. 2010).

MATERIAL AND METHODS

Plant material, growth conditions, drought treatment and sampling protocol

Transgenic tobacco (*Nicotiana tabacum* cv. Samsun) genotypes were used in this study. The transgenic system consists of lines that are homozygous for a *Populus alba* isoprene synthase (isoprene-emitting; referred to here as IE), and azygous null segregants (isoprene non-emitting; referred to here as NE) within each line (Vickers *et al*. 2009b). Fourth generation transgenic plants from two different lines (lines 6 and 12) were used. Ten days after seed germination, the seedlings were transferred to Klasmann compost (Klasmann-Deilmann GmbH, Germany) in 2 L pots. Plants were grown in two climate-controlled phytotrons with an ambient day/night temperature of 30 ± 5°/20 ± 3°C, a daily photoperiod of 10 h with a photosynthetic photon flux density (PPFD) of 800 ± 100 µmol m^{-2} s^{-1}, and a relative humidity of 50 – 60%. Fully expanded leaves were used for all measurements.

For drought experiments, pots were wrapped in plastic film and weighed daily during the experiment. Drought was imposed by withholding water from 30-d-old plants. We used as a drought indicator the fraction of transpirable soil water (FTSW, %) as explained in Brilli *et al*. (2007). This parameter normalizes differences in the velocity of drought stress occurrence, due to uneven plant growth and canopy transpiration. During drought stress, gas exchange measurements were taken prior to stress (FTSW = 98%), and when the stress was mild (FTSW = 68%), severe (FTSW = 34%), and at FTSW endpoint (FTSW = 24%). Plants reached FTSW endpoint 7-8 days after interrupting irrigation. At FTSW endpoint drought-stressed plants were re-watered to pot capacity, and measurements were made after a 2-d recovery period (FTSW = 96%). Biochemical measurements for ABA, non-structural carbohydrates, pigments and phenylpropanoids were taken prior to stress, at FTSW endpoint and after recovery.

Gas-exchange measurements

Gas exchange was measured using a LI-6400 portable photosynthesis system (Li-Cor, Lincoln, NE, USA). Measurements were performed at a PPFD of 1000 µmol m^{-2} s^{-1}, 400 µmol mol^{-1} [CO_2] and a leaf temperature of 30°C. Photosynthesis (P_n), transpiration (T), and stomatal conductance (g_s) were calculated using the LI-6400 software, and the instantaneous water use efficiency (WUE) was calculated as P_n/T ratio.

Responses of P_n to changes in intercellular CO_2 (C_i) concentration were measured at a PPFD of 1000 µmol m^{-2} s^{-1} and [CO_2] between 50 and 1600 µmol mol^{-1}, starting with the lowest [CO_2] to remove stomatal limitations on P_n (Centritto, Loreto & Chartzoulakis 2003). The method of Sharkey *et al*. (2007) was used to calculate the electron transport rate and the maximum carboxylation rate allowed by Rubisco (V_{cmax}), from the response of P_n to C_i.

Chlorophyll fluorescence imaging

Images of chlorophyll fluorescence were obtained using a MAXI-Imaging-PAM Chlorophyll Fluorometer (Walz, Effelrich, Germany). A charge-coupled device (CCD) camera with a resolution of 640×480 pixels collected the fluorescence signal emitted by leaves in response to measuring, actinic and saturating light. Leaves were dark-adapted for 15 min prior to determination of minimal (F_0) and maximal (F_m) chlorophyll fluorescence. These measurements were used to calculate the maximum quantum yield of PSII photochemistry ($F_v/F_m = (F_m–F_0)/ F_m$). Leaves then were exposed to actinic, moderate light intensity (371 µmol m^{-2} s^{-1}) and, after steady-state fluorescence (F_s) was reached, a saturating pulse was applied to determine the maximum fluorescence in the light (F_m'). Chlorophyll fluorescence images taken from illuminated leaves were used to calculate the operating photochemical efficiency of PSII ($\Phi PSII = F_m'–F_s)/F_m'$ (Genty, Briantais & Baker 1989)). The non-photochemical quenching (NPQ) then was calculated as $NPQ = (F_m–F_m')/F_m'$ (Bilger & Björkman 1991).

Analysis of isoprene

Measurements were performed by partially diverting the outlet of the cuvette of the gas-exchange system previously described into a silico-steel cartridge packed with 200 mg of Tenax (Agilent, Cernusco sul Naviglio, Italy). A volume of 5 L of air was pumped through the trap at a rate of 150 mL min^{-1}. The cartridge was analyzed using a Perkin-Elmer Clarus 580 gas-chromatograph coupled with a Clarus 560 Mass-Selective-Detector and a thermal-desorber TurboMatrix (Perkin Elmer Inc., Waltham, MA, USA). Column was an Elite-5-MS capillary column (30 m length). The column oven temperature was kept at 40°C for the first 5 min, then increased with a 5°C min^{-1} ramp to 250°C, and finally maintained at 250°C for 2 min. Isoprene was identified using the NIST library provided with the GC/MS Turbomass software. Isoprene was quantified, and peak/fragmentation identity confirmed, using a calibration curve prepared with an authentic isoprene standard (Rivoira, Milan, Italy).

Analysis of ABA and non-structural carbohydrates

ABA was determined by an indirect ELISA test based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Trivellini *et al*. 2011). ABA was measured after extraction in distilled water overnight at 4 °C. Plates were coated with 200 µl per well ABA-4'-BSA conjugate and incubated overnight at 4 °C, then washed three times with 75 mM PBS buffer, pH 7.0, containing 1 g L^{-1} BSA and 1 ml L^{-1} Tween 20. Then 100 µL ABA standard solution or sample and, subsequently, 100 µL DBPA1 solution (50 µg mL^{-1}) were added to each well and competition was allowed to occur at 37 °C for 30 min. The plates were then washed and 200 µL per well of a secondary antibody [alkaline phosphatase-conjugated rabbit anti-mouse (Sigma-Aldrich, Milano, Italy) in PBS buffer at a final dilution of 1:2000] were added and incubated for 30 min at 37 °C. The plates were washed again and 200 µL per well of p-nitrophenyl phosphate were added and incubated for 30 min at 37 °C. Absorbance at 415 nm was recorded with a microplate reader (Perkin-Elmer MDL 680).

Soluble carbohydrates were identified and quantified by HPLC-RI analysis following the protocol of Tattini *et al*. (1996). Briefly, 200 mg of fresh tissue were extracted with 3 x 5 mL of 75% ethanol, the ethanol fraction reduced to dryness under vacuum, and finally rinsed 2 mL water (pH 7). The aqueous extract was purified through -CH and -SAX Bond-Elute cartridges (Varian, Harbor City, CA), and the eluate was reduced to dryness. Samples were rinsed with ultrapure water and injected in a Series 200 HPLC equipped with 200-RI detector (Perkin-Elmer), and separated on an 8 × 300 mm SC1011 column (Showa Denko, Tokyo, Japan) maintained at 88 ± 1 °C. Eluent was ultrapure water at a flow rate of 0.8 mL min^{-1}. Starch was quantified as reported in Chow & Landhäusser (2004) on the pellet resulting from ethanol extraction for the analysis of soluble carbohydrates.

Analysis of photosynthetic pigments and phenylpropanoids

Individual carotenoids were identified and quantified as reported in Beckett *et al*. (2012). Fresh leaf material was extracted with 2 × 5 mL acetone (added with 0.5 g L^{-1} $CaCO_3$) and 15 µL aliquots were injected in a Perkin Elmer Flexar HPLC equipped with a quaternary 200Q/410 pump and a LC 200 photodiode array detector. Pigments were separated in a 250 × 4.6 mm Waters Spherisorb ODS1 (5 µm) column operating at 30°C, eluted with a linear gradient solvent system, at a flow rate of 1.2 mL min^{-1}. Violaxanthin cycle pigments (i.e., violaxanthin, antheraxantin, zeaxantin), lutein and β-carotene were identified by comparison of their retention times and UV spectra with those of authentic standards. Total chlorophyll was quantified as in Lichtenthaler & Buschmann (2001).

Individual phenylpropanoids were extracted and purified following the protocol of Tattini *et al*. (2004). Briefly, 800 mg fresh material were extracted with 3 × 6 mL of 75% EtOH/H2O adjusted to pH 2.5 with formic acid. The supernatant was partitioned with 4 × 5 mL of n-hexane, reduced to dryness and finally

rinsed with 2 mL of CH_3OH/H_2O (8:2). Aliquots of 10 µL were injected into the Perkin Elmer liquid chromatography unit reported above. Phenylpropanoids were separated using a 250 × 4.6 mm Polaris C-18 Ether column (5 µm) operating at 30°C. The eluent was H_2O (added with 0.1% triethylamine and adjusted to pH 2.5 with H_3PO_4) and CH_3CN. Phenylpropanoids were separated using a linear gradient solvent system as reported in Tattini *et al*. (2004). Phenylpropanoids were identified using retention times and UV-spectral characteristics of authentic standards (Extrasynthese, Lyon-Nord, Genay, France) as well as by mass spectrometric data. HPLC-MS analysis was performed with an Agilent LC 1200 chromatograph coupled with an Agilent 6410 triple-quadrupole MS-detector equipped with an ESI source (all from Agilent Technologies, Santa Clara, CA). Quercetin 3-O rutinoside (m/z 610) and kaempferol 3-O rutinoside (m/z 594) were quantified at 350 nm. Chlorogenic acid isomers (namely 3-, 4-, and 5-caffeoylquinic acid, m/z 354), were quantified at 330 nm.

Epidermal flavonoids were optically estimated *in vivo* using the portable fluorimetric sensor Multiplex 3 (Mx) (FORCE-A, Orsay, France), as detailed in Agati *et al.* (2011) and Ben Ghozlen *et al*. (2010). The fluorescence signals were integrated over a 8-cm^2 leaf area. The Chl fluorescence signals under red light excitation (λ_{exc} = 625 nm), FRF_R, and UV-excitation (λ_{exc} = 375 nm), FRF_{UV}, were used to calculate the flavonoid index (FLAV), FLAV = FRF_R/FRF_{UV}. Chlorophyll in the first layer of mesophyll was also estimated by calculating the Chl index (CHL), CHL = FRF_R/RF_R, where RF_R is the red emission excited under red light.

Experimental design and statistical analysis

The experimental design was a complete random with 20 replicate plants per each genotype. Data were analyzed using repeated measures analysis of variance (ANOVA) with between-subjects factors (Girden 1992). Differences of emissions in IE plants subjected to the stress and after re- watering were statistically analyzed using a Student's t-test. For all other parameters, isoprene emission (IP) was the between-subjects factor and water treatment (T) was the within-subjects factor. For parameters showing a significant IP × T interaction, differences between NE and IE plants were analyzed, within each water treatment (FTSW), using one-way ANOVA. Results for line 12 are shown in the main text, whereas line 6 data are shown in supplementary material (Table S1).

RESULTS

Isoprene emission responses under drought stress conditions

Under well-watered conditions at the start of the experiment, isoprene emission levels in IE plants were ~ 2 nmol m^{-2} s^{-1} (Figure 1, and Table S1). This was less than 0.1% of the carbon fixed by photosynthesis. Under mild drought stress (68 % FTSW), isoprene emission almost doubled; emission gradually decreased as drought stress increased, and under severe water stress (24 % FTSW) levels were around half those of the pre-stressed plants (Fig. 1). Emissions recovered to levels slightly higher (line 12, Fig. 1) or similar (line 6, Table S1) than they were prior to drought stress upon re-watering. During drought and after re-watering the carbon allocated to isoprene was 2-3 times higher than before stress. However, this fraction never exceeded 0.3% of the photosynthetic carbon. No isoprene was detected from NE plants.

Effects of isoprene production on metabolism under unstressed conditions

Isoprene production had no effect on most physiological and biochemical traits in tobacco plants under well-watered conditions (see data at 98 % FSTW, before stress, Figs. 2-7, and Table S1). No significant differences were observed in photosynthetic parameters (Fig. 2; Table S1), with the exception of stomatal conductance, which was slightly higher in IE than in NE leaves in both lines (Fig. 2b, Table S1). There was also no significant difference in chlorophyll fluorescence between IE and NE lines (Fig. 3; Table S1). Isoprene production did not affect the concentration of chlorophylls and total violaxanthin-cycle pigments (VAZ, Fig. 4a; Table S1), as already reported (Vickers *et al*. 2009b). No significant variation in the concentration of ABA (Fig. 5; Table S1), starch (Fig. 6a; Table S1) or phenylpropanoids (Fig. 7; Table S1) were observed between IE and NE plants. Violaxanthin levels were not significantly different (Fig. 4b, Table S1); however, antheraxanthin levels were higher in IE than in NE plants (Fig 4c, Table S1), and zeaxanthin levels were slightly lower in IE plants than in NE plants (Fig 4d). The latter difference was not significant in line 6 (Table S1). In addition, hexoses (glucose and fructose) were slightly higher in NE than in IE plants (Fig. 6b,c).

Drought-induced effects on gas exchange and PSII performance

Drought stress resulted in a decrease in P_n and g_s in both IE and NE plants, the magnitude of which increased as drought was more severe (Fig. 2 a,b and Table S1). Drought caused a stronger inhibition of P_n and g_s in NE than in IE plants (Table 1, Fig. 2a,b, Table S1). Differences were particularly evident and statistically significant when the drought stress was severe (FTSW = 34%), and at the FTSW endpoint (Fig. 2 and Table S1). The WUE increased during the drought stress period in both genotypes, indicating control of stomatal closure over water losses by transpiration; no significant difference between WUE in IE and NE plants of line 12 was observed (Table 2), whereas WUE was significantly higher in NE than in IE plants of line 6, due to stomatal closure in NE plants (Table S1). Stomatal limitation was one of the factors causing photosynthesis inhibition during drought stress both in IE and NE plants, as shown by the decline of C_i in

288 both genotypes; however, this limitation was similar in both genotypes over the duration of the experiment
289 (Table 1, Fig. 2c, Table S1). The apparent maximum rate of carboxylation by Rubisco (V_{cmax}) also decreased
290 in a similar way in IE and NE plants during and after drought stress (Table 2, Table S1).

291 The photochemistry of photosynthesis, as monitored by the PSII efficiency estimated by chlorophyll
292 fluorescence, showed contrasting results. In IE plants of line 12, the maximal quantum yield of PSII (F_v/F_m)
293 was not significantly affected under drought stress (Fig. 3a). However, throughout the drought stress
294 period, F_v/F_m in NE plants was lower than in IE plants (Fig. 3a). At FTSW endpoint, IE plants had a
295 significantly higher F_v/F_m than NE plants in both lines (Fig. 3a and Table S1). Conversely, the actual efficiency
296 of PSII photochemistry (ΦPSII) behaved similarly in both IE and NE plants. ΦPSII was not significantly
297 affected until drought became severe, but dropped remarkably at FTSW endpoint, both in NE and IE plants
298 (Fig. 3b and Table S1). Declines in ΦPSII were matched by increases in non-photochemical quenching (NPQ)
299 in both genotypes, with no significant difference in the response of IE and NE plants (Fig. 2c and Table S1).

300 When the FTSW was reconstituted to > 90% by re-watering, an incomplete recovery was observed in P_n and
301 g_s in both IE and NE leaves (Fig. 2a,b, and Table S1). However, recovery was better in IE plants than in NE
302 plants, which allowed attaining photosynthesis rates significantly higher in IE plants. Interestingly, C_i
303 recovered to the pre-stress level (FTSW 98%) by the end of re- watering in all lines and genotypes (Fig. 2c
304 and Table S1), whereas the V_{cmax} was further inhibited in both IE and NE leaves of the two lines (Table 2 and
305 Table S1). The recovery of F_v/F_m of NE plants was also only partial (Fig. 3a), indicating that drought damaged
306 the photochemical apparatus of tobacco leaves that do not emit isoprene. However, ΦPSII surprisingly
307 dropped even further, and NPQ increased even further, upon re-watering, both in IE and NE plants (Fig. 3b,c
308 and Table S1), indicating the onset of biochemical damage affecting the linear transport and inducing
309 dissipation of energy non-radiatively.

310

311 **Drought-induced effects on photosynthetic pigments**

312 While total chlorophyll levels decreased slightly during drought stress and remained slightly lower than in
313 pre-stressed plants after re-watering (Fig. 4a and Table S1), there was no significant difference between
314 sampling time or between IE and NE plants over the duration of the experiment (Table 1, Table S1). The
315 significant decrease of chlorophyll observed in the top mesophyll cells (as indicated by CHL index, Table 4)
316 probably is related to the small and non-significant decrease in total chlorophyll observed during and after
317 drought stress (Fig. 4a).

318 Xanthophyll cycle pigments showed differing responses to drought. In both genotypes, violaxanthin was
319 unchanged under severe water deficit but decreased by ~ 50% after re-watering to FTSW > 90% (Fig. 4b).
320 There was no significant difference between violaxanthin responses in IE and NE plants (Table 1).

Antheraxanthin levels remained unchanged under severe drought stress in NE plants, but increased slightly in IE plants, resulting in a larger difference between the two genotypes relative to pre-stress levels (Fig. 4c, Table 1 and Table S1). By the time soil moisture levels had been returned to FTSW >90%, antheraxanthin levels had doubled in both genotypes relative to FTSW endpoint (24%), further increasing the difference between the two genotypes. Similarly, zeaxanthin levels became higher in IE plants under drought stress while they remained the same in NE plants (Fig. 4d). Also similarly, by the time soil moisture levels had been returned to FTSW > 90%, zeaxanthin levels had increased in both genotypes, but while the increase was double in NE plants, it was ~ 3-fold in IE plants, again further increasing the difference between the genotypes.

Overall, xanthophyll cycle pigments relative to total chlorophyll were significantly higher in IE plants than in NE plants under drought stress, as the ratio remained unchanged in NE plants, but increased significantly in IE plants (Fig. 4e, Table 1 and Table S1). This reflected the higher levels of de-epoxidated pigments antheraxanthin and zeaxanthin in the IE plants. The ratio VAZ Chl tot^{-1} decreased in both genotypes after re-watering, but remained much higher in IE than in NE. Again, much higher levels of de-epoxidated pigments in IE plants contributed to the difference between genotypes. Reflecting these observations, the de-epoxidation status was significantly higher in IE plants than in NE plants both during and after drought stress (Fig. 4f, Table 1 and Table S1). Lutein and β-carotene increased similarly in drought-stressed IE and NE plants (Table 3), and there was no significant difference between the two genotypes before or during water stress. However, after re-watering, the concentrations of the two carotenoids further increased in IE plants, whereas they decreased again in NE plants such that levels were similar to pre-stress plants.

Drought-induced effects on ABA production and non-structural carbohydrate metabolism

Drought stress induced biosynthesis of leaf ABA in both genotypes, but significantly more in IE leaves, in this case reaching concentrations > 700% with respect to well-watered plants (Fig. 5 and Table S1). The concentration of ABA decreased steeply after re-watering, but remained at least four times higher than before stress, and significantly higher in IE plants. Drought induced a significant decrease in starch concentration and a significant increase in concentrations of hexoses (Fig. 6 and Table S1). The decrease in starch was significantly higher in IE plants under severe drought stress than in NE plants, and the increase in fructose and glucose was significantly higher in IE plants than in NE plants under severe drought stress (Fig. 6 and Table S1). Sucrose, which represents just a small fraction of soluble carbohydrates in tobacco leaves (about 11% in well-watered, and 5% in drought-stressed plants), was not affected by drought (data not shown). Re-watering induced full recovery of starch levels, with no difference between IE and NE plants, whereas the concentration of glucose and fructose remained as high as during severe stress in NE plants and increased even further with respect to severe-stress levels in IE plants (Fig. 6 and Table S1).

355

356 **Drought-induced effects on phenylpropanoid accumulation**

357 Severe drought stress had no effect on phenylpropanoid biosynthesis in NE plants, whereas production was
358 significantly stimulated in IE plants (Table 1 and Fig. 7a). The increase was mostly due to a stimulation of
359 quercetin 3-O-rutinoside biosynthesis (Fig. 7c and Table S1), whereas smaller increases were observed in
360 the concentrations of chlorogenic acid isomers (Fig. 7b), and kaempferol 3-O-rutinoside (Fig. 7d).
361 Phenylpropanoid biosynthesis was stimulated after re-watering in both genotypes, but much more so in IE
362 plants. During re-watering, the rate of phenylpropanoid biosynthesis normalized to the net carbon gain
363 accounted for to 2.36 mmol phenylpropanoid mol^{-1} CO_2 in IE plants and 0.59 mmol phenylpropanoid mol^{-1}
364 CO_2 in NE plants. The ratio of quercetin to kaempferol derivatives increased in IE plants during both
365 drought stress and the subsequent re-watering, from 1.8 (pre-stress) to 2.45 (severe stress) to 3.32 (re-
366 watering).

367 The flavonol index (FLAV), which estimates the epidermal concentration of quercetin and kaempferol
368 derivatives (Agati *et al*. 2011) and provides a good measurement of the epidermal concentration of
369 chlorogenic acid (the absorbance at 375 nm of chlorogenic acid is ~40% of its maximum absorbance at 328
370 nm, (Agati *et al*. 2013), was not affected by drought stress, but increased in re-watered plants (Table 4).
371 FLAV did not differ between NE and IE line 12 plants during drought stress and re-watering.

372

373 **DISCUSSION**

374 Isoprene emitters are better protected against thermal (Singsaas *et al*. 1997; Velikova *et al*. 2006) and
375 oxidative stresses (Peñuelas *et al*. 2005; Vickers *et al*. 2009b). As for the mechanism by which isoprene
376 protects leaves against these stresses, it has been suggested that isoprene stabilizes photosynthetic
377 membranes (Velikova *et al*. 2011) and/or quenches ROS (Affek & Yakir 2002; Loreto & Schnitzler 2010). The
378 two mechanisms are not mutually exclusive, as a better membrane stabilization allows the photosynthetic
379 apparatus to work better under stress conditions, in turn avoiding ROS formation and accumulation
380 (Velikova, Sharkey & Loreto 2012). Thus, isoprene should make plants more resistant against virtually all
381 stresses that impair the photosynthetic apparatus, including drought. Isoprene biosynthesis is resistant to
382 drought even when photosynthesis is heavily repressed (Brüggemann & Schnitzler 2002), with the result
383 that significantly higher carbon loss in the form of isoprene as a proportion of fixed carbon is experienced
384 by the drought-stressed plants (Brilli *et al*. 2007). Moreover, isoprene emission is often stimulated in plants
385 recovering from drought, implying activation of isoprenoids metabolism under stress (Sharkey & Loreto
386 1993, Brilli et al. 2007).

We have found that transgenic, isoprene emitting tobacco plants are also better protected against drought, compared to non-emitting plants. Isoprene emission levels in non-stressed IE plants were slightly lower than previously observed in these transgenic lines (Vickers *et al*. 2009b), probably due to variation in experimental conditions. However, they were still in the range of species that naturally emit high levels of isoprene (Sharkey & Loreto 1993). Isoprene emission in IE plants did not affect a wide range of biochemical, physiological and morphological properties in non-stressed plants relative to NE plants, as reported previously (Vickers *et al*. 2009b, 2011). The exceptions included slight increases in stomatal conductance and slight decreases in hexoses levels in IE plants relative to NE plants. These exceptions are discussed further below.

In this study, isoprene emission from IE plants almost doubled during early stages of drought stress, dropping below pre-stress levels only when the stress became most severe, when photosynthesis was also severely repressed. This is consistent with a previous study performed using the same transgenic plants (Ryan *et al*. 2014), and with isoprene emission from naturally-emitting plants under drought stress (Brüggemann & Schnitzler 2002, Brilli *et al.* 2007; Fortunati *et al.* 2008). Considering the very severe inhibition of photosynthesis at severe drought (FTSW = 24%) the maintenance of isoprene emission, even at reduced levels, is remarkable. Our results expand previous results on natural emitters (Funk *et al.* 2004, Fortunati *et al.* 2008), showing that isoprene emission is resistant to severe drought even when the trait has been artificially introduced. As photosynthesis was not completely suppressed, it is suggested that photosynthesis still supplied the majority of carbon to isoprene biosynthesis of severely stressed IE tobacco plants, although labeling experiments should be performed to confirm our suggestion (Brilli et al. 2007). In re-watered plants, isoprene emission was higher than (line 12) or similar to (line 6) pre-stressed plants. As photosynthesis recovery was incomplete, plants recovering from drought stress were clearly investing a greater proportion of their overall carbon budget into isoprene production than prior to stress occurrence. The carbon investment both during the stress and after the original stress source is removed suggests a derived benefit from increased isoprene production. In support of this, P_n was significantly higher in IE plants throughout the experiment.

While stomatal conductance was also slightly higher in IE plants throughout the experiment, this did not have a significant effect on intracellular CO_2 concentrations (C_i) or V_{cmax} and is thus unlikely to be primarily responsible for the higher rates of P_n observed in IE than in NE plants under severe drought stress. On the other hand, the photochemistry of photosynthesis (namely F_v/F_m) was negatively affected by drought stress only in NE plants, and it only partially recovered on re-watering. This is consistent with responses observed under mild water stress (Ryan *et al*. 2014), and supports previous observations that isoprene protects the photosynthetic apparatus from oxidative stress (Vickers *et al*. 2009b). The mechanism might be through improved stabilization of photosynthetic membranes, possibly enhancing stability of PSII light harvesting

complexes (Velikova *et al*. 2011). Protection of membranes in drought-stressed tobacco are responsible for drought-tolerance (Ryan *et al*. 2014), lending credence to this possibility.

Interestingly, the partial recovery of photosynthesis after re-watering was not accompanied by a similar recovery of biochemical efficiency as V_{cmax} and ΦPSII further decreased in re-watered plants, indicating that recovery of photosynthesis might only be due to attenuation of CO_2 diffusive limitations. Our fluorescence data indicate an interesting temporal shift of the photochemical damage caused by drought, which is not revealed by gas-exchange analysis. The large decrease of ΦPSII and the concurrent increase of NPQ after two days of re-watering indicate that the photochemical reactions leading to linear electron transport were impaired by the drought stress, and reveal that plants needed to dissipate an increasing amount of energy radiatively, despite the partial recovery of photosynthesis. This seems to be independent of isoprene biosynthesis (NPQ did not vary between IE and NE plants), and conform to recent evidence that isoprene does not act directly as a sink for excess energy under drought conditions (Ryan *et al*. 2014).

The xanthophyll cycle is involved in NPQ formation, and in determining the oxidative status of the plant (Ramel *et al*. 2012). Drought stress had a minor effect on foliar carotenoids in NE plants, whereas in IE plants it stimulated the biosynthesis of antheraxanthin and zeaxanthin, and consequently enhanced de-epoxidation (DES) of violaxanthin-cycle pigments, during severe drought and on re-watering. Thus, we conclude that drought-induced isoprene biosynthesis may up-regulate MEP-derived isoprenoid metabolism, which also produces carotenoids. Recent reports suggest that DMADP accumulation might cause feedback inhibition of 1-deoxy-D-xylulose-5-phosphate synthase (DXS) activity, which may in turn negatively affect the entire MEP pathway (Banerjee et al. 2014; Ghirardo et al. 2014). Therefore, it may be suggested that in IE tobacco plants, isoprene production by DMADP also reduces DMADP chloroplast concentration to levels that may stimulate other components of the MEP pathway.

Higher DES should contribute to quench the energy-dependent component of the non-photochemical quenching of fluorescence (Demmig-Adams 1990; Niinemets *et al*. 2003), but NPQ increased similarly in IE and NE plants, and together with increasing DES in re-watered plants. Our data suggest that drought stress stimulated components of NPQ that were not dependent on xanthophylls, most likely slowly relaxing components of the quenching (Demmig-Adams *et al*. 1999). This may have limited complete recovery of photosynthesis in re-watered plants but does not explain the higher photosynthesis in IE than NE during both drought stress and re-watering. We hypothesize that, in IE plants, the large increase of antheraxanthin and zeaxanthin, which is not mirrored by a concurrent de-epoxidation of violaxanthin may be involved in a more general mechanism of membrane strengthening (Havaux, Dall'Osto & Bassi 2007). In particular, the increase of zeaxanthin when isoprene started to decrease may indicate cooperation between volatile and non-volatile isoprenoids in preserving chloroplast membrane structural intactness (Singsaas *et al*. 1997; Beckett *et al*. 2012), perhaps limiting membrane peroxidation (Ramel *et al*. 2012). We further note that not

455 only de-epoxidated xanthophylls but also lutein and β-carotene largely increased in re-watered IE plants,
456 which supports general stimulation of the MEP pathway in leaves in which isoprene biosynthesis occurs. It
457 is possible that the increase in lutein during re-watering may also have sustained NPQ in IE leaves (Dall'Osto
458 *et al*. 2006), thus allowing zeaxanthin to serve other functional roles in chloroplast membranes, i.e.
459 minimizing lipid peroxidation (Ramel *et al*. 2012).

460 It is well known that ABA builds up during drought and is responsible for drought-induced stomatal closure,
461 in turn limiting photosynthesis (Davies & Zhang 1991; Tardieu & Davies 1992). Indeed, we show that,
462 overall, photosynthesis and stomatal conductance were inversely associated with ABA content, in heavily
463 stressed leaves and after re-watering. In plants under mild drought, no difference is observed between ABA
464 levels in IE and NE plants (Ryan *et al*. 2014). However, here IE plants accumulated significantly higher levels
465 ABA than NE plants under severe drought and re-watering. In leaves, isoprene and ABA are both
466 synthesized through the same biosynthetic pathway (the MEP pathway in the chloroplast; see Lichtenthaler
467 *et al*. 1997). Isoprene emission levels correlate with ABA levels in leaves (Barta & Loreto 2006) and fruits
468 (Botton *et al*. 2011), suggesting possible co-ordinate regulation of production. In absence of transport of
469 ABA from roots, which is known to contribute a fraction of foliar ABA, especially under drought stress
470 (Davies & Zhang 1991), isoprene indeed mirrors a labile pool of foliar ABA that is associated with stomatal
471 closure under certain environmental conditions (Barta & Loreto 2006). In our experiment, ABA
472 accumulated in stressed leaves in large concentrations. This might be due to both the stimulation of the
473 MEP pathway (Barta & Loreto 2006), possibly also indirectly produced by tighter control of DMADP
474 concentration in IE plants, as explained above, and the transport of ABA synthesized by roots (Simonneau,
475 Barrieu & Tardieu 1998), or shoots (Christmann *et al*. 2005). However, the significantly higher accumulation
476 of ABA in the leaves of IE plants was not due to higher transpiration rates (Fig. 2b), thus suggesting that the
477 MEP pathway largely contributed to the foliar ABA pool in drought-stressed leaves, and in leaves after re-
478 watering.

479 Interestingly, the higher ABA levels in the IE plants did not result in increased stomatal closure, as also
480 recently observed in different lines of transgenic tobacco (Ryan *et al*. 2014). This is in apparent contrast
481 with current understanding of ABA effects under drought stress. However, we note that a tight correlation
482 between 'foliar ABA' and stomatal conductance during water stress has been rarely observed (Brodribb &
483 McAdam 2013). This is the case of anisohydric leaves, in which the hydraulic component of stomata
484 opening may prevail over the chemical component (Damour *et al*. 2010; Brodirrib & McAdam 2013). This
485 makes ABA concentration in the xylem sap (rather than 'foliar ABA') actually correlated with g_s (Tardieu &
486 Simmoneau 1998; Pantin *et al*. 2012). Data of our study and those of Ryan *et al*. (2014) indicate that
487 tobacco plants switched from isohydric to anisohydric behavior (Domec & Johnson 2012) as drought stress
488 progressed, thus making stomata opening less sensitive to ABA. We also speculate that the stomatal

response may be non-linear at the very high ABA concentrations observed in our study (as well as in Ryan *et al*. 2014). At very high concentrations, ABA may tightly affect hydraulic regulation of the leaf upstream of stomata (Pantin *et al*. 2012).

Taken together, these observations suggest a central role of ABA in the metabolic readjustment during and after a severe drought stress (Rook *et al*. 2006; Fujita *et al*. 2011). *First*, in drought-stressed leaves, ABA may be responsible for the alteration of non-structural carbohydrate metabolism. Early effects of drought-induced ABA accumulation include an increase in the activities of β-amylase (consistent with starch degradation observed here) and of vacuolar invertase (Pelleschi *et al*. 1999; Kempa *et al*. 2008). The higher ABA levels we observed in IE plants relative to NE plants under severe drought correlates with the increased starch degradation and increased hexoses levels observed in IE plants. The high hexose levels are sustained after re-watering, though starch levels recover. This is consistent with the partial recovery of photosynthesis, which is clearly then sufficient to re-stock the starch pool. *Second*, ABA and soluble sugars can act individually and/or in concert in promoting the biosynthesis of phenylpropanoids, particularly flavonoids (Koch 1996; Abe *et al*. 1997; Tossi, Lamattina & Cassia 2009; Luo *et al*. 2012). ABA accumulation up-regulates the expression of myb genes and the activity of chalcone synthase (CHS) and chalcone isomerase (CHI) (Tossi, Lamattina & Cassia 2009; Perrone *et al*. 2012). In our IE tobacco leaves the concentration of effective antioxidant phenylpropanoids, namely chlorogenic acid isomers and quercetin 3-O-rutinoside was 50 – 150% higher than in NE plants during and after drought stress. These data are consistent with the down-regulation of polyphenol biosynthesis observed in isoprene-suppressed transgenic poplar (Behnke *et al*. 2010). Differences in flavonol concentrations between IE and NE leaves are not due to changes at the epidermal level (Table 4), and therefore must be due to an increase of flavonols in the mesophyll. As with xanthophyll-cycle pigments discussed above, mesophyll-located dihydroxy phenylpropanoids serve functions as ROS scavengers (Agati *et al*. 2009; Agati & Tattini 2010). While ABA and hexose concentrations have already peaked at severe drought, phenylpropanoid biosynthesis mostly occurred during re-watering, when ABA concentration started to decline. Fisher *et al*. (2006) and Gagnè *et al*. (2011) already reported a similar out-phasing between metabolites. Perhaps, xanthophylls and especially flavonoids represent a more long term adaptive antioxidant response (Havaux & Klopptesch 2001; Agati *et al*. 2013) than the lipophilic and hydrophilic antioxidant compounds measured under mild drought stress conditions (Vickers *et al*. 2011; Ryan *et al*. 2014). *Third*, sugar signaling plays a role in the accumulation of MEP pathway, not only of phenylpropanoid pathway products. In Arabidopsis with mutations in PRL1 (Pleiotropic Regulatory Locus 1), a gene encoding a global regulator of sugar, stress, and hormone responses, MEP pathway products increase in the absence of any up-regulation of the core MEP pathway (Flores-Perez *et al*. 2010). Feeding sucrose to wild type plants also results in increased production

of carotenoids, suggesting that the observed phenotype is a result of increased sugar accumulation in the mutant line. This is consistent with the data presented here, showing increased hexose concentrations (Fig. 6) coupled with increased carotenoids. This mechanism may also be responsible for increases in other MEP-derived products, and act in a feed-forward loop through increasing ABA production (Cazzonelli & Pogson 2010). In addition to sugar responses, PRL1 also regulates stress and hormone responses, further supporting a potential role for this mechanism during drought stress.

CONCLUSIONS

Transgenic plants that emit isoprene may better tolerate severe drought stress. This experiment helps understanding why isoprene emission is preserved or stimulated in natural emitters undergoing or recovering from drought (Sharkey & Loreto 1993; Brilli *et al*. 2007), suggesting an indirect role for isoprene in the response of plants to drought stress. We confirm that isoprene emission is stimulated by mild drought stress, and is maintained as a high proportion of assimilated carbon under severe drought stress. As observed previously for ozone stress (Vickers *et al*. 2009b), isoprene-emitting plants seem to have a more rapid and stronger protective response to mild drought stress, suggesting that isoprene may render plants in a pseudo-state of stress hyper-responsiveness (sensu Enfissi *et al*. 2010). This 'priming for stress response' idea might explain the general protective effect of isoprene (Vickers *et al*. 2009a). Under prolonged drought conditions, isoprene synthesis also triggers production of non-volatile isoprenoids, mostly ABA and carotenoids, and then up-regulates phenylpropanoid metabolism. Isoprene-emitting plants also have perturbations in non-structural carbohydrate metabolism, which may be related to the overall stress response network. We have postulated that ABA accumulation may trigger most of the metabolic readjustment when isoprene is produced. However, it should be mentioned that isoprenoid metabolites are generally used as hormones and therefore regulate plant physiology (Puligo, Perello & Rodrigo-Concepcion 2012), and that at least one isoprenoid metabolite can also act as a signal modulating the expression of stress-related genes (Xiao *et al.* 2012). Irrespective of the mechanism, our findings expand knowledge on the defensive mechanisms triggered by isoprene, unveiling cooperation with multiple metabolic pathways in protection from stress events, and highlighting the central role of isoprene in stress-induced metabolic tuning.

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Table 1. Summary of two-way ANOVA for the effects of isoprene emission (IP) and water treatment (T), with their interaction factor (IP × T), on physiological- (total degree of freedom, d.f. = 59) and biochemical-related traits (d.f. = 23) in tobacco plants (line 12). Abbreviations as shown in the text.

Trait	F_{IP}	F_T	$F_{IP \times T}$
P_n	215.4***	1012.5***	9.7**
g_s	124.5***	1345.6***	6.7**
C_i	0.6ns	151.1***	0.9ns
F_v/F_m	566.8***	52.6***	51.8***.
Φ_{PSII}	5.8*	614.5***	4.4*
NPQ	93.7***	845.8***	2.8ns
ABA	38.6***	104.3***	8.8*
Starch	7.8*	251.4***	16.4**
Sucrose	2.7ns	2.3ns	2.5ns
Glucose	29.8***	125.4***	20.9**
Fructose	59.3***	206.1***	65.7***
Chl_{tot}	1.2ns	10.4*	1.7ns
Violaxanthin	13.1*	101.9***	0.2ns
Antheraxanthin	195.6***	190.8***	26.7***
Zeaxanthin	36.5**	116.8***	34.5***
(VAZ) Chl_{tot}^{-1}	644.6***	421.3***	50.3***
(A+Z) $(V+A+Z)^{-1}$	25.1**	216.2***	8.1*
Total Phenylpropanoids	12.8*	19.9**	2.4ns
Chlorogenic acid isomers	13.5*	20.6**	2.3ns
Que 3-O-rutinoside	17.8**	18.6**	2.8ns
Kae 3-O-rutinoside	10.7*	7.5*	1.8ns

Data were analyzed using ANOVA with between-subject factors. Isoprene emission (IP) was the between-subjects factor, and water treatment (T) was the within-subjects factor. Gas exchange and PSII performance were estimated on plants at 98, 68, 34, and 24% of fraction of transpirable soil water (FTSW), before and during drought stress, and at 96% FSTW during re-watering. Biochemical analyses were performed on plants at 98 (before stress), 24 (during severe stress), and 96% (during re-watering) FTSW. $P < 0.05$ = *;$P < 0.001$ = **; $P < 0.0001$ = ***; not significant = ns.

778 Table 2. Water use efficiency (WUE, µmol CO_2 $mmol^{-1}$ H_2O), apparent rate of maximum carboxylation by Rubisco (V_{cmax}, µmol m^{-2} s^{-1}) and
779 electron transport rate (ETR, µmol m^{-2} s^{-1}) in non-emitting (NE) and isoprene-emitting (IE) tobacco plants before drought stress (FTSW
780 98%), at the end of drought stress (FTSW 24%) and after re-watering (FTSW 96%).

781

Parameter	FTSW = 98%		FTSW = 24%		FTSW = 96%		F_{IP}	F_T	$F_{IP \times T}$
	NE	IE	NE	IE	NE	IE			
WUE	3.4 ± 0.23	3.6 ± 0.19	4.3 ± 0.35	4.6 ± 0.28	3.5 ± 0.18	3.4 ± 0.22	1.5ns	7.5*	1.2ns
V_{cmax}	82.4 ± 5.4	88.5 ± 4.7	58.4 ± 6.1	62.8 ± 6.2	42.8 ± 4.4	46.6± 5.0	2.5ns	17.8**	1.8ns
ETR	126 ± 8	135 ± 7	101 ± 12	107 ± 13	77 ± 8	80 ± 9	3.1ns	14,5*	1.9ns

782
783 Data (means ± SD, *n* = 6) were analyzed using repeated measures analysis of variance (ANOVA) with between-subject factors. Isoprene emission
784 (IP) was the between-subjects factor, and water treatment (T) was the within-subjects factor. $P < 0.05$ = *; $P < 0.001$ = **; not significant = ns.
785

Table 3. Ratio of carotenoid to total chlorophyll (Car Chl_{tot}^{-1}), and concentration (µmol g^{-1} DW) of lutein and β-carotene in non-emitting (NE) and isoprene-emitting (IE) tobacco leaves before drought stress (FTSW 98%), at the end of drought stress (FTSW 24%) and after re-watering (FTSW 96%).

Pigment	FTSW = 98%		FTSW = 24%		FTSW = 96%		F_{IP}	F_T	$F_{IP \times T}$
	NE	IE	NE	IE	NE	IE			
Car Chl_{tot}^{-1}	0.21 ± 0.02	0.22 ± 0.01	0.30 ± 0.02	0.32 ± 0.03	0.27 ± 0.02	0.32 ± 0.02	4.5ns	98*	7.5*
Lutein	0.78 ±0.04	0.81 ± 0.05	0.96 ± .06	0.94 ±0.04	0.77 ±0.06	1.13 ± 0.09	2.5ns	17.9**	1.8ns
β-carotene	0.51± 0.05	0.49 ± 0.04	0.67 ± 0.03	0.66 ± 0.02	0.50 ± 0.05	0.86 ± 0.07	3.1ns	14.5*	1.9ns

Data (means ± SD, $n = 4$) were analyzed using ANOVA with between-subject factors. Isoprene emission (IP) was the between-subjects factor, and water treatment (T) was the within-subjects factor. $P < 0.05$ = *; $P < 0.001$ = **; not significant = ns.

794 Table 4. Flavonoid (FLAV) and Chlorophyll (CHL) indexes in non-emitting (NE) and isoprene-emitting (IE) tobacco leaves before drought
795 stress (FTSW 98%), at the end of drought stress (FTSW 24%), and after re-watering (FTSW 96%).

796

Index	FTSW = 98%		FTSW = 24%		FTSW = 96%		F_{IP}	F_T	$F_{IP \times T}$
	NE	IE	NE	IE	NE	IE			
FLAV	0.45 ± 0.07	0.42 ± 0.04	0.48 ± 0.05	0.46 ± 0.07	0.63 ± 0.03	0.64 ± 0.04	1.5ns	10.7*	1.2ns
CHL	2.65 ± 0.11	2.54 ± 0.09	1.48 ± 0.10	1.39 ± 0.11	1.53 ± 0.09	1.51 ± 0.12	1.4ns	19.4**	1.4ns

797

798 Indexes (in Multiplex units) of adaxial and abaxial surfaces were pooled together prior to statistical analysis. Data (means ± SD, *n* = 6) were
799 analyzed using ANOVA with between-subjects factors. Isoprene emission (IP) was the between-subjects factor, and water
800 treatment (T) was the within-subjects factor. $P < 0.05$ = *, $P < 0.001$ = **; not significant = ns.

Figure captions

Figure 1. Isoprene emission from IE tobacco leaves (line 12) during drought stress and subsequent re-watering. Asterisks denote significant differences with respect to pre-stressed plants (FTSW = 95%, first bar from the left). Data are means ± SD (n = 4) of measurements carried out on the first fully expanded leaf (3rd to 4th leaf from the apex). The same leaves were used for all non-destructive measurements. Differences of emissions were statistically analyzed using a Student's t-test. Asterisks denote differences at $P < 0.05$.

Figure 2. Photosynthesis (P_n, a), stomatal conductance (g_s, b) and intercellular CO_2 concentration (C_i, c) in non-emitting (NE, open bars) and isoprene-emitting (IE, grey bars) tobacco plants (line 12) during drought stress and subsequent re-watering. Data are means ± SD (n = 6). Analysis of variance (ANOVA) with between-subjects factor was carried out to assess statistical significance of mean differences (Table 1). Asterisks denote significant differences ($P < 0.05$) between NE and IE plants at each FTSW.

Figure 3. Maximal (F_v/F_m, a) and actual (Φ_{PSII}, b) efficiency of PSII photochemistry, and non-photochemical quenching (NPQ, c) in non-emitting (NE, open bars) and isoprene-emitting (IE, grey bars) tobacco plants (line 12) during drought stress and subsequent re-watering. Data are means ± SD (n = 6). Statistical treatment of data is given in Table 1. Asterisks denote significant differences ($P < 0.05$) between NE and IE plants at each FTSW.

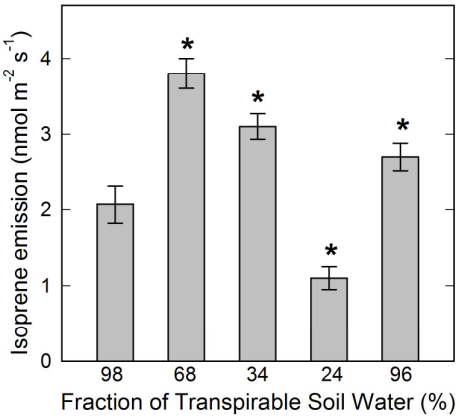
Figure 4. Concentration of chlorophyll (a), violaxanthin (b), antheraxanthin (c), zeaxanthin (d) in non-emitting (NE, open bars) and isoprene-emitting (IE, grey bars) tobacco leaves (line 12) during drought stress and subsequent re-watering. The total concentration of violaxanthin, antheraxanthin and zeaxanthin (V+A+Z) on a chlorophyll basis (d) and the de-epoxidation state of violaxanthin-cycle pigments (DES = (A+Z) (V+A+Z)-1 , f) are also shown. Data are means ± SD (n = 4). Statistical treatment of data is given in Table 1. Asterisks denote significant differences ($P < 0.05$) between NE and IE plants at each FTSW.

Figure 5. Concentration of abscisic acid (ABA) in leaves of non-emitting (NE, open bars) and isoprene- emitting (IE, grey bars) tobacco during drought stress and subsequent re-watering. Data are means ± SD (n =4). Statistical treatment of data is given in Table 1. Asterisks denote significant differences ($P < 0.05$) between NE and IE plants at each FTSW.

Figure 6. Concentration of starch (a), glucose (b) and fructose (c) in leaves of non-emitting (NE, open bars) and isoprene-emitting (IE, grey bars) tobacco (line 12) during drought stress and subsequent re-watering. Data are means ± SD (n = 4). Statistical treatment of data is given in Table 1. Asterisks denote significant differences ($P < 0.05$) between NE and IE plants at each FTSW.

840

841 Figure 7. Concentration of total (a) and individual (chlorogenic = chlorogenic acid isomers, b; Que
842 3-O-rut = quercetin 3-O-rutinoside, c; Kae 3-O- = kaempferol 3-O-rutinoside, d) phenylpropanoids
843 in leaves of non-emitting (NE, open bars) and isoprene-emitting (IE, grey bars) tobacco (line 12)
844 during drought stress and subsequent re-watering. Data are means ± SD (n = 4). Statistical
845 treatment of data is given in Table 1. Asterisks denote significant differences ($P < 0.05$) between
846 NE and IE plants at each FTSW.



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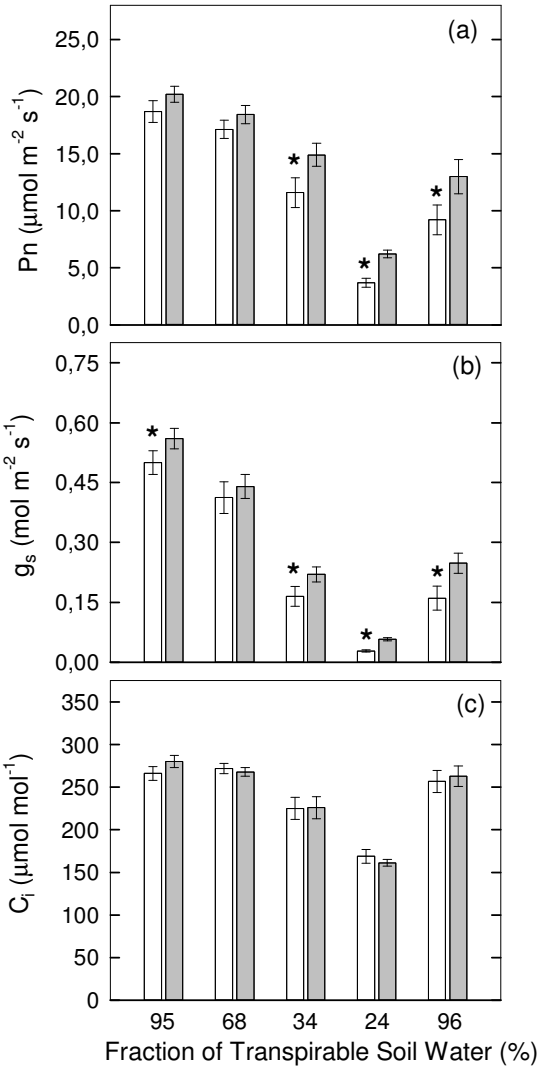
850 Fig. 1



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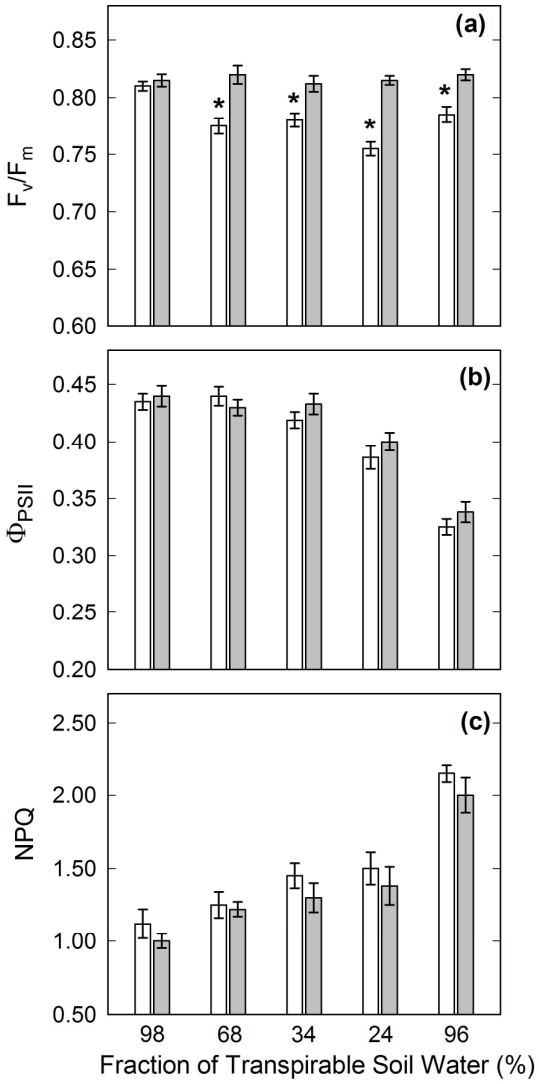
854 Fig. 2



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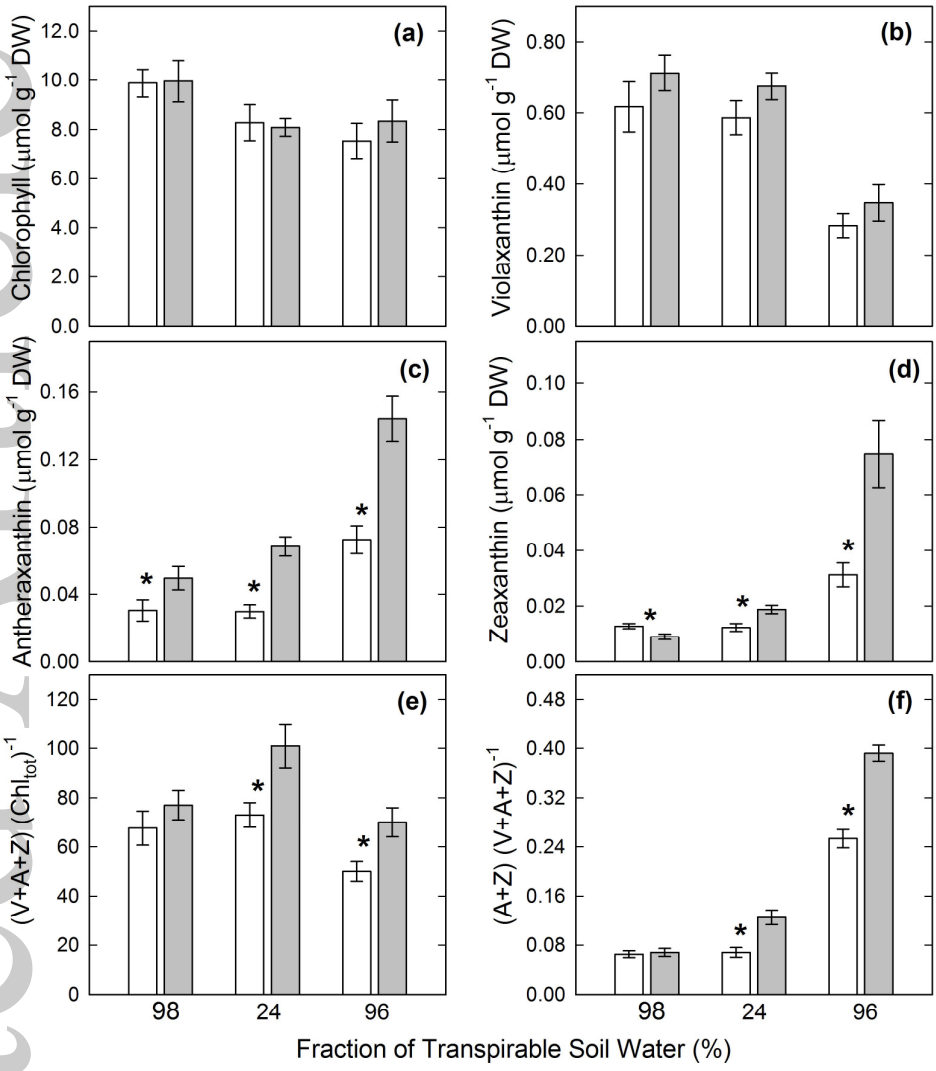
858 Fig. 3



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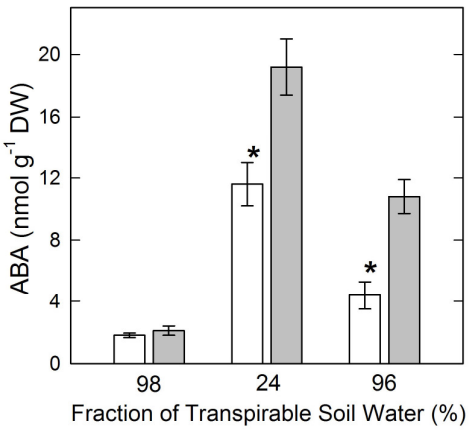
862 Fig. 4



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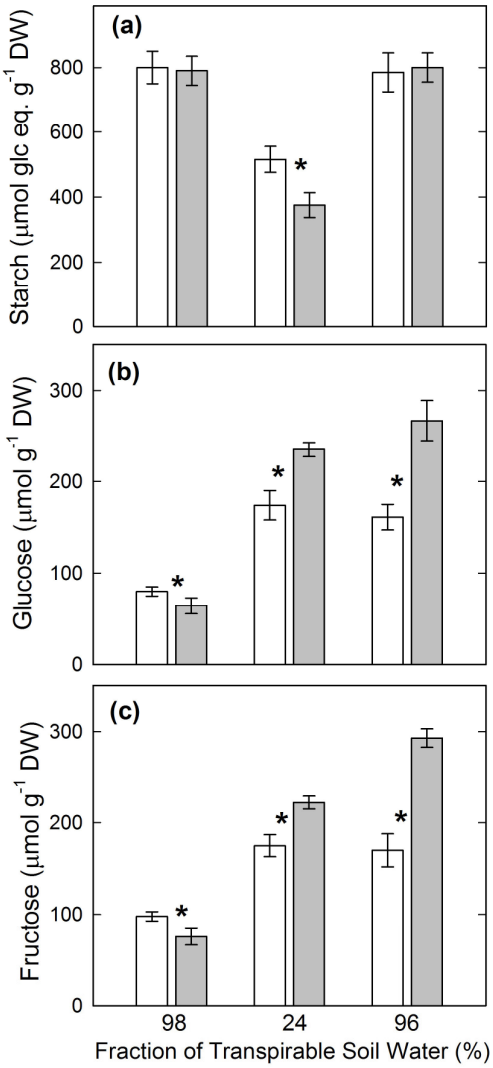
866 Fig. 5



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870 Fig. 6

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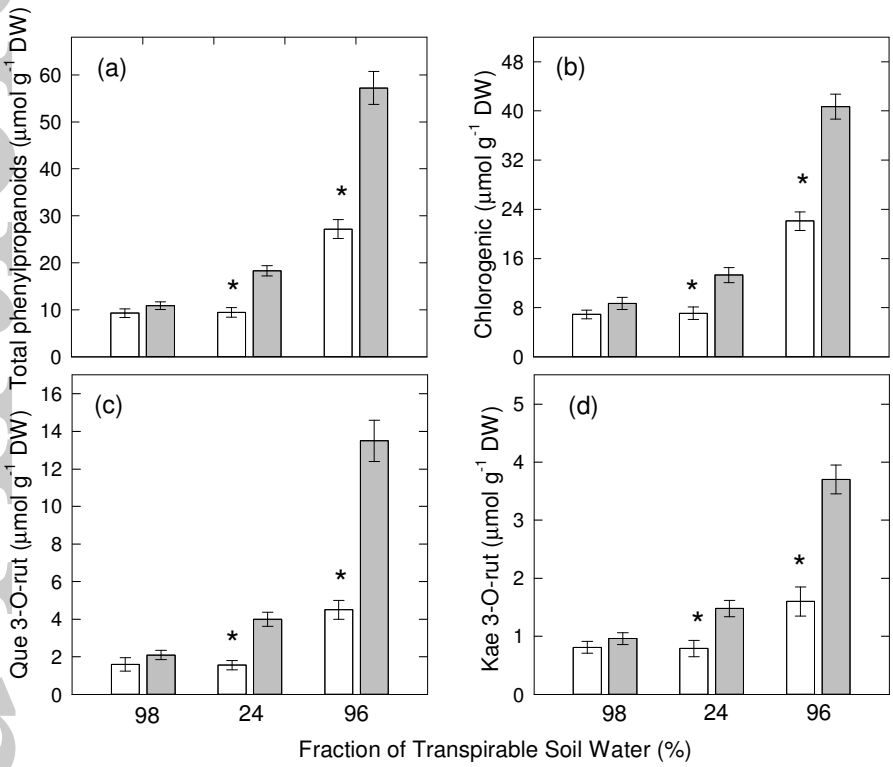

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874 Fig. 7