Running title:

Absence of isoprene modifies chloroplastidic membranes in poplar

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Knocking down of isoprene emission modifies the lipid matrix of thylakoid membranes and influences the chloroplast ultrastructure in poplar

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Summary

The biological function of isoprene emission in plants is closely associated with the structural organization and functioning of plastidic membranes.
ABSTRACT

Isoprene is a small lipophilic molecule with important functions in plant protection against abiotic stresses. Here, we studied the lipid composition of thylakoid membranes and chloroplast ultrastructure in isoprene emitting (IE) and non-isoprene emitting (NE) poplars. We demonstrated that the total amount of mono- (MGDG), di-galactosyldiacylglycerols (DGDG), phospholipids (PL), and fatty acids is reduced in chloroplasts when isoprene biosynthesis is blocked. A significantly lower amount of unsaturated fatty acids, particularly linolenic acid (18:3) in NE chloroplasts was associated with the reduced fluidity of thylakoid membranes, which in turn negatively affects PSII photochemical efficiency ($\Phi_{\text{PSII}}$). The low $\Phi_{\text{PSII}}$ in NE plants was negatively correlated with non-photochemical quenching (NPQ) and the energy-dependent (qE) component of NPQ. Transmission electron microscopy revealed alterations in the chloroplast ultrastructure in NE compared with IE plants. NE chloroplasts were more rounded and contained less grana stacks and longer stroma thylakoids, more plastoglobules, and larger associative zones between chloroplasts and mitochondria. These results strongly support the idea that in isoprene-emitting species, the function of this molecule is closely associated with the structural organization and functioning of plastidic membranes.

INTRODUCTION

Isoprene is globally the most abundant biogenic hydrocarbon constitutively emitted from many plant species (Guenther et al., 2012). It has been proposed that leaf isoprene emission is an important adaptation for plants, conferring tolerance to different environmental constraints (Vickers et al., 2009; Loreto and Schnitzler, 2010; Loreto and Fineschi, 2014). However, biogenic isoprene emission represents a non-trivial carbon loss in plants, particularly under stress conditions (Fang et al., 1996; Brilli et al., 2007; Teuber et al., 2008; Ghirardo et al., 2014), and the reason(s) why plants emit isoprene are still ambiguous, and the true role of isoprene emission remains elusive. Different approaches and techniques have been used to determine whether and how the cost of this “expensive” carbon emission is matched by the accomplishment of the physiological function in planta. It has been shown that isoprene might quench and/or regulate reactive oxygen and nitrogen species formation (Behnke et al., 2010a; Velikova et al., 2012), therefore indirectly providing a general antioxidant action (reviewed in Vickers et al., 2009; Loreto and Schnitzler, 2010) and stabilizing thylakoid
membrane structures due the lipophilic properties of this molecule (Sharkey et al., 2001; Velikova et al., 2011).

Protein and pigment-protein complexes are assembled and embedded in a lipid matrix, which has a unique lipid composition. The thylakoid lipid bilayer of chloroplasts is characterized as a high proportion of galactolipids with one (monogalactosyldiacylglycerol, MGDG) or two (digalactosyldiacylglycerol, DGDG) galactose molecules (Joyard et al., 2010). MGDGs are the primary constituents (~50%) of thylakoid membrane glycerolipids, followed by DGDGs (~30%), sulfoquinovosyl diacylglycerol (SQDG, ~5-12%) and phosphatidylglycerol (PG, 5-12%) (Kirchhoff et al., 2002). Galactolipids contain a large proportion of polyunsaturated fatty acids, and consequently the thylakoid membrane is a relatively fluid system (Gounaris and Barber, 1983) compared with other biological membranes. The fluidity of the thylakoid membrane is essential for photosynthetic processes.

The thylakoid membranes are highly organized internal membrane chloroplast systems that conduct the light reactions of photosynthesis. These membranes comprise pigments and proteins organized in complexes. Thylakoid membranes are arranged into stacked and unstacked regions called grana and stroma thylakoids, respectively, differentially enriched in photosystem I (PSI) and photosystem II (PSII) complexes (Mustárdy et al., 2008). The spatial separation of the PSI and PSII complexes in the stacked and unstacked membrane regions and the macromolecular organization of PSII in stacked grana thylakoids are self-organizing processes and important features to maintain the functional integrity of the photosynthetic apparatus (Kirchhoff et al., 2007).

It is not known how changes in the lipid matrix affect lipid-protein interactions and vice versa, and how membrane macro-organization ensures the efficient diffusion of protein complexes, associated with plant adaptation to the changing environment remains elusive. The isoprene impact on thylakoid intactness and functionality has been assessed using different biophysical techniques (Velikova et al., 2011). Thermoluminescence data demonstrated that the position of the main peak (Q_b peak) was up-shifted approximately 10°C in isoprene emitting plants, suggesting modifications in the lipid environment due to the presence of isoprene in heterologous Arabidopsis plants expressing the isoprene synthase gene from poplar. It was also shown that isoprene improves the stability of PSII light-harvesting complexes (LHCCI-PSII) through the modification of pigment-protein complex organization in thylakoid membranes (Velikova et al., 2011). Moreover, we recently showed that knocking down isoprene emission in poplar remodels the chloroplast proteome (Velikova et al., 2014). The lack of isoprene resulted in the down-regulation of proteins associated with
the light reactions of photosynthesis, redox regulation and oxidative stress defenses and several proteins responsible for lipid metabolism (Velikova et al., 2014).

In the present study, we focused on the lipid composition of thylakoid membranes in isoprene-emitting (IE) and non-emitting (NE) poplar leaves. Specifically, we determined whether the translational suppression of isoprene synthase in NE leaves influences the lipid matrix of thylakoids and how this phenomenon affects membrane structure and function. Here we provided evidence that the suppression of isoprene biosynthesis in poplar (i) reduced total galactolipids, phospholipids, and linolenic fatty acid (18:3), (ii) altered the chloroplast ultrastructure, and (iii) stimulated photoprotective mechanisms.

RESULTS

Lipid, fatty acid and malondialdehyde analyses

Chloroplast membranes isolated from NE poplar had significantly lower (-53%, P<0.01) lipid contents than the membranes of IE plants (Fig. 1). In both NE and IE plants, the major molecular species of MGDG were 18:2-18:3 and 18:3 dimers. In DGDG the major molecular species were n16:0-18:3 and 18:3 dimers. Linolenic acid (18:3) was the major fatty acid of both IE and NE chloroplast membranes, but the content of this fatty acid was consistently much lower in NE than in IE plants (Table 1, Fig. S1). The fatty acid analysis also revealed significantly lower palmitic (n16:0), linoleic (18:2) and stearic (n18:0) acid contents in NE plants compared with IE plants. In the fraction of the phospholipids (PL) the phosphatidic acid (18:1) levels were lower in NE plants.

The concentration of malondialdehyde (MDA), the principal product of polyunsaturated fatty acid peroxidation, was lower in chloroplasts isolated from NE plants than in those isolated from IE poplars (Fig. 2), associated with a lower concentration of polyunsaturated fatty acids in NE chloroplasts (Table 1, Fig. S1).

Chloroplast ultrastructure observations and protein abundance in photosynthetic membranes

To determine whether the different lipid concentrations and changes in lipid composition affect the chloroplast structure, thin leaf segments obtained from the middle region of IE and NE leaves were subjected to Transmission Electron Microscopy (TEM) analyses.
Representative micrographs of chloroplasts from IE and NE specimens are shown in Fig. 3 and 4.

The typical elliptic shape of mesophyll chloroplasts was more oval in NE than in IE specimens (Fig. 3). The mesophyll cells of IE leaves are characterized by a well-developed inner membrane system, comprising grana of different sizes and relatively long stromal thylakoids. IE chloroplasts contained single, midsize starch granules, less numerous peroxisomes, and these organelles were associated with relatively small-sized mitochondria (Fig. 3A, B and 4A, B). Conversely, the chloroplasts of NE plants were characterized by a less developed membrane system, with shorter and fewer grana stacks and longer stroma thylakoids (Fig. 4 C, D, E). NE chloroplasts contained more plastoglobules and smaller starch grains than IE chloroplasts (Fig. 3C, D). NE chloroplasts were also in close structural contact with mitochondria through relatively large associative regions (Fig. 4C). A relatively large number of NE chloroplasts were undeveloped (data not shown).

To further understand how the structural changes were related with the protein enrichment in photosynthetic membranes we extracted MS data from our recent proteome study (Velikova et al., 2014). The concentrations of PSI-RCI and PSII-RCII were strongly decreased in NE chloroplasts compared to IE (Fig. 5A, B). Lower protein abundance of PSII-RCII correlated with less number of stacks (Fig. 4F). Chlorophyll concentrations in the NE lines RA1 and RA2 were also significantly reduced (Fig. 5C).

**Chlorophyll fluorescence**

We measured light- and dark-adapted states of chlorophyll fluorescence in IE and NE poplar plants grown under ambient greenhouse conditions (Fig. 6). There was no significant difference in maximal PSII activity between IE and NE plants (data not shown), suggesting that the efficiency of PSII, when all reaction centers were open, was similarly high in both groups of plants. However, NE leaves exhibited significantly lower Φ_{PSII} and qL and higher NPQ (non-photochemical quenching) and qE (energy-dependent quenching) (Fig. 6). Importantly, the true efficiency of PSII (Φ_{PSII}) was lower in NE compared with IE, indicating that a smaller fraction of the absorbed light energy was used for photochemistry. Indeed, the accurate indicator of the PSII redox state, qL, (Baker, 2008), was significantly lower in NE, suggesting that the fraction of open PSII reaction centers was much lower in these mutants (Figs 5, 7, Fig. S2).

**Multivariate data analyses**
We examined the involvement of lipid content and fatty acid compositions in NE and IE chloroplasts, compared with chlorophyll fluorescence measurements, MDA contents and previously described proteomic differences (Velikova et al., 2014). The principal component (PC) analysis showed that the isoprene emission traits reflected the largest variance of the measured data, indicated by the separation between NE and IE samples in the first two principal components (Fig. S2; explained variance: PC1=51%, PC2=16%). Additionally, there were no appreciable differences within the two groups, as both RA1 and RA2 lines and WT and EV lines clustered together in the NE and IE groups, respectively.

We performed a discriminant analysis to determine which of the analyzed parameters were significantly affected by the suppression of isoprene biosynthesis and assess the relative importance of these parameters in distinguishing NE from IE plants (Fig. 7). The OPLS analysis indicated, overall, that differences between NE and IE chloroplasts reflect the lipid composition, fatty acid and MDA content, chlorophyll fluorescence parameters and chloroplastic proteins associated with photosynthesis or cell structure. Each singular factor had a different importance (Fig. 7C). Specifically, the most important (high VIP values) variables negatively correlated with NE (positive and high correlation coefficient values) were MDA, qL, PL, saturated and unsaturated fatty acids, the MGDG and DGDG, and photosynthetic proteins (Fig. 7B, C). Importantly, the unsaturated fatty acid 18:3 (linolenic acid) was strongly negatively correlated with NE in all lipids (MGDG, DGDG and PL). Additionally, linolenic acid was well correlated with the lipid degradation product MDA, detected in both MGDG and DGDG. The PL content was highly correlated with photosynthetic proteins, namely PSI proteins, ATP synthase, cytochrome b_{6f} and PsbP. Conversely, NPQ and qE were strongly and positively correlated with the NE genotype.

The computed OPLS model was reliable, resulting in a significant (P = 0.00061, CV-ANOVA) cross-validated predictive ability of $Q^2(Y) = 84\%$ to distinguish NE from IE samples and a cross-validated goodness of $R^2(X) = 44\%$, $R^2 = 93\%$, $R^2(Y) = 100\%$ using only the first PC.

DISCUSSION

Suppression of isoprene biosynthesis decreases the chloroplastic lipid content and alters chloroplast ultrastructure
One of the proposed biological functions of isoprene is the stabilization of thylakoid membrane structures through the modification of the lipid environment and organization of the pigment-protein complexes in thylakoid membranes (Velikova et al., 2011). Indeed, several clear alterations were evident in thylakoid membrane lipids and fatty acid composition due to the translational suppression of isoprene synthase activity in poplar plants. The most important changes in NE chloroplasts were the absolute decrease in the contents of galactolipids (MGDG, DGDG) and phospholipids (PL) through the down-regulation of the unsaturated fatty linolenic acid (18:3). The functional role of MGDGs in the bioactivity of various membrane proteins is well known (Lee 2003, 2004): a mutant with a defective MGDG synthase 1 (mgd1) is unable to produce photosynthetically active membranes (Kobayashi et al., 2013). MGDGs are essential for the efficient activity of violaxanthin de-epoxidase (VDE) (Yamamoto and Higashi, 1978). The ability of the lipid mixture to segregate into bilayer and non-bilayer phases might regulate the protein content in chloroplast membranes (Garab et al., 2000). Indeed, we could demonstrate that the proteins related to photosynthesis were strongly down-regulated in NE compared to IE plants (Fig. 7 and Fig. 8; Velikova et al., 2014).

DGDGs are the predominant bilayer lipid species in thylakoid membranes of higher plants (Joyard et al., 2010). They exert structural functions and improve the thermal stability of membranes, particularly at high temperatures (Krumova et al., 2010). DGDGs bind to PSII (Loll et al., 2007) through the formation of hydrogen bonds with tyrosine in PSII (Gabashvili et al., 1998), and DGDGs are also important for binding of extrinsic proteins required for the stabilization of the oxygen-evolving complex (Sakurai et al., 2007). Our data clearly indicate that the suppression of isoprene biosynthesis significantly diminished the level of DGDGs in poplar chloroplasts, which was accompanied with a reduction of the RCI and RCI concentration (Fig. 5A, B), PsbP and PsbQ protein subunits of PSII, and LHCI and LHCII (Velikova et al., 2014). When the thylakoid membrane protein complexes were resolved by blue native-PAGE, the protein patterns of the two groups of poplar lines looked quite similar in content and intensity of the individual bands (Velikova et al., 2014, see supporting information). However, semi-quantitative analysis of the individual protein bands showed that the levels of PSI, the PSII dimer, ATP synthase, the PSII monomer, and the cytochrome b6f complex were slightly reduced in NE compared with IE chloroplasts (Velikova et al., 2014).

Decreased lipid and protein levels were associated with changes in the ultrastructure of the chloroplasts from NE plants, suggesting a role for isoprene biosynthesis in the structural organization of plastidic membranes. These results are consistent with previous studies that...
indicated a role for MGDGs and DGDGs in the structure of thylakoid membranes (Dörmann et al., 1995; Jarvis et al., 2000). In the present study we observed significant reduction of grana stacks per chloroplasts in NE compare to IE poplar lines (Fig. 4E, F), which was related to important decrease in PSI and PSII proteins (Fig. 5A, B). Alterations in protein stoichiometry could exert a direct influence on the thylakoid membrane ultrastructure (Pribil et al., 2014), in particular, the assembly of PSII and LHCII into super- and megacomplexes (Kouřil et al., 2012). It was demonstrated (Labate et al., 2004) that the constitutive expression of the pea \textit{Lhcb 1} gene in transgenic tobacco plants leads to increased grana stacking indicating that increased concentrations of LHCII results in more stacking.

Typically, chloroplast membranes have a unique lipid composition characterized by a high proportion of galactolipids containing a large portion of tri-unsaturated fatty acids (C\textsubscript{16} or C\textsubscript{18}) (Joyard et al., 2010). The high content of tri-unsaturated fatty acids guarantees the high fluidity of the thylakoid membranes and the precise allocation of the photosynthetic machinery to efficiently acquire light energy (Gounaris and Barber, 1983). The level of membrane viscosity is an important factor for the photosynthetic performance, e.g., providing the optimal conditions for the diffusion of hydrophobic molecules, such as plastoquinol (Kirchhoff et al., 2000, 2002) or membrane intrinsic protein complexes (e.g., during state transitions) (Allen and Forsberg, 2001; Tikkanen et al., 2008). The low linolenic acid (18:3) content in all lipid fractions from NE chloroplasts indicates that in the absence of isoprene, the thylakoid membrane fluidity is reduced, which in turn negatively affects the efficiency of PSII photochemistry (Fig. 6). A low level of unsaturation in thylakoid membranes makes PSII extremely susceptible to photoinhibition and causes a significant reduction in the content of D1 protein (the reaction center protein) at high irradiance (Kanervo et al., 1995), suggesting that membrane fluidity is a critical factor for PSII D1 protein turnover. Moreover, we detected in NE chloroplast lower amounts of phosphatidic acid (18:1), an important intermediate in lipid biosynthesis (Joyard et al., 2010) with functions as signaling lipid (Testerink and Munnik, 2005, 2011; Horváth et al., 2012; McLoughlin and Testerink, 2013).

We observed that the lower level of linolenic acid (18:3) detected in NE chloroplasts was associated with significantly lower MDA chloroplast content in NE compared with IE poplars (Fig. 2, Table 1). Previous studies have reported that MDA is primarily derived from tri-unsaturated fatty acids in chloroplasts (Yamauchi et al., 2008; Schmid-Siegert et al., 2012). MDA can be used as an oxidative stress marker when plants are exposed to unfavorable conditions (Esterbauer et al., 1991), but is also present in healthy plants (Weber et al., 2004; Mène-Saffrané et al., 2007, 2009). On a whole leaf extract level, MDA levels were found
higher in NE (Behnke et al., 2010b), which agree with their higher concentrations of linolenic acid (Way et al., 2013). The production of MDA from tri-unsaturated fatty acids serves to adsorb a portion of the reactive oxygen species (ROS) (Mène-Saffrané et al., 2009) and therefore MDA is a by-product in the mechanism of cell protection.

Another remarkable observation in the present study was the increased abundance of plastoglobules in NE compared with IE chloroplasts. Plastoglobules are lipoprotein particles containing isoprenoid-driven metabolites (primarily prenylquinones, including plastoquinone and phyloquinone), tocopherols (Vidi et al., 2006) and structural proteins (plastoglobulins) (Bréhélin et al., 2007). The increased number of plastoglobules in NE compared with IE chloroplasts might reflect the higher levels of α-tocopherol in leaves of these lines as previously demonstrated (Behnke et al., 2010b).

Electron tomography revealed that plastoglobules are physically coupled to thylakoid membranes via a half-lipid bilayer, providing a direct lipid conduit for metabolite channeling between plastoglobules and thylakoid membranes (Austin et al., 2006). Moreover, plastoglobules are involved in different secondary metabolism pathways, stress responses, and in the development of thylakoids (Bréhélin et al., 2007). In a previous study, we showed that the PAP fibrillin content, comprising lipid-associated proteins (PAPs) and fibrillins, is negatively correlated with the NE plants (Velikova et al., 2014). This observation suggests the involvement of isoprene in the maintenance of thylakoid membranes.

Interestingly, we observed larger associative zones between chloroplasts and mitochondria in NE plants. Mitochondria are instrumental for the generation of metabolic energy in eukaryotic cells, and these organelles deliver intermediates to support different metabolic pathways, including photosynthesis (Jacoby et al., 2012). One of the important benefits of mitochondria-chloroplast interactions is the optimization of photosynthetic carbon assimilation through the coordinated production and utilization of ATP and NADPH, the induction of photosynthesis, the activation of enzymes and the maintenance of metabolite levels (Raghavendra and Padmasree, 2003). We propose that the larger associative zones between chloroplasts and mitochondria in NE plants reflect a higher demand for assimilatory power (ATP and NADPH) compared with IE. Indeed, the down-regulation of the cytochrome b$_6$f complex in NE chloroplasts indicates the inhibition of ATP production, associated with the down-regulation of extrinsic subunits of ATP synthase in isoprene-suppressed lines (Velikova et al., 2014). Because isoprene functions as a protective molecule against oxidative stress (Loreto and Schnitzler, 2010), the isoprene suppression in NE plants might be balanced...
by enhancing other compensatory protective mechanisms such as photorespiration and oxidative electron transport, which are both mediated by mitochondria.

Recent analyses demonstrated that the suppression of isoprene biosynthesis dramatically reduces carbon fluxes throughout the MEP pathway (Ghirardo et al., 2014), followed by the re-allocation of carbon to other pathways, which in turn induces profound metabolic changes, particularly in lipid biosynthesis (Way et al., 2013; Kaling et al., 2015). Thus, at the cellular level, lipid metabolism is up-regulated in NE leaves, whereas at the subcellular level, as shown herein, low levels of galactolipids and phospholipids comprise the structure of NE chloroplasts. These results suggest that the crucial needs of NE plants to maintain the correct fluidity of thylakoid membranes induces the up-regulation of lipid metabolism, including lipid intermediates, likely compensating for the low levels of galactolipids and phospholipids packed into chloroplast membranes. Thus, isoprene might (i) directly improve the fluidity of thylakoid membranes in synergy with galactolipids or (ii) indirectly affect lipid biosynthesis or trafficking into the chloroplast. Whether, the lack of isoprene function or the alteration of the plastidic isoprenoid pathway itself induces changes in the chloroplastidic lipid levels, thereby affecting membrane fluidity, should be examined in future studies.

**Functional changes relate to structural alterations in NE chloroplasts**

We measured light- and dark-adapted states of chlorophyll fluorescence in IE and NE poplar plants grown under unstressed conditions in order to assess whether the structural changes have functional significance as regards the differences in ability to emit isoprene. Our results showed that \( \Phi_{\text{PSII}} \) was lower in NE plants than in IE plants, consistent with previous observations that the proteins involved in photosynthetic processes are down-regulated in NE, potentially decreasing the efficiency of photochemistry of photosynthesis (Fig. 5; Velikova et al., 2014). The lower \( \Phi_{\text{PSII}} \) values in NE were negatively correlated to NPQ, a protective mechanism for the removal of excess excitation energy within pigment complexes and the inhibition of the formation of free radicals (Demmig-Adams and Adams, 2006).

Higher NPQ levels in concert with restricted ETR between both photosystems and a reduced plastoquinone pool has been shown (Härtel et al., 1998) to be accompanied by DGDG modifications in the *Arabidopsis* mutant (*dgd1*). Moreover, in this mutant PSI showed an increased capacity for cyclic electron transfer and a reduced capacity for state transitions (Ivanov et al., 2006). Similar to the *dgd1* mutant (Dörmann et al., 1995), the NE poplar plants showed a lower DGDG content, modified chloroplastic ultrastructure, increased NPQ,
restricted ETR (Behnke et al., 2007), and decreased total chlorophyll content (Fig. 5C) (Behnke et al., 2013; Way et al., 2013; Ghirardo et al., 2014).

The NPQ comprises energy-dependent (i.e., dependent on the energization of thylakoid membranes) quenching (qE), state transitions (qT) (Minagava, 2011), photoinhibition quenching (qI) (Müller et al., 2001), and zeaxanthin-dependent quenching (qZ) (Nilkens et al., 2010). Energy-dependent quenching is the most important and well-characterized component of NPQ. This transition is triggered through the acidification of the thylakoid lumen (Ruban et al., 2012), which in turn leads to the protonation of VDE, for the conversion of violaxanthin to zeaxanthin, and PsbS, a polypeptide of the PSII-associated LHC (Kiss et al., 2008; Murchie and Niyogi, 2011).

Here we showed higher values of qE in NE plants, which might reflect a particular conformation of the LHCII complex resulting from chlorophyll and/or xanthophyll/protein interactions (Horton et al., 2005). Indeed, we observed that many proteins associated with photosynthesis are less abundant in NE chloroplasts (Fig. 5 and Fig. 7; for detail see Velikova et al., 2014). This lack of photosynthetic proteins could lead to specific conformational changes, which in turn could determine the higher qE in NE poplars. However, the supramolecular organization of the PSII antenna involves numerous interactions between proteins, suggesting that the changes in these interactions (Garab and Mustardy, 1999; Horton et al., 2005) could be responsible for the increase in NPQ we observed in NE plants. Indeed, with CD spectroscopy it has been shown that isoprene deficiency inhibits the formation of the chirally organized macrodomains. This effect in turn decreases the thermal stability of thylakoid membranes (Velikova et al., 2011). We also observed the significant down-regulation of the cytochrome b6f complex in NE lines (Velikova et al., 2014), which might inhibit the production of ATP in isoprene-suppressed plants. The increase of qE in NE lines might reflect the optimization of electron transport and ATP synthesis through the modulation of the cyclic electron transfer around PSI, the activation state of ATP synthase, and the partitioning of the proton motive force between ΔpH and the membrane electrical potential (Horton et al., 2005).

CONCLUSION

The proposed biological functions of isoprene in plants have been associated with the ability of this molecule to affect thylakoid membrane organization and reduce the formation of ROS, conferring tolerance to heat and oxidative stress. It has been hypothesized that isoprene improves the thermal stability of thylakoid membranes by affecting the membrane
lipid composition (Velikova et al., 2011). Herein, we provided direct evidence of the relationship between isoprene emission and the level of main lipid classes and their fatty acid composition, and we characterized the structural organization of the photosynthetic machinery in IE and NE poplar genotypes. The suppressed isoprene production in NE plastids was associated with the reduced amount of galacto- and phospholipids, lower level of the major fatty acid (18:3), and altered chloroplast ultrastructure (Fig. 8). The suppression of isoprene biosynthesis causes considerable metabolic changes, particularly in lipid biosynthesis (Way et al., 2013; Kaling et al., 2015) and significant alterations in the chloroplast proteome (Velikova et al., 2014). The majority of the plastidic and mitochondria proteome is encoded in the nuclear genome, and there is a continuous exchange of “forward” information from nucleus-to-organelle (anterograde) and of “backward” information from organelle-to-nucleus (retrograde) (Pfannschmidt, 2010). According to the retrograde signaling concept, based on the available experimental data, signals originating in chloroplasts and/or mitochondria modulate nuclear gene expression (Leister, 2012). These signals originate from carotenoid biosynthesis, ROS, photosynthetic redox processes, and changes in the pool of metabolites (Pfannschmidt, 2010; Leister, 2012). The plastidic signals identified so far have been associated with specific stress conditions. It is likely that the comprehensive changes in the metabolome (e.g., Way et al., 2013; Kaling et al., 2015), liposome, proteome (Velikova et al., 2014) and ultrastructure of the chloroplasts in non-isoprene emitting poplars (Fig. 8) as well as the distinct physiological behavior of these plants reflects finely tuned retrograde signaling. The precise mechanism(s) for the transmission of the changes in chloroplast to the nucleus in NE plant cells remain elusive.

MATERIAL AND METHODS

Plant material

In the present study, we used the same gray poplar (Populus x canescens; syn. Populus tremula x P. alba) genotypes as utilized in previous chloroplast proteome research (Velikova et al., 2014), namely two isoprene-emitting lines (IE: wild type and empty vector control, WT/EV) and two non-isoprene emitting (NE: RA1/RA2) lines. EV was used to ensure that the differences between NE and IE reflected specific alterations in the isoprene synthase gene and not to a more general genetic manipulation effect. The plants were grown in a greenhouse as previously described (Velikova et al., 2014). Briefly, the ambient temperature was 25/20°C, with a relative humidity of 50/60% and a photoperiod of 16/8 h (day/night). The plants were fertilized weekly with Triabon (Compo, Münster, Germany) and Osmocote (Scotts Miracle-Gro, Marysville, OH) (1:1 v/v; 10 g per liter of soil).
Four-month-old plants were used for the experiments. Fully expanded leaves (9th node from the apical meristem) from 6-7 different plants, considered as biological replicates, were used for physiological, biochemical and structural studies. The chloroplasts were isolated as previously described (Velikova et al., 2014) and used for lipid and malondialdehyde (MDA) analyses.

Lipid extraction procedure

The total lipids from chloroplasts were extracted according to Bligh and Dyer (1959). All procedures were performed in dim light using chilled solvents (containing BHT, 0.01% w/v) and glassware. The chloroplast samples (0.5 mL) were mixed with chloroform/methanol (1:2 v/v; 1.9 mL) for approximately 2 min, and subsequently 0.625 mL of chloroform and 0.625 mL of distilled water were added. The lower chloroform phase, containing the lipids, was removed, and aliquots were transferred into vials and exsiccated under N₂. The residues were weighed and calculated for total lipids.

GC-MS analysis of phospholipid fatty acid composition

Phospholipid fatty acids (PLFAs) were analyzed as previously described (Way et al., 2013; Behnke et al., 2013). Briefly, the PLFAs were separated from other lipids using a silica bonded phase column (MEGA-BE-SI, 2 g 12 mL⁻¹, 20/PK, Bond ELUT, Agilent Technologies, CA, USA). Fatty acid methyl esters (FAMEs) were obtained after mild alkaline hydrolysis. Myristic acid was used as internal standard for gas chromatography analysis. Un-substitute FAMEs were measured using a 5973MSD GC-MS (Agilent Technologies, Oberhaching, Germany) coupled with a combustion unit to an isotope ratio mass spectrometer (DeltaPlus; Thermo Electron Corporation, Bremen, Germany) and identified using the established fatty acid libraries and characteristic retention times of pure standards. The fatty acids were named according to the total number of C atoms and double bonds. Saturated straight-chain fatty acids are indicated as ‘n’.

UPLC-ESI-Qq-ToF-MS of galactolipids

Lipids were dissolved in 1 mL of LC-MS grade methanol (Fluka). MGDG and DGDG contents were analyzed using the UPLC-ESI Qq-ToF-MS System (Ultra performance liquid chromatography electrospray time of flight mass spectrometry, maXis Bruker, Bremen, Germany). Aliquots of 2.5 μL of each sample were analyzed in three technical replicates in randomized order.
The chromatographic separation was achieved on a C18 ACQUITY UPLC BEH column, 50 mm, 2.1 mm, and 1.7 µm (Waters), using a gradient elution. The composition was changed from 50% to 92% B for 10 min and maintained for an additional 10 min, then change to 100% B for 1 min and maintained for 5 min. The flow was set to 0.4 mL h⁻¹. Mobile phase A comprised water/isopropyl alcohol (95:5, v/v), and mobile phase B comprised acetonitrile/isopropyl alcohol (95:5, v/v). A format of 0.001 mM sodium (Sigma-Aldrich, Taufkirchen, Germany) was added to both mobile phases. This method has been previously published for profiling photosynthetic glycerol lipids (Xu et al., 2010).

MGDGs and DGDGs were detected as sodium adducts through positive electrospray ionization. The instrument was calibrated with ESI tune mix (Agilent Technologies). Acquired spectra were internally calibrated and exported to GENE DATA software for chromatographic alignment and peak picking. MGDGs and DGDGs were identified based on the retention time and detected exact mass (mass error < 0.01 Da).

MGDG and DGDG standards (Larodan) were used to evaluate the analytical performance and determine the QC, which was injected 10 times in the beginning for column conditioning and after every 10th sample to validate the measuring performance.

Malondialdehyde (MDA) content

The lipid peroxidation level in extracted chloroplast samples was quantified after measuring the MDA content using the thiobarbituric acid-reactive substances (TBARS) assay according to Hodges et al. (1999). The chloroplast sample (0.100 mL) was mixed with 1.2 mL of 80% ethanol (containing 0.01% BNT w/v) and sonicated in a water bath sonicator for 3 min, followed by centrifugation at 5,000 g for 10 min at 4ºC. An aliquot of the obtained supernatant (0.5 mL) was mixed with the same volume of 0.65% (w/v) thiobarbituric acid (TBA) solution containing 20% (w/v) trichloroacetic acid (TCA). Another aliquot of the supernatant (0.5 mL) was mixed with 0.5 mL of 20% (w/v) TCA, representing the zero-control. The mixture was heated at 95ºC for 30 min. The reaction was terminated after incubation in an ice bath. The cooled mixture was centrifuged at 10,000 g for 10 min at 4ºC, and the absorbance of the supernatant was measured at 532, 600 and 440 nm (Perkin Elmer, Walthman, MSC, USA). MDA equivalents were calculated according to Hodges et al. (1999), namely:

1) \[ \text{[A}_{532+\text{TBA}} - \text{A}_{600+\text{TBA}} - (\text{A}_{532-\text{TBA}} - \text{A}_{600+\text{TBA}}) \] = A

2) \[(\text{A}_{440+\text{TBA}} - \text{A}_{600+\text{TBA}}) \times 0.0571 \] = B

3) MDA equivalents (nmol ml⁻¹) = \((A - B)/157000\) \times 10⁶
Protein and chlorophyll analysis

For the calculation of the abundance of reaction center proteins in PSI and PSII we used the chloroplast proteome data published in Velikova et al. (2014). Peak intensities of peptides identified as RCI (protein accession numbers: POPTR_0008s15100.1, POPTR_0006s27030.1, POPTR_0003s14870.1, POPTR_0002s25510.2) and RCII (protein accession numbers: POPTR_0011s03390.1, POPTR_0004s03160.1, POPTR_0005s22780.1, POPTR_0002s05660.1, POPTR_0005s01430.1, POPTR_0005s27800.3, POPTR_0002s05720.1, POPTR_0002s25810.1, POPTR_0001s44210.1, POPTR_0006s26270.1), respectively, were summed, and expressed per mg chlorophyll.

The chlorophyll content was measured in isolated chloroplast suspension after extraction with 80% of ice-cold acetone. Absorbance at 663 and 646 nm was detected to determine chlorophyll a and chlorophyll b concentrations, calculated according to Porra et al. (1989).

Chlorophyll fluorescence measurements

The chlorophyll fluorescence parameters were measured on intact leaves using a MINI-PAM Photosynthesis Yield Analyzer (Heinz Walz GmbH, Effeltrich, Germany). The leaves were dark-adapted for 15 min prior to the determination of the minimal (F_o) and maximal (F_m) chlorophyll fluorescence, and subsequently the leaves were exposed to actinic light (430 μmol m^-2 s^-1). After steady-state fluorescence was obtained, a saturating pulse was applied to determine the maximum fluorescence in the light (F_m'). The operating efficiency of PSII photochemistry (Φ_{PSII}) was calculated from (F_m' – F')/F_m' (Genty et al., 1989). The redox state of PSII was assessed based on the parameter q_L = (F_q'/F_v')/(F_o'/F'), where F' is the fluorescence emission from the light-adapted leaf, F_v' - variable fluorescence from the light-adapted leaf, and F_q' is the difference in fluorescence between F_m' and F' (Baker, 2008); F_o' was estimated using the following equation: F_o' = F_o/[(F_v/F_m) + F_o/F_m'] (Oxborough and Baker, 1997). The non-photochemical quenching (NPQ) was calculated as NPQ = (F_m - F_m')/F_m' (Bilger and Björkman, 1991). The NPQ relaxation kinetics in the dark was used to calculate energy-dependent (qE) quenching. qE was assigned as a fast-relaxing component (within the first 2 minutes of dark relaxation after switching off the actinic light), calculated as qE = (F_m'' – F_m'')/F''_m,2min dark (Zaks et al., 2013).

Transmission Electron Microscopy (TEM)
Leaf segments (1 mm$^2$) were cut from the middle of the leaves for TEM analyses. The segments were fixed in 2.5% (v/v) glutaraldehyde (electron microscopy grade) in 0.1 M sodium cacodylate buffer, pH 7.4 (Science Services, Munich, Germany), post-fixed in 2% (v/v) aqueous osmium tetroxide (Dalton, 1955), dehydrated in gradual ethanol (30–70%), stained with uranyl acetate (2% in 70% ethanol), dehydrated in gradual ethanol (70-100%) and propylene oxide (100%), embedded in Epon (Merck, Darmstadt, Germany) and cured for 24 h at 60°C. Semi-thin sections (300 nm) were cut and stained with toluidine blue. Ultrathin sections of 50 nm were collected onto 200 mesh copper grids before examination using transmission electron microscopy (Zeiss Libra 120 Plus, Carl Zeiss NTS GmbH, Oberkochen, Germany). The images were acquired using ‘Slow Scan CCD-camera’ and ‘iTEM’ software (Olympus Soft Imaging Solutions, Münster, Germany).

**Statistical analyses**

Correlation analyses between different data sets of phospholipid (PL) and galactolipid (MGDG, DGDG) contents, fatty acid compositions, chlorophyll fluorescence parameters, MDA content, the data groups and IE or NE genotypes were performed using Principal Component Analysis (PCA) and Orthogonal Partial Least Square regression (OPLS) from the software package ‘SIMCA-P’ (v13.0.0.0, Umetrics, Umeå, Sweden). In addition, we included the chloroplastic protein contents associated with photosynthesis and structure (Velikova et al., 2014) to correlate lipids to proteins. Because the proteomic data originated from 3 samples for each plant genotype (containing 6 - 7 different leaves each of the three samples), multivariate analyses were performed using only the data matching the same 3 samples used for both proteomic and PL analyses. Galactolipids, MDA, and chlorophyll fluorescence measurements were obtained from more and different extracts, and therefore only the data from 3 samples were taken randomly and used for these analyses. We added the means of all biological replicates to examine the correlations between genotypes using data originating from different leaf extracts. The resulting matrix size was therefore 78-by-16 (variables-by-observations). Thus, the present analyses could correlate any data value with an isoprene emission trait (plant genotype IE and NE), but correlations between/within variables could be achieved only using data from the same leaf material, i.e., within PL and proteins, MGDG, DGDG and MDA, and within chlorophyll fluorescence data.

The multivariate data analyses followed the established procedures to analyze MS data as previously described (Ghirardo et al., 2005; Ghirardo et al., 2012; Kreuzwieser et al., 2014; Vanzo et al., 2014; Velikova et al., 2014). The isoprene emission trait was selected as the Y-
variable for the OPLS analysis by setting NE = 1 and IE = 0. The X variables were centered, and each type of data was block-wise scaled with 1 sd\(^{-1}\), considering the different number of X-variables in each group of data. Each calculated significant principal component was validated using ‘full cross validation’, with 95\% confidence level on parameters. The regression model OPLS was further tested for significance using CV-ANOVA (Eriksson et al., 2008). Variables showing variable of importance for the projection (VIP) values greater than 1 and jack-knifing method uncertainty bars smaller than the respective VIP values were defined as discriminant variables to distinguish IE from NE samples. For the proteomics data, containing a much higher number of variables, the VIP threshold was set to 0.5. The statistical significance of the differences between the means of discriminant variables and the functional and structural parameters measured in NE and IE plants were additionally evaluated using Student’s \(t\)-test and at an \(\alpha\) level of 0.05, unless otherwise stated.

Legend to figures:

**Figure 1.** Lipid content in isolated chloroplasts of isoprene emitting (IE, WT/EV) and non-isoprene emitting (NE, RA1/RA2) poplar. Error bars display the SE (n=4). Asterisks indicate significant differences with WT; ** \(P < 0.01\).

**Figure 2.** MDA level in isolated chloroplasts of isoprene emitting (IE, WT/EV) and non-isoprene emitting (NE, RA1/RA2) poplar. Error bars display the SE (n=4). Asterisks indicate significant differences with WT; * \(P < 0.05\).

**Figure 3.** Transmission electron micrographs of representative chloroplast cross-sections taken from the intact leaves of isoprene emitting (IE, WT/EV) (A, B) and non-isoprene emitting (NE, RA1/RA2) (C, D) poplar. Height / Length ratio (E) and average number of starch grains in IE and NE chloroplasts (F). CW - cell wall; GT - granal thylakoids; M, mitochondrion; P, plastoglobuli; S, stroma; SI, starch gain. Scale bar = 1 \(\mu\)m in A, B, C and D; at x 6,300 magnification.

**Figure 4.** Transmission electron micrographs of representative chloroplast cross-sections taken from the intact leaves of isoprene emitting (IE, WT/EV) (A, B) and non-isoprene emitting (NE, RA1/RA2) (C, D) poplar. Average number of stacks per chloroplast (E) and correlation between PSII – RCII protein abundance (10 peptides; for protein accession
numbers see Materials and Methods) and number of stacks (F). CW - cell wall; GT - granal thylakoids; M, mitochondrion; P, plastoglobuli; S, stroma; SI, starch gain. Scale bar = 500 nm in A, and C, at x10,000 magnification; and 200 nm in B and D, at magnification x20,000.

**Figure 5.** Protein abundance of PSI-RCI (4 peptides; for protein accession numbers see Materials and methods) (A), PSII-RC (10 peptides; for protein accession numbers see Materials and methods) (B) and chlorophyll content (C) in isoprene emitting (IE, WT/EV) and non-isoprene emitting (NE, RA1/RA2) poplar plants. Protein abundance represents sum of MS data extracted from our proteome study (Velikova et al., 2014). Error bars display the SE (n=4). Asterisks indicate significant differences with WT; **P* < 0.01; ***P* < 0.001.

**Figure 6.** PSII photochemical efficiency (A), redox state of PSII (B), non-photochemical quenching (C) and NPQ energy-dependent component (D) of isoprene emitting (IE, WT/EV) and non-isoprene emitting (NE, RA1/RA2) poplar plants at growth conditions. Values represent means of 5-7 different plants out of three independent experiments (n = 15–21, ± SE is given). Photosynthetic parameters are described in “Material and Methods”. Asterisks indicate significant differences with WT; *P* < 0.05, **P* < 0.01, ***P* < 0.001.

**Figure 7.** Score (A), loading (B) and correlation coefficient plots (C) of Orthogonal Partial Least Squares (OPLS) of lipid classes, fatty acid composition and MDA contents in isolated chloroplasts, chlorophyll fluorescence parameters measured in intact leaves (NPQ, $\Phi_{\text{PSII}}$, qE and qL), and chloroplast proteins related to photosynthesis and proteins with structural activity. (A) IE (WT/EV), grey circles; NE (RA1/RA2), white triangles. (B) Each parameter is indicated with different symbol. Dark grey circles, MGDG; dark gray square, DGDG; gray triangle, PL; dark gray circles with a dot, MGDG – fatty acids; dark gray square with a dot – DGDG – fatty acids; gray triangle with a dot, PL – fatty acids; green diamond, MDA; red star, NPQ; red triangle-down, qE; blue square, qL; blue star, proteins with structural activity; green star, proteins related to photosynthesis. (C) Parameter colors as plot legend B. Only discriminant data with VIP > 1 (for all except proteins) and VIP > 0.5 (proteins) are presented. Model fitness: $Q^2(Y) = 84\%$; $R^2(X) = 44\%$, $R^2 = 93\%$, $R^2(Y) = 100\%$ using one PC; P = 0.00061, CV-ANOVA.
Figure 8. Schematic overview of the changes in chloroplast ultrastructure, lipid composition, change, protein abundance and PSII fluorescence triggered by the suppression of isoprene biosynthesis and emission in poplar plants.

Acknowledgements

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Supporting information

Supplemental Figure S1 provides lipid content and fatty acid composition in isolated chloroplasts of isoprene emitting (IE, WT/EV) and non-emitting (NE, RA1/RA2) poplar. Supplemental Figure S2 provides score and loading plots of PCA all parameters analyzed (lipid and fatty acid composition, MDA, NPQ, $\Phi_{PSII}$, qE, qL, proteins related to photosynthesis and proteins with structural activity.

LITERATURE CITED


Table 1. Fatty acid composition (μg mg⁻¹ Chl) of the main lipid classes in C₁₆-C₂₃ saturated (:0) and unsaturated (:1,:2,:3) compounds in chloroplasts of isoprene emitting (IE, WT/EV) and non emitting (NE, RA1/RA2) poplar plant lines (n16:0, palmitic acid; 16:1, palmitoleic acid; n18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; n23:0, tricosanoic acid). Fatty acids are designated as the total number of C atoms followed by the number of double bonds and their location (omega) after the colon. Saturated straight-chain fatty acids are indicated by ‘n’. Means ± SE and shown; n=4. Asterisks indicate significant differences with WT; * P < 0.05, ** P < 0.01, *** P < 0.001.

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Figure 1. Lipid content in isolated chloroplasts of isoprene emitting (IE, WT/EV) and non-isoprene emitting (NE, RA1/RA2) poplar. Error bars display the SE (n=4). Asterisks indicate significant differences with WT; ** $P < 0.01$. 
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Figure S1. Lipid content and fatty acid composition in isolated chloroplasts of isoprene emitting (IE, WT/EV) and non-emitting (NE, RA1/RA2) poplar. (A) Monogalactosyldiacylglycerols (MGDG), (B) digalactosyldiacylglycerols (DGDG), (C) phospholipids (PL). Error bars display the SE (n=4). Asterisks indicate significant differences with WT; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
Supplemental Figure 2

Fig. S2. Score and loading plots of PCA all parameters analyzed (lipid and fatty acid composition, MDA, NPQ, $\Phi_{\text{PSII}}$, qE, qL, proteins related to photosynthesis and proteins with structural activity. (A) IE (WT/EV), grey circles; NE (RA1/RA2), white triangles. (B) Each parameter is indicated with different symbol. Dark grey circles, MGDG; dark gray square, DGDG; gray triangle, PL; dark gray circles with a dot, MGDG – fatty acids; dark gray square with a dot – DGDG – fatty acids; gray triangle with a dot, PL – fatty acids; dark gray square with a dot – DGDG – fatty acids; gray triangle with a dot, PL – fatty acids; green diamond, MDA; red star, NPQ; red triangle-down, qE; blue square, qL; blue star, proteins with structural activity; green star, proteins related to photosynthesis. Data with VIP > 1 (all data except proteins) and VIP > 0.5 (proteins) are presented.
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