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School of Doctoral Studies in Biological Sciences

Ph.D. Thesis

Plant Physiology

Carbon dioxide transport within the leaf mesophyll: physico-chemical and biological aspects

by

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Annotation

Stomatal conductance and mesophyll conductance for CO_2 transport are two key components of diffusive limitations of photosynthesis, since they restrict CO_2 flux from the leaf surface to the sub-stomatal cavity and from there to the sites of carboxylation. This thesis summarizes our findings in the field of nature of mesophyll conductance to CO_2 transport and its regulation *per se* and in respect to stomatal conductance.

Declaration [*in Czech*]

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Ostrava 19.9.2013 Daniel Vrábl

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Besides that I would like to thank Jiří Šantrůček, my supervisor, for introduction me to the phenomenon of mesophyll conductance and stable isotopes. It was honor and pleasure for me to supervise Dan Hisem's and Jitka Neuwirthova's master and bachelor thesis respecitvely. I would like to thank them for their confidence. I am very grateful to all people from Laboratory of Department of Experimental Plant Biology at University of South Bohemia, particularly to Marie Hronková for helping me with gas exchange measurements and Jiří Květoň and Vladislav Marek for technical assistance in IRMS measurements.

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List of Publications

The thesis is based on the following articles which are referred to in the text by their Roman numerals:

I. **Vrábl D, Vašková M, Hronková M, Flexas J, Šantrůček J** (2009) Mesophyll conductance to CO_2 transport estimated by two independent methods: effect of variable CO_2 concentration and abscisic acid. *Journal of Experimental Botany,* **60**, 2315-–2323.

D. Vrábl designed the experiment, carried out the gas exchange, fluorescence measurements and gas sampling for IRMS analysis. He was responsible for data analysis, their evaluation,writing the manuscript and complete the revisions.

II. **Štroch M, Vrábl D, Podolinská J, Kalina J, Urban O, Špunda V** (2010) Acclimation of Norway spruce photosynthetic apparatus to the combined effect of high irradiance and temperature. *Journal of Plant Physiology,* **167**, 597–605.

D. Vrábl participated designing the experimental setup. He was responsible for gas exchange measurements and data evaluation. He participated in writing and revision of the manuscript.

III. **Hisem D, Vrábl D** (2013) Low concentration of abscisic acid enhances mesophyll conductance to CO_2 transport. Submitted to *Journal of Experimental Botany.*

D. Vrábl designed the experimental setup, he participated in writing and revision of the manuscript.

Confirmation of co-authors:

We confirm that contribution of Daniel Vrábl to the above listed articles was as mentioned.

Michal Štroch

Daniel Hisem

Content

Introduction

Importance of CO_2 in the photosynthesis

Carbon dioxide diffusion from the ambient atmosphere to the chloroplast stroma is crucial for plants as photosynthesis is highly limited by CO_2 availability at the sites of carboxylation in chloroplasts (C_c). While the recent CO_2 concentration in atmosphere is 400 μmol mol^{-1}(NOAA, National climatic data center, 2013) and is progressively rising, the effective CO_2 concentration at the site of carboxylation is much lower and below saturated concentration due to several resistances restricting diffusion from leaf surface into the chloroplast stroma. Here we would like to contribute to understanding of the role of CO_2 not only as a substrate of photosynthesis and the major source of carbon, but as pathway of energy dissipation as well. Due to poor affinity of Rubisco enzyme to CO_2, the decrease in C_c increases the rate of energy-wasting within the photorespiration pathway. Moreover as a result of lower total energy use by carboxylation and oxygenation at the low C_c, chloroplasts are more liable to be over reduced and photoinhibited (Terashima *et al.*, 2011).

CO_2 diffusion is on its pathway from leaf surface into the chloroplast restricted by several resistances (defined as: $r = 1/D * dx$, where D is diffusional coefficient and x is length of diffusional pathway). The first of them is boundary layer at the leaf surface, where air molecules are decelerated and thus thin layer of the slow laminar flow is created. The resistance of the boundary layer is related to the wind speed and leaf length. Subsequently CO_2 has to pass through stomata. Opened stomata facilitate diffusion of CO_2 to reach the mesophyll but simultaneously let water vapour exit the leaf to the atmosphere. Hence by opening and closing, stomata regulate not only the carbon assimilation but also water loss and leaf water status. Therefore stomatal resistance is the first of adjustable resistances affecting CO_2 diffusion. Resistance to CO_2 diffusion in the mesophyll intercellular airspace is given by the length of the CO_2 diffusion pathway from sub-stomatal cavity to the mesophyll cell surface. Then CO_2 dissolves in the cell wall water and diffuses across the cell wall, plasma membrane, cytosol, chloroplast envelope, and stroma to Rubisco. The pathway comprising CO_2 transport from sub-stomatal cavity into the chloroplast is termed mesophyll conductance (g_m) (the resistance is expressed here as conductance: $g = 1/r$) (Fig.1). The rate of diffusion through the mentioned composite segments of the diffusion pathway depends on the effective thickness and

diffusivity of each component section (Terashima *et al.*, 2011). The diffusion of CO_2 within

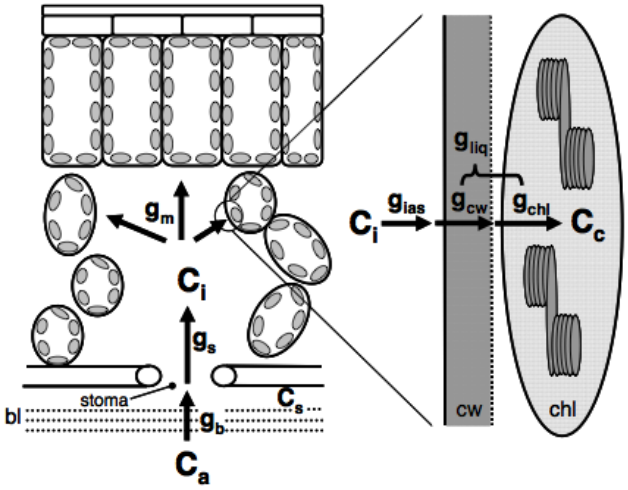

Figure 1: Schematic illustration of diffusion pathway of CO_2 in terms of concentration from the ambient (C_a) through leaf surface (C_s) and intercellular air spaces (C_i) to the chloroplast (C_c) concentration. Boundary layer conductance (g_b), stomatal conductance (g_s) and mesophyll conductance (g_m) are figured. The partition of mesophyll conductance into three components - intercellular air space conductance (g_{ias}), cell wall conductance (g_w) and chloroplast conductance (g_{chl}) - is figured on the right side of the figure where the cell wall (cw) and the chloroplast (chl) with granum is featured in detail. g_w and g_{chl} are often being joined and termed as liquid phase conductance (g_{liq}) since CO_2 has to diffuse through liquid phase inside the cell. Adapted from Terashima *et al.* (2011) and Hisem (2011)

the leaf mesophyll would be possible to describe purely in physical and mathematical way, but it becomes apparent that it is not as straightforward. The previously used term "effective resistance" denotes that circumstance that the diffusion path length is generally longer than the linear distance from sub-stomatal cavity to chloroplast due to tortuosity and/or limited porosity of the diffusional pathway (Evans *et al.*, 2009). Tabulated diffusion coefficients for CO_2 in free water cannot be directly applied to leaves due to presence of solutes and macromolecules in liquid-phase components of the diffusion pathway and changes in pH, temperature etc. Moreover, CO_2 can interconvert with HCO_3^- inside leaf cells in a reversible reaction catalyzed by carbon anhydrases.

In the past it was assumed that CO_2 concentration in the chloroplast is almost the same

as CO_2 concentration in the sub-stomatal cavity, thus that g_m is large enough and not limiting the rate of photosynthesis. With development of methods estimating g_m in the last two decades, it has become more and more clear that g_m is finite and significantly restricts CO_2 diffusion into the chloroplast and consequently it becomes one of the limiting factors of photosynthesis.

Variability of mesophyll conductance to CO_2

Nature of g_m has been mostly attributed to that structural properties of the leaf (mesophyll anatomy), which consequently cause most of the g_m variations (von Caemmerer and Evans, 1991; Lloyd *et al.*, 1992). Leaf mesophyll thickness is often discussed as one of the parameters that could affect g_m at structural level influencing the effective path length for CO_2 diffusion from the sub-stomatal cavity to the uppermost mesophyll cell surface (e.g. Terashima et al. 2001). In purpose to characterize impact of mesophyll structure on g_m more precisely, parameters as mesophyll porosity, mesophyll area exposed to intercellular airspaces or the surface area of chloroplasts facing intercellular airspaces have been used (Hanba *et al.*, 1999). g_m is usually expressed on leaf area basis, however the real surface of the mesophyll cell walls across which CO_2 diffuses is considerably larger. Based on i) negative correlation between mesophyll porosity and g_m and ii) positive correlation between mesophyll area exposed to intercellular airspaces and g_m, Kogami (2001) suggested that g_m would be primarily determined by variation of the conductance in liquid phase rather than that in gas phase.

Recent knowledge of variation in g_m proves that leaf cellular and sub-cellular layout is the factor which affects particularly long term changes in g_m. But fast response of g_m to CO_2, temperature, light and other factors has been observed as well by Flexas *et al.* (2008). Moreover the authors claim, that response of g_m is even faster then g_s. Thus long term variation of g_m due to leaf structure can not be the only one factor affecting g_m.

Real or apparent regulation of g_m

Considerable number of observations of large and rapid response of g_m to various environmental factors which have been published in last decade evokes the effort to uncover other then structural properties of the mesophyll which affect variability of g_m. Analysis of temperature response of g_m provided first insight of the character of the diffusion of CO_2 within

the g_m. If the processes determining g_m are driven purely by diffusion, then g_m should have a temperature coefficient (Q_{10}) close to that of the diffusivity of CO_2 in pure water. The Wilke-Chang equation predicts a Q_{10} of 1.25 at 25 °C, varying little across the biologically relevant temperature range. This is in close agreement with range of measurements (Tamimi *et al.*, 1994). If an enzyme or contribution of a metabolic process is required for the effective transfer of CO_2 to the site of carboxylation, then g_m should be more sensitive to temperature, with a Q_{10} value close to or above 2. Based on a Q_{10} of approximately 2.2 for g_m in tobacco leaves, Bernacchi *et al.* (2002) speculated that enzymatic or protein facilitated diffusion of CO_2 controls g_m. The most likely candidates for this effect would be carbonic anhydrase (CA) and aquaporins.

But it has to be pointed out that argument based on the high Q_{10} is defensible in the case of *N. tabacum*, which had the expected temperature response for a protein-facilitated process. However, the relative constancy of g_m from 20°C to 35°C in *Q. cannariensis* and from from 28°C to 38°C in *Eperua grandiflora* (Pons and Welschen, 2003) argue against g_m being determined by only protein facilitated diffusion. It is more likely that g_m is determined by multiple processes with different temperature sensitivities which result in complex temperature response. It is more likely in relation to the miscellaneous nature of g_m.

Several authors demonstrate that the respiration processes can not be neglected in the calculation of g_m. Primarily because respiration processes are very sensitive to temperature. The errors of g_m estimation result from incorrect estimation of the rate of photorespiration and electron transport via PSII (when using simultaneous measurements of gas exchange and fluorescence - variable J method). Particularly electron transport rate is strongly affected by temperature as was shown in **II**–Štroch *et al.* (2010). Similarly, when stable isotope method is used forg_m estimation , assumption of constant fractionation factor within the whole temperature range may lead to errouneous g_m.

Gillon and Yakir (2000) developed advanced method providing the estimation of CO_2 concentration in chloroplast (C_c) and at the chloroplast surface (C_{cs}). This method is based on different discrimination of isotopes ^{13}C and ^{18}O in CO_2 and H_2O in the leaf mesophyll. While ^{13}C is discriminated within the whole diffusional pathway (leaf surface - chloroplast) then discrimination of ^{18}O occures only between chloroplast surface and chloroplast stroma due to oxygen exchange between ^{18}O-enriched water and CO_2 in the chloroplast, a process catalyzed

by carbonic anhydrase (CA). We applied this approach for segmentation of overall g_m to conductances of cell wall (g_w) and chloroplast (g_{chl}) and evaluation of the temperature response of the particular conductances within the g_m (Fig.2). By fitting the relationships with Arrhenius equation Q_{10} has been observed as the indicator of diffusion character. Q_{10} for g_w, which in fact involves CO_2 transport not only within the cell wall but from sub-stomatal cavity to the chloroplast surface, was 1.63 which indicates, that simple diffusion dominated the CO_2 transport. On the other hand Q_{10} for overall g_m was 2.5 and particularly Q_{10} for g_{chl} was 2.58, implying that facilitation of CO_2 diffusion should be located within one of the chloroplast envelopes. These findings are in agreement with Uehlein *et al.* (2008), who localized aquaporins proteins (NtAQP1) of PIP1 family particularly in inner membrane of chloroplast. Since the ability to transport CO_2 has been proved in PIP1 aquaporin subfamily (Uehlein *et al.*, 2003), at least part of the g_m variability seems to be localized in the inner chloroplast membrane.

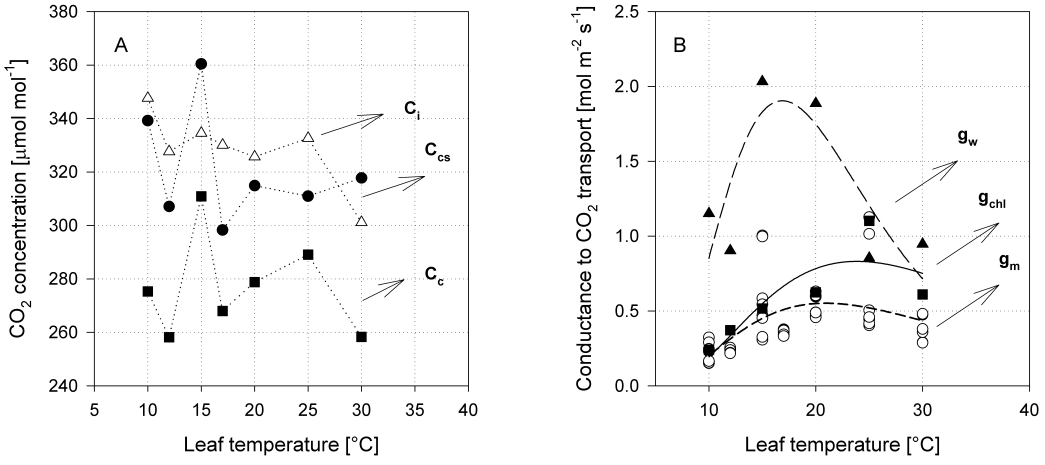

Figure 2: Temperature response of A) CO_2 concentration in sub-stomatal cavity (C_i), chloroplast surface (C_{cs}) and chloroplast stroma (C_c). B) Temperature response of overall mesophyll conductance to CO_2 transport (g_m) and particular conductances of cell wall (g_w) and chloroplast (g_{chl}). (Vrábl *et al.,unpublished results*)

Previous studies of temperature response of g_m reported typical pattern with initial exponential increase (Bernacchi *et al.*, 2002) followed by peak and subsequent plateau (Yamori *et al.*, 2006) or decrease (Bernacchi *et al.*, 2002). But the most recent findings of Evans and

von Caemmerer (2013) evoke questions in terms of effects of photorespiration on temperature response of g_m. The authors observed linear relationship between g_m and temperature with g_m increasing from 0.5 to 1.75 $\mathrm{mol\,m^{-2}\,s^{-1}\,bar^{-1}}$, at the temperature ranging from 15°C to 40°C. The measurements were made under 2 and 21% O_2, so it was possible to derive both the fractionation factor of ^{13}C associated with photorespiration as well as the overall effect of discrimination of ^{13}C associated with photorespiration. Latter increased from 1.1 to 2.7‰ between 20 and 40°C. Thus as the isotopic discrimination associated with g_m declined at higher temperatures, this was offset by increased discrimination associated with photorespiration. By the modeling of temperature response of g_m the particular mechanisms behind the sensitivity of g_m to temperature can be revealed. Evans and von Caemmerer (2013) has shown that diffusivity of CO_2 in water increases with temperature. Therefore they predict a decrease of CO_2 solubility with temperature if g_m is solely determined by diffusion through water in the cell wall, cytosol and chloroplast stroma.

What can the CO_2 response of mesophyll conductance tell us about its nature?

It was observed that g_m is sensitive to large amount of external stimuli. Most of them directly or indirectly affect CO_2 concentration in the mesophyll and/or chloroplast by changes in diffusional conductances and/or consumption of CO_2 within Calvin-Benson cycle. Thus it can be speculated that CO_2 concentration in the mesophyll or chloroplast can be the one of many or the only factor which drive the variability of g_m.

At low C_i, g_m in most of the studies increased with subsequent peak and an exponential decrease until steady state was reached at high C_i. Such type of response was initially suggested by During (2003) who observed six-fold decrease of g_m when C_i increased from 300 to 1000 ppm air in grapevine. Later, Flexas *et al.* (2007) provided more detailed analysis of g_m response to CO_2 in six different species supporting previous findings. Similar pattern of g_m-C_i response has been observed by **I**–Vrábl *et al.* (2009); Hassiatou *et al.* (2009); Yin *et al.* (2009); Bunce (2010) in measurements on sunflower, banksia, wheat, and bean and soybean, respectively. Likewise in more recent study Tazoe *et al.* (2011) found significant decrease of g_m when CO_2 increased. In summary, here presented and previously published data support the hypothesis that g_m is affected by CO_2 concentration. So far, two types of g_m/CO_2 relation-

ship have been published (if the study of Tazoe *et al.* (2009) is omitted). Firstly, g_m increases at low CO_2 concentrations, peaks, and declines exponentially thereafter. Secondly, only exponential decay without the initial growth and peak was observed. Although Loreto *et al.* (1992) and later Tazoe *et al.* (2009) found no sensitivity of g_m to varying CO_2 concentration, their data could be insufficient since they measured g_m over the three times smaller range of CO_2 concentrations (from 100 to 500 ppm) than in studies proving CO_2 dependency of g_m. Bunce (2010) has recently shown that sensitivity of g_m to CO_2 increases with lower measuring PPFD and even more when using lower PPFD during plant growth. Nevertheless, this response differed between studied species with bean being more sensitive than soybean. Interestingly, Bunce (2010) was able to record g_m response to CO_2 on very small range of CO_2 concentration (from 100 to 300 ppm). Therefore, neither methodological nor species-dependent variation in g_m response to CO_2 can be disclaimed. This should further be tested on the leaf and single plant level. On canopy-scale, no changes in g_m were found in response to varying CO_2 concentration in sunflower grown in growth cabinet (Schaufele *et al.*, 2011) but g_m exerted high sensitivity to CO_2 in plants treated by ABA. It may be possible that application of ABA can increase sensitivity to CO_2 as diffusional conditions in leaf interior are changed due to lower g_s.

But it has to be admited that up to now we are still quite far away from understanding of the response of g_m to variable CO_2 concentration. Several authors speculate that the pattern of the g_m/CO_2 relationship is more significantly affected by errors in methodology of g_m estimation then the real variation in mesophyll conductance affecting CO_2 transport inside the leaf.

Looking for the link between stomatal and mesophyll conductance

Within CO_2 transport from leaf surface into the chloropast, g_s and g_m are connected in series. Thus one may speculate whether change of g_s will induce the change in the g_m and vice-versa. If so, there should exist a factor, which drives g_s as well as g_m. Most of the so far elucidated factors affecting g_m (see Flexas et al., 2008 for review) simultaneously affected also g_s. In most of the published studies the responses to various stimuli, of both g_s and g_m, are of approximately the same extent and similar pattern, then g_m/g_s relationship acquires the character of linear increase. On the other hand, several studies presented variations in only

one of the conductances, either g_s or g_m, while the other remained unaffected. Up to now, inverse pattern of g_s and g_m to the given treatment has not been published, except for the work of Pons and Welschen (2003), showing that during midday photosynthesis depression at 28 - 33°C, initial increase of g_m was accompanied by simultaneous decrease of g_s.

The link between g_s and g_m can be evaluated by manipulation of g_s and observing the impact upon g_m. g_s can be easily restrained by exogenous application of abscisic acid (ABA). Although the closure of stomata in presence of ABA is well characterized even without induction of water stress (Dodd and Davies, 2004; Flexas et al., 2006a), direct effect of ABA on g_m on the leaf scale is very scarce and contradictory. Flexas et al. (2006a) presented reduction of g_m after ABA addition in soybean and tobacco while Vrábl et al. (2009) found no effect in sunflower using five times lower exogenous ABA concentration (20 μM). On canopy scale, Schäufele et al. (2011) showed that g_m decreased in presence of ABA, especially at higher ambient CO_2 concentrations (C_a), while controls showed hardly any response to increasing C_a.

Buckley and Warren (2013) modeled the nitrogen and water use in the term of g_s and g_m relationship. They conclude that: i) g_m cannot respond as sensitively to irradiance as g_s can. Thus with increasing irradiance the optimal g_s will be greater at high light than it would be if it was physiologically possible for g_m to track g_s perfectly. ii) constraints on g_m require a compensatory increase in g_s , the result is that C_i goes down thus C_i -C_c increases. But the authors are unaware of any data showing this C_i increase up through the canopy thus it is likely, that compensatory effect of g_s is offset by other factor that reduce g_s for example reduced water potential in upper canopy levels. Moreover from **I**–Vrábl *et al.* (2009) and Warren (2008) the authors deduce, that g_s–g_m relationship may reflect a tight coordination between the rate of photosynthesis and g_m, or a tendency for g_m to compensate the reduction in g_s.

Non-parallel g_s and g_m variation induced by abscisic acid treatment

Previous results from **I**–Vrábl *et al.* (2009) motivated us for further experiments designed to reveal the effect of abscisic acid on g_s-g_m link (**III**–Hisem and Vrábl, 2013). Abscisic acid (ABA) as the stress plant hormone with wide range of physiological effects is synthesized during the water shortage conditions. The primary effect of ABA is closing of the stomata

to reduce water loss during transpiration. Flexas et al. (2006) showed a reduction of g_s and g_m after addition of ABA in concentration 100 μM. Contrary to their finding, our results (**I**–Vrábl *et al.*, 2009) indicated that addition of ABA in lower concentration (20 μM) induced reduction of g_s and C_i which is not accompanied by reduction of neither A_N nor g_m. This evokes the hypothesis, that different concentration of ABA induced different response of g_s and g_m. We grew the *Helianthus annuus* plants in Hoagland nutrient solution while plants was exogenously treated with 10 and 20 μM ABA. Both treatments induced reduction of g_s and consequently decrease of CO_2 concentration in sub-stomatal cavity. Contrary to that, reduction of A_N has been observed only at 20 μM ABA treatment. Maintenance of A_N at 10 uM ABA treatment has been likely caused by significant enhancement of g_m, which increases CO_2 concentration in chloroplast, C_c. This is very promising result, which should be further evaluated. Under the condition of reduced g_s and enhanced g_m water loss through stomata is strongly reduced but rate of CO_2 assimilation does not change or is even enhanced (Fig. 3). This leads to enhancement of instantaneous water use efficiency without any depression of photosynthesis. It can be hypothesized that low concentration of ABA could positively affect carbon gain under moderate water shortage conditions.

Possible role of aquaporins in regulation of mesophyll conductance

Our measurements with variable CO_2 concentration and abscisic acid and the results of other authors indicate high variability of g_m, which can be partly attributed to the involvement of a CO_2 transporter in the processes underlying g_m. The family of major intrinsic proteins (MIPs) aquaporins are often suggested as the most likely gated channels, which are able to affect CO_2 transport in mesophyll cells. Our experiments with ABA indirectly support this suggestion similarly to Wan *et al.* (2004) who demonstrated, using pressure probe technique, that ABA treatment enhances water permeability of leaf mesophyll cells. According to Uehlein *et al.* (2008) AQP1, which is PIP1 isoform of aquaporin belonging to PIP (plasma membrane intrinsic protein) subfamily, is able to transport not only molecules of water but molecules of CO_2 as well.

There are several questions which need to be answered before we will get more clear picture of the importance of aquaporins in regulation of mesophyll conductance. i) Each subunit of the aquaporin tetramer consist of six transmembrane helixes which are connected

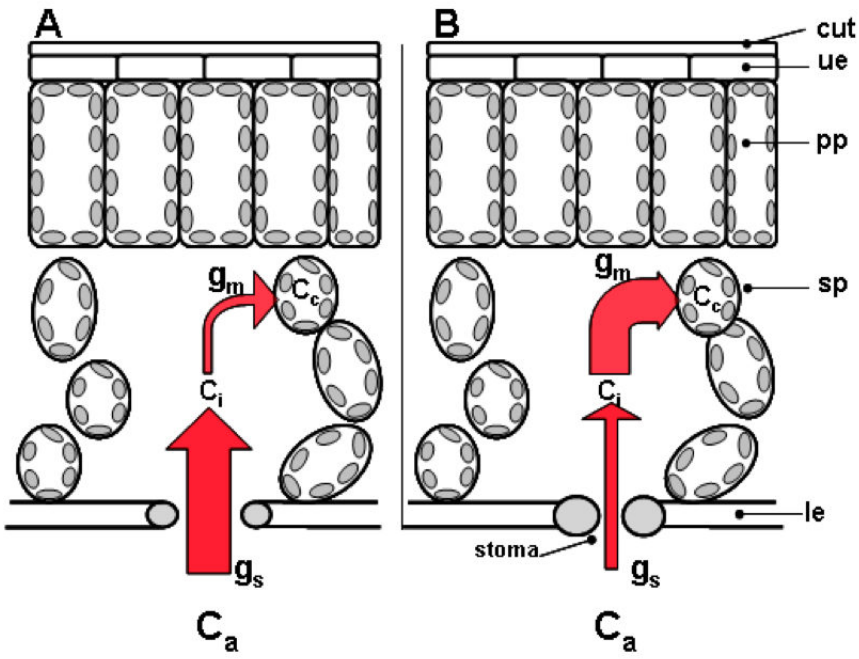

Figure 3: Simplified model of leaf section showing possible link between g_m and g_s in the case when stomata are fully opened as in controls (A) and closed as in ABA-treated plants (B). Red arrows represent a magnitude of CO_2 flux from ambient (C_a) to sub-stomatal cavity (C_i) and from sub-stomatal cavity to chloroplast stroma (C_c). The cuticle (cut), upper epidermis (ue), palisade parenchyma (pp), sponge parenchyma (sp), lower epidermis (le) and stoma are shown.

by five loops and form hydrophilic pore through the lipid bilayer. Two of the loops, containing co-called NPA motifs (Asn-Pro-Ala), meet at the center of the pore and constitute a size exclusion zone, with a diameter of 3 Å. The size of the water molecule is 2.78 Å but the size of CO_2 molecule is 3.23 Å. Besides that the geometry of the water and CO_2 molecule differs as well. ii) The previous point should be answered by clarification of the site, where CO_2 is transported within the aquaporin. Single aquaporins are organized into the tetramer structure thereby central pore without any specific binding site of the transported molecules is formed. Thus it was suggested that CO_2 molecules are able to pass the protein central pore (Wang *et al.*, 2007). iii) Molecules of CO_2 will follow the pathway with the highest conductance, thus for evaluation of the aquaporin significance it is crucial to know particular conductance to CO_2 transport through the individual barriers (cell wall, plasmatic membrane

and chloroplast membranes). Albeit Evans *et al.* (2009a) and Terashima *et al.* (2011) tried to evaluate the significance of particular diffusional barriers in mesophyll to CO_2 transport the measurements on particular cellular and sub-cellular structures are still missing. iv) While water transport through the particular aquaporins is quite well explored, it is not clear whether CO_2 molecules are transported in the same pathway. Moreover we are still far away from complete understanding of the sensitivity of particular aquaporins and/or aquaporin tetramers to various external stimuli like CO_2 concentration, pH, temperature, etc. v) Last but not least just one paper (Uehlein *et al.*, 2008) clarifies the localization of PIP1 aquaporin isomer which is able to transport CO_2 molecules. For better understanding of the role of aquaporins in CO_2 transport it would be helpful to know the localization of the different aquaporin isomers, composition of tetramers within the mesophyll cells in different plants and various growth regimes.

Summary

As was mentioned above, mesophyll conductance is one of the major factors limiting photosynthesis. It was shown that this parameter is not rigid but variable, which would allow tune up the CO_2 and water movements in mesophyll. The phenomenon of mesophyll conductance is very actual and interesting for growing community of plant physiologists. The number of publications addressing various aspects of mesophyll conductance to CO_2 transport is increasing in the last 23 years and number of reports increases exponentially from 2001 up to 2009 since the introduction of commercial devices capable of simultaneous gas exchange and chlorophyll fluorescence measurements and the progress in isotope-ratio mass spectrometry (IRMS) instrumentation and particularly very recent advancement in tunable diode laser spectrometry (TDLS) (Fig.4). The exponential increase of reports culminated by the special issue in Journal of Experimental Botany in 2009 (vol. 60), where the most of previous research and original papers has been reviewed. Up to year 2009 most of the papers have been focused on the sensitivity of mesophyll conductance to various internal and external factors, but understanding of the mechanisms behind the g_m responses was missing. Presently, research on mesophyll conductance is more focused on processes which stand behind the variability of g_m and prefers the comprehensive view of the CO_2 transport in mesophyll. It is obvious, that the recent approach to mesophyll conductance requires more focused same as the sophisticated broader

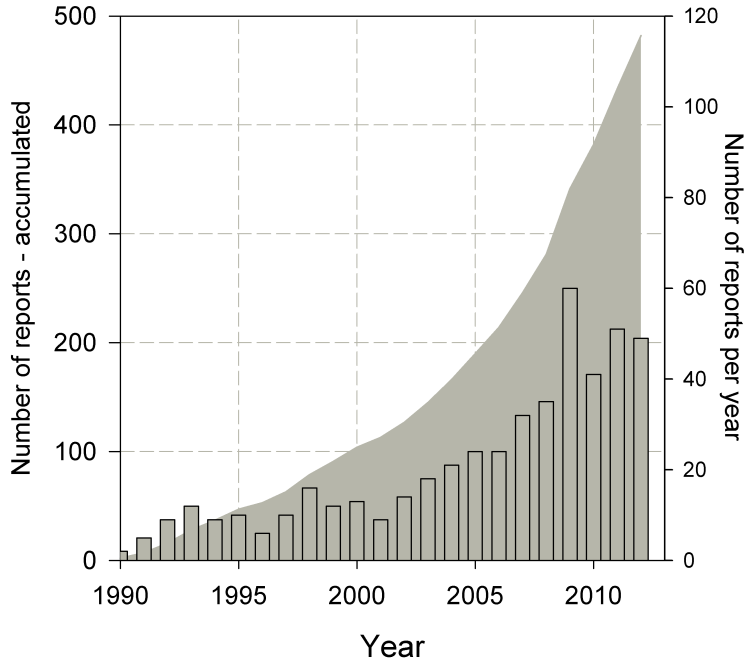

Figure 4: Evolution of publications on mesophyll conductance to CO_2 transport over the last 23 (1990–2012) years. Bars represent number of reports published in each year and the gray area represents accumulated number of publications.

experiments and the production of specific mutants and improvements of g_m estimation, thus it would be expected that the increase of number of papers related to the phenomenon of mesophyll conductance is not going to be as steep.

Very recently several, novel papers (Thollen *et al.*, 2012; Buckley and Warren, 2013), has been published. The first of them evaluates phenomenon of mesophyll conductance in a complex view including the impact of mitochondria respiration and photorespiration on variability of mesophyll conductance in perspective of the three-dimensional nearness. On the other hand Buckley and Warren (2013) are considering short term and long term variability of mesophyll conductance from the view of economics of nitrogen and water use efficiency and pointed out that the stomatal and mesophyll conductance link can be broken under specific condition. It is promising that both of this papers confirm our major results of variability of mesophyll conductance to CO_2 concentration (**I**–Vrábl *et al.*, 2009) and the non-uniform response of stomatal and mesophyll conductance after low concentration of abscisic acid (**III**–

Hisem and Vrábl, 2013).

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RESEARCH PAPER

Mesophyll conductance to CO_2 transport estimated by two independent methods: effect of variable CO_2 concentration and abscisic acid

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Abstract

Mesophyll conductance (g_m) and stomatal conductance (g_s) are two crucial components of the diffusive limitation of photosynthesis. Variation of g_m in response to CO_2 concentration was evaluated by using two independent methods based on measurements of variable electron transport rate (J) and instantaneous carbon isotope discrimination, respectively. Both methods of g_m estimation showed a very similar shape of the g_m/C_i relationship, with an initial increase at low substomatal CO_2 concentrations (C_i), a peak at 180–200 μmol mol^{-1} C_i, and a subsequent decrease at higher C_i. A good correlation was observed between values of g_m estimated from the two methods, except when C_i <200 μmol mol^{-1}, suggesting that the initial increase of g_m at low C_i was probably due to unreliable estimates over that range of C_i. Plants were also treated with abscisic acid (ABA), which induced a reduction in g_s without significantly affecting the rate of photosynthesis, g_m or the photosynthetic capacity. The present results confirm, using two independent methods, that g_m is strongly sensitive to C_i, and that the relationship between g_s and g_m is not conservative, differing between control and ABA-treated plants.

Key words: Abscisic acid, carbon dioxide, *Helianthus annuus*, mesophyll conductance, photosynthesis.

Introduction

Mesophyll conductance restricts the influx of carbon dioxide from the leaf internal airspace to the site of carboxylation and, therefore, may be a crucial component of the diffusive limitation of photosynthesis besides stomatal conductance (Evans *et al.*, 1986; Di Marco *et al.*, 1990; Flexas *et al.*, 2008; Warren, 2008*b*). Early gas exchange studies assumed that mesophyll conductance (g_m) was large and constant and, hence, that CO_2 concentrations in substomatal cavities (C_i) and in chloroplasts (C_c) were nearly the same (Farquhar *et al.*, 1980). However, a number of studies indicate that g_m may significantly limit photosynthesis and several sources of variation in g_m have been described, including water and salt stresses and changes in leaf temperature (see Flexas *et al.*, 2008, for a review). Several studies have investigated the response of g_m to changes in CO_2 concentration. Harley *et al.* (1992) argued that the observed reduction of g_m when C_i was increased from 100 μmol mol^{-1} to 300 μmol mol^{-1} in *Quercus rubra* was due to unreliable values, owing to the great sensitivity of the g_m estimation to errors in the determination of the electron transport rate (J). However, some decrease in g_m, when estimated by the isotopic method, was also observed by Loreto *et al.* (1992), especially in *Xanthium strumarium*. Düring (2003) analysed the relationship between photosynthetic rate (A_N) and C_i measured simultaneously by chlorophyll fluorescence and gas exchange and observed

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a clear decline of g_m with increasing C_i. Centritto *et al.* (2003) showed that keeping salt-stressed leaves showing decreased g_m at low C_i for 1 h resulted in a restoration of control values for g_m. Flexas *et al.* (2007*a*) have provided the most detailed analysis yet of g_m variation in response to changes in CO_2 concentration. Six different C_3 species showed between 5-fold and 9-fold variations in g_m with changes in substomatal CO_2 concentration. The pattern of the g_m/C_i dependency was species-dependent, and g_m strongly declined at high C_i. Moreover, Flexas *et al.* (2007*a*) verified these CO_2 responses of g_m using the variable J method, the curve-fitting method, and the isotopic method under both photorespiratory and non-photorespiratory conditions (although the latter two only at 400, 1000, and 1500 μmol mol^{-1} CO_2 ambient concentrations). However, more recent studies have yielded contrasting results. For instance, Hassiotou *et al.* (2009) observed a CO_2-dependency of g_m in *Banksia* species using the fluorescence method, which has also been suggested by Yin *et al.* (2009) using the same method as well as a novel A_N/C_i curve-fitting approach in wheat. However, Tazoe *et al.* (2009), also working with wheat, did not observe any CO_2-dependency of g_m using the isotopic method.

Since all methods rely on certain assumptions, the simultaneous application of several techniques is useful in order to increase the reliability of g_m estimation (Pons *et al.*, 2009, this issue). Several authors have obtained similar values of g_m when comparing the isotopic and variable J methods (Warren *et al.*, 2004; Flexas *et al.*, 2006, 2007*a*), or the curve-fitting (Ethier and Livingston, 2004; Ethier *et al.*, 2006) and variable J methods (Flexas *et al.*, 2007*a*). Although all the methods share some assumptions—so that it is not surprising that they yield similar results—Warren (2006) pointed out that the isotopic method is the one sharing the fewest common assumptions with any of the other methods, which makes it a prime candidate for any study aiming to use multiple methods.

To the best of our knowledge, no comprehensive analysis of the CO_2-induced variation of mesophyll conductance by simultaneously using the variable J method and the isotopic method has been published. Therefore, the aim of the present work was the detailed evaluation of the g_m/CO_2 relationship by using two independent methods without overlapping assumptions. Also, potential interactions between stomatal and mesophyll conductances were analysed by comparison of control plants and plants whose stomatal conductance was reduced by the exogenous application of abscisic acid.

Materials and methods

Plant material and growth conditions

Plants of *Helianthus annuus* were individually grown from seeds in 3.0 l pots filled with perlite in a growth cabinet (Snijders Scientific, Jumo Imago F3000, Netherlands) with a 16/8 h day/night cycle. Day and night temperatures were 25 °C and 18 °C, respectively, and the relative humidity was 70%. Photosynthetically active photon flux density (*PPFD*) during the light period was held constant at 300–400 μmol m^{-2} s^{-1} depending on the height of the individual plants. Throughout the experiment a commercial nutrient solution (Kristalon Start, NU3 BV Vlaardingen, The Netherlands) was supplied every 2–3 d. 30 d after sowing, one-half of the plants was exogenously supplied with 20 μM abscisic acid (ABA; Sigma-Aldrich, Seelze, Germany) dissolved in 1 ml of methanol. An aliquot of pure methanol was added to the nutrient solution of the control plants.

Gas-exchange and chlorophyll fluorescence measurements

All measurements were made on young fully expanded leaves, 3 days after ABA treatment. Day respiration (R_d) and the apparent CO_2 photocompensation point (C_c^*) were determined according to the method of Laisk (1977). To estimate R_d and C_c^* simultaneously, A_N/C_i curves at five different *PPFD*s (50, 100, 150, 300, and 500 μmol m^{-2} s^{-1}) were determined for six different CO_2 levels ranging from 50–300 μmol CO_2 mol^{-1} with an open gas-exchange system, Li-6400 (Li-Cor Inc., Lincoln, NE, USA), equipped with a 2×3 cm broadleaf chamber and an integrated light source (Li-6400-02B; Li-Cor, Inc.). The point where the five A_N/C_i plots intersect represents C_c^* (x-axis) and R_d (y-axis). C_c^* was used as a proxy for the chloroplastic photocompensation point (Γ*), according to Warren (2006).

Calibration of the relationship between chlorophyll fluorescence and rates of electron transport was carried out using the Li-6400 with an integrated fluorescence chamber head (Li-6400-40; Li-Cor, Inc.). Photochemical efficiency of photosystem II (Φ_{PSII}) was calculated following the procedures of Genty *et al.* (1989) from steady-state fluorescence (F') and maximal fluorescence (F_m') during a light-saturating pulse:

$$\Phi_{PSII} = \frac{(F_m' - F')}{F_m'} \quad (1)$$

The electron transport rate J_f is then related to Φ_{PSII}:

$$J_f = \Phi_{PSII} PPFD \times \alpha \times \beta \quad (2)$$

where *PPFD* is the photosynthetically active photon flux density, α is the total leaf absorptance, and β represents the partitioning of absorbed quanta between photosystems II and I. The product α×β was determined, following Valentini *et al.* (1995), from the relationship between ϕ_{PSII} and ϕ_{co_2} (where $\phi_{co_2} = (A + R_d)/PPFD$) obtained by varying either ambient CO_2 concentration under non-photorespiratory conditions in an atmosphere containing less than 1% O_2. The R_d determined via the Laisk method (Laisk, 1977) was used to calculate ϕ_{co_2}.

Mesophyll conductance (g_m) was determined at different CO_2 concentrations from simultaneous measurements of A_N/C_i and J_f/C_i curves. CO_2-response curves were

performed in three light-adapted leaves of four ABA-treated and four control plants at *PPFD* of 800 μmol m^{-2} s^{-1} with a 10% fraction of blue light to maximize stomatal aperture. Leaf temperature was kept close to 23 °C and leaf-to-air vapour pressure deficit was approximately 0.75 kPa during all measurements. 20–30 min after clamping the leaf, once steady-state was reached, a CO_2-response experiment was performed. Gas exchange and chlorophyll fluorescence were first measured at 400 μmol mol^{-1} ambient CO_2 (C_a), then C_a was decreased stepwise to 50 μmol mol^{-1}, and after that returned to 400 μmol mol^{-1} to restore the original A_N value. Subsequently, C_a was increased stepwise to 1500 μmol mol^{-1}. C_a was changed in 14 steps and the time lag between two consecutive measurements at different C_a was 3–6 min.

Leakage of CO_2 into and out of the leaf cuvette was determined for the range of CO_2 concentrations used in this study with photosynthetically inactive leaves enclosed in the leaf chamber (obtained by heating the leaves until no variable chlorophyll fluorescence was observed) and used to correct the measured leaf fluxes (Flexas *et al.*, 2007*b*).

Estimation of g_m by gas exchange and chlorophyll fluorescence

Mesophyll conductance (g_m) was estimated by a single-point method according to Harley *et al.* (1992) as:

$$g_m = A_N / \left(C_i - \left(\Gamma^* \left(J_f + 8\left(A_N + R_d\right)\right)\right) / \left(J_f - 4\left(A_N + R_d\right)\right)\right) \quad (4)$$

where A_N and C_i are taken from gas-exchange measurements of CO_2-response curves and Γ^* and R_d were estimated using the method of Laisk (1977) (see above).

Estimation of g_m by instantaneous carbon isotope discrimination

Simultaneous measurements of gas-exchange parameters and carbon isotope composition were carried out with a Li-6400 leaf area meter. Owing to the small ^{13}C discrimination, a 6×2 cm narrow leaf chamber (Li-6400-11; Li-Cor, Inc.) was used to maximize the draw-dawn in CO_2 between chamber inlet and outlet. The leaf was illuminated with a laboratory-made LED red/blue light source. *PPFD* was 800 μmol m^{-2} s^{-1} with a 10% fraction of blue light. 20–30 min after clamping the leaf, when steady-state was reached, CO_2-response curves were performed. The chamber exhaust tube was connected to a gas sampling container with a Swagelok Y-piece connection. Under steady-state conditions, air exiting the cuvette was collected in the 100 ml container for 10 min. After that period, the chamber exhaust tube was reconnected to the instrument's match valve and the matching procedure was carried out before recording the actual gas-exchange parameters at the various CO_2 concentrations of the CO_2-response curve. The flow rate through the leaf chamber was 350 μmol air s^{-1}, and the air was collected for 10 min to ensure the air inside the gas-sampling container was exchanged 15–20 times. In order to collect a reference air sample, the same procedure was carried out with the empty cuvette.

Carbon isotope composition was estimated with a continuous flow stable isotope ratio mass spectrometer (DeltaPlus XL, ThermoFinnigan, Bremen, Germany) coupled via GasBenchII (ThermoFinnigan, Bremen, Germany) with PreCon (ThermoFinnigan, Bremen, Germany) ensuring CO_2 trapping in liquid N_2. This made it possible to estimate $\delta^{13}CO_2$ at low as well as high CO_2 concentrations.

Carbon isotope discrimination was calculated according to Evans *et al.* (1986) as:

$$\Delta^{13}C_{obs} = \left[\xi\left(\delta^{13}C_{out} - \delta^{13}C_{in}\right) / \left(1000 + \delta^{13}C_{out} - \xi\left(\delta^{13}C_{out} - \delta^{13}C_{in}\right)\right)\right] \quad (5)$$

where $\xi = C_{in}/(C_{in} - C_{out})$ and C_{in} and C_{out} are the CO_2 concentrations of the air entering and leaving the chamber, respectively. For $\delta^{13}C_{in}$ the value obtained from air leaving the empty chamber was used.

Mesophyll conductance values were determined by comparing predicted and observed discrimination values. Predicted discrimination (Δ_i) was calculated according to Evans *et al.* (1986) as

$$\Delta_i = a + (b - a) \times c_i / c_a \quad (6)$$

where a is the fractionation occurring due to diffusion in air (4.4‰), b is the net fractionation by Rubisco and phosphoenolpyruvate carboxylase (PEPC) (29‰), and C_i and C_a are the intercellular and ambient concentrations of CO_2, respectively.

Finally, g_m was calculated from equation 7 (Evans and von Caemmerer, 1996)

$$\Delta_i - \Delta^{13}C_{obs} = \left(29 - 1.8\right)\left(A_N / g_m\right) / c_a \quad (7)$$

where 1.8‰ is the discrimination due to dissolution and diffusion of CO_2 in water. Fractionation resulting from respiration and photorespiration was assumed to be negligible (Warren *et al.*, 2003; Flexas *et al.*, 2007*a*).

Results

A strong linear relationship (R^2=0.90) between ϕ_{co_2} and ϕ_{PSII} and J_{CO_2} at different CO_2 concentrations (measured in air containing less then 1% O_2 with control and ABA-treated plants) was found, indicating a constant non-assimilatory electron flow. The slope of this relationship was 9.13, in agreement with the range described for other species (Warren and Dreyer, 2006; Flexas *et al.*, 2007*a*; Warren, 2008*a*) and no significant differences were observed between treatments.

Using the methods of Laisk (1977) and Warren (2006), no difference in C_c^* between ABA-treated and control plants was found (38±4 and 39±4 μmol mol^{-1}, respectively). By contrast, R_d significantly differed between ABA-treated (0.9±0.1 μmol CO_2 m^{-2} s^{-1}) and control plants (2.0±0.5 μmol CO_2 m^{-2} s^{-1}) (Table 2).

At ambient CO_2, ABA-treated plants presented significantly lower g_s than control plants. A_N in ABA-treated plants was slightly lower than in control plants, but these differences were non-significant (Table 1). Consequently, they showed lower C_i and C_c (Table 1).

The response of net photosynthesis to substomatal CO_2 concentration (C_i) of control and ABA-treated plants of *Helianthus annuus* (Fig. 1A) shows the typical non-rectangular hyperbolic relationship with an initial, almost linear part followed by a near-constant part. The maximum rate of photosynthesis was approximately 37 μmol m^{-2} s^{-1}. Analysis of A_N/C_i curves and their parameters showed very slight non-significant differences between control and ABA-treated plants (Table 2). The linear electron transport rate (J_f) initially increased with C_i, peaked at 300 μmol mol^{-1}, and decreased thereafter (Fig. 1B), probably due to feedback limitation from the utilization of end-products (Sharkey *et al.*, 1988). No differences in the rate of linear electron transport between control and ABA-treated plants were found. A reduction of g_s over the entire C_i range was the only clear effect induced by ABA treatment (Fig. 1C). Stomatal conductance rose at low C_i in both control and ABA-treated plants, with maximum values of 0.43 and 0.28 mol m^{-2} s^{-1}, respectively, at C_i of 200 μmol mol^{-1}. Stomatal conductance was reduced to 38% of control in ABA-treated plants at 150–400 μmol mol^{-1} ambient CO_2 concentration. The maximum reduction (60%) was observed at high CO_2 concentrations.

The dependency of g_m on C_i by using two independent methods was evaluated by the variable J and isotopic methods. Data from simultaneous measurements of gas exchange and chlorophyll fluorescence were used to calculate g_m for most C_i values except for very low ones, where A_N was close to zero or negative. Non-linear proportionality between g_m and C_i was observed (Fig. 2A) with the shape of decline being exponential at C_i >200 μmol mol^{-1}. The initial part of the g_m/C_i relationship was also analysed using the isotopic method (Fig. 2B). Within the range of ambient CO_2 of 100–1000 μmol mol^{-1}, mesophyll conductance showed a pattern similar to the g_s/C_i dependency, with an initial increase at low CO_2 concentrations followed by a decline at high C_i. The isotopic method gave slightly different absolute values of g_m, but the pattern of the g_m/C_i relationship was the same.

Table 1. Mean values of rate of photosynthesis, A_N (μmol m^{-2} s^{-1}), stomatal conductance, g_s (mol m^{-2} s^{-1}), substomatal CO_2 concentration, C_i (μmol mol^{-1}), chloroplastic CO_2 concentration, C_c (μmol mol^{-1}), and mesophyll conductance, g_m (mol m^{-2} s^{-1}); at CO_2 400 μmol mol^{-1} for control and ABA-treated plants

	A_N	g_s	C_i	C_c	g_m
Control	28.9±2.1	0.45±0.06	318.8±7.2	240.3±21.9	0.41±0.16
ABA-treated	27.5±2.4	0.27±0.05	278.9±19.9	206.1±22.0	0.42±0.17

Discussion

Water and salt stresses often result in simultaneous decreases of g_s and g_m (Bongi and Loreto, 1989; Flexas *et al.*, 2002; Centritto *et al.*, 2003; Peeva and Cornic, 2009), and there is some indication that this could be related to ABA (Flexas *et al.* 2006, 2008). The simultaneity of these responses, together with the suggestion that g_s and g_m also show similar responses to light and CO_2 (Flexas *et al.*, 2007*a*; Hassiotou *et al.*, 2009; Yin *et al.*, 2009), have led to the hypothesis that g_s and g_m are intrinsically co-regulated (Flexas *et al.*, 2008; Peeva and Cornic, 2009). However, depression of g_m at low g_s would increase the shortage of CO_2 at the sites of carboxylation and thus exacerbate the CO_2 limitation to photosynthesis. Therefore, one may speculate that there might be an advantage in terms of carbon gain in enhancing g_m at low g_s, but, in that case, oscillation of photosynthesis may occur when falling C_i triggers stomatal opening (Šantrůček *et al.*, 2003).

Despite these antecedents, the effects of ABA on g_m are far from clear, and there are certainly some controversies regarding the effects of light and CO_2 on g_m (Tazoe *et al.*, 2009). The effects of ABA and CO_2 on g_m, and the co-regulation of g_s and g_m in sunflower are addressed here, using two independent methods, i.e. chlorophyll fluorescence and isotope discrimination.

The ABA treatment applied in the present study was sufficient to decrease g_s significantly over the entire range of C_i, but it did not induce significant changes in either A_N, g_m or the photosynthetic capacity of leaves at ambient CO_2. Therefore, the relationship between g_s and g_m strongly differed between control and ABA-treated plants (Fig. 3A), and the only difference observed between control and ABA-treated plants in A_N/C_i curves consisted of a general displacement of ABA-plants data towards lower C_i. This means that all values (for both control and ABA-treated plants) were close to the saturated part of the well-known curvilinear relationship between A_N and g_s, i.e. that at ambient CO_2 A_N in these plants was more limited by photosynthetic capacity than by CO_2 availability. This was expected since A_N/C_i curves were measured at a *PPFD* double that of the growing *PPFD* (see Materials and methods), and it is indeed consistent with the relatively low V_{cmax} presented by these plants (Table 2) and by the fact that, at ambient CO_2, they present C_i values that are close to the transition point between CO_2-limited and RuBP-limited photosynthesis (compare C_i values in Table 1 with A_N/C_i curves in Fig. 1). It may be argued that the application of exogenous ABA may induce patchy stomatal closure, therefore inducing errors in the estimation of C_i, and hence g_m, V_{cmax}, etc. (Terashima, 1992; Buckley *et al.*, 1997). However, although the reduction of g_s observed was substantial, g_s values in ABA-treated plants were still very high (i.e. higher than 0.25 mol m^{-2} s^{-1}) and they did not significantly affect A_N. Using chlorophyll fluorescence imaging, Flexas *et al.* (2006) showed in other herbaceous species that exogenous ABA did not induce patchy stomatal closure even when g_s dropped to much lower values than

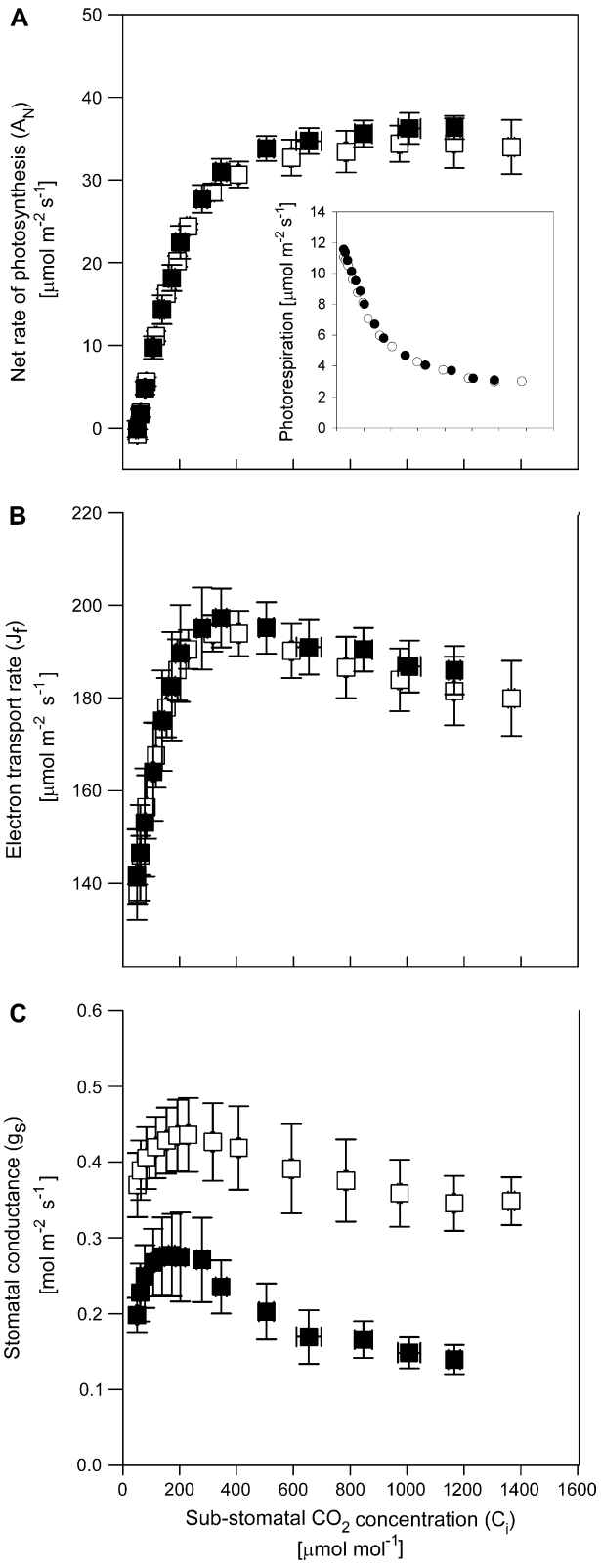

Fig. 1. CO_2 response of gas exchange and chlorophyll fluorescence parameters of control (open symbols) and ABA-treated (closed symbols) leaves of sunflower. (A) Response of net photosynthesis to substomatal CO_2 concentration, for parameters of mechanistic analysis see Table 1.; inset: rate of photorespiration estimated as R_p=1/12[*ETR*–4(A_N + R_d)] of control (open symbols) and ABA-treated plant (closed symbols). (B) Response of electron transport rate to substomatal CO_2 concentration. (C) Response of stomatal conductance to substomatal CO_2 concentration. Each point represents the mean of 12 replicates while error bars show standard deviations.

those here (i.e. lower than 0.05 mol m^{-2} s^{-1}), and it has been shown even in several heterobaric species that patchy stomatal closure does not induce a significant bias in C_i calculations until average g_s is lower than 0.03 mol m^{-2} s^{-1} (Buckley *et al.*, 1997; Flexas *et al.*, 2002). Moreover, the close similarity in the curvature of the A_N/C_i dependency itself has been taken as an indication for the absence of patchy stomatal closure in ABA-treated plants (Pospíšilová and Šantrůček, 1994, 1996; Grassi and Magnani, 2005). Therefore, since a homobaric species was used, and even the lower values of g_s induced here are *c.* 10-fold higher than those known to induce patchiness-related errors in C_i, we firmly believe that our C_i estimations are accurate.

Despite the lack of effect of ABA on g_m, both g_s and g_m were apparently responsive to CO_2, increasing from low C_i to a maximum at about 200 μmol CO_2 mol^{-1} air, and subsequently decreasing with increasing C_i (Fig. 1). Regarding stomatal conductance, this biphasic pattern is not the most common, but it has been already described for some species like *Eucalyptus grandis* (Leuning, 1995), *Chenopodium album* (Šantrůček and Sage, 1996), or *Xanthium strumarium* (Messinger *et al.*, 2006) and its inflection point related to the inflection between Rubisco-limited and electron transport-limited photosynthesis (Messinger *et al.*, 2006), which is in complete agreement with the data presented here (Fig. 1). Regarding g_m, the pattern fully agrees with that reported by Flexas *et al.* (2007*a*) for *Nicotiana tabacum* and Richter-110 grapevine (a hybrid of *Vitis berlandieri*×*rupestris)*. Remarkably, the two independent methods used showed a similar pattern of g_m dependency on C_i. The two methods rely on quite a few assumptions and technical difficulties (Pons *et al.*, 2009), which may to some extent cause these apparent changes of g_m with C_i. However, the only assumption shared by the two methods is the accuracy of the gas exchange measurements, including the incidence of patchiness discussed above. Therefore, the similarity of the patterns probably reflects the true behaviour of g_m in response to C_i. Still, a closer examination of the assumptions used and the results obtained may be worthy.

Harley's variable *J* method requires a proper balance between photosynthesis and photorespiration, and it is sensitive to the values of *J*, Γ* and, to a lesser extent, R_d used (Harley *et al.*, 1992; Loreto *et al.*, 1992; Pons *et al.*, 2009). The extent of photorespiration was calculated in the C_i region from 50–1500 μmol mol^{-1} from gas exchange and fluorescence data according to Valentini *et al.* (1995), indicating that photorespiration rates are still significant even at the highest CO_2 concentrations used (Fig. 1a, inset). Subtraction of the rate of photosynthesis measured in air

containing less then 1% O_2 (A) and 21% O_2 (A_N) yielded similar results (data not shown). Therefore, the main assumption underlining the use of Harley's variable J method was not violated over the range of C_i studied. Concerning J values, these were properly calibrated at low O_2, as recommended (Valentini *et al.*, 1995; Pons *et al.*, 2009). C_c^* was used as a proxy for Γ*, as in other studies, which has been proved not to affect g_m estimates significantly (Warren and Dreyer, 2006). Moreover, since the values obtained for both control and ABA-treated plants were similar, any deviation from the 'true' value would have resulted in proportionally similar effects in the estimation of g_m for both groups of plants. By contrast, R_d differed significantly between control and ABA-treated plants, but a sensitivity analysis using either one or another value of R_d in both groups of plants showed negligible differences in the estimates of g_m (data not shown). To account for the possible artefacts caused by these variables, Harley *et al.* (1992) established a criterion of reliability, which states that only data with values of dC_c/dA_N (i.e. the relative variation of chloroplast CO_2 concentration over the variation of photosynthesis) between 10 and 50 can be considered to be reliable. According to this criterion, in the present study data for C_i <200 μmol mol^{-1} and C_i > 800 μmol mol^{-1} were not reliable. Still, a substantial effect of CO_2 for a C_i range between these two extremes was observed, in agreement with Flexas *et al.* (2007*a*).

The plants used in the present study presented substantially high rates of photosynthesis, which places them at the limit of accuracy for the use of the Harley's variable J method (Evans and Loreto, 2000). By contrast, the isotope discrimination method works better with high photosynthesis rates (Evans and Loreto, 2000; Pons *et al.*, 2009). High photosynthesis brings a high draw-down in CO_2 concentration (and H_2O). This is an important factor to minimize the errors induced by the estimation of particular constants (R_d, Γ*) and variables (C_i, ξ). Moreover, in conjunction of using a larger leaf cuvette (12 cm^2, see Materials and methods), high photosynthesis minimizes the errors caused by leaf chamber leaks (Flexas *et al.*, 2007*b*), edge effects (Pons and Welschen, 2002). For the isotopic method, a high ξ parameter was obtained during these experiments, and thus the difference in $\delta^{13}CO_2$ in and $\delta^{13}CO_2$ out was high. Therefore, the error of single $\delta^{13}CO_2$ estimations was small and thus the subsequent estimation of $\Delta^{13}CO_2$ was very precise.

In summary, the precautions taken when using each of the two methods, as well as the good agreement between both (Fig. 3B) suggest that the data were sufficiently reliable. The fact that the isotopic estimates of g_m gave slightly higher values than the single point variable J method (Fig. 2), is in contrast to the observations obtained by Loreto *et al.* (1992) but in agreement with those of Flexas *et al.* (2007*a*, *c*). These discrepancies could be due to numerous reasons, such as slightly biased Γ* estimations in the Harley method, ignoring ^{13}C fractionation during respiration and photorespiration, or

Table 2. Mean values of mechanistic analysis of A_N/C_i curves of control and ABA-treated plants

V_{cmax} (μmol m^{-2} s^{-1}), maximum carboxylation capacity; J_{max} (μmol m^{-2} s^{-1}), maximum capacity for electron transport rate; Γ* (μmol mol^{-1}), CO_2 compensation concentration in the absence of mitochondrial respiration; R_d (μmol m^{-2} s^{-1}), day respiration at the apparent CO_2 photocompensation point (C_c^*). Values are averages ±SE of 12 replicates.

	V_{cmax}	J_{max}	Γ*	R_d
Control	56.7±6.7	57.3±7.2	38±4	2.0±0.5
ABA-treated	62.2±3.3	65.3±2.3	39±4	0.9±0.1

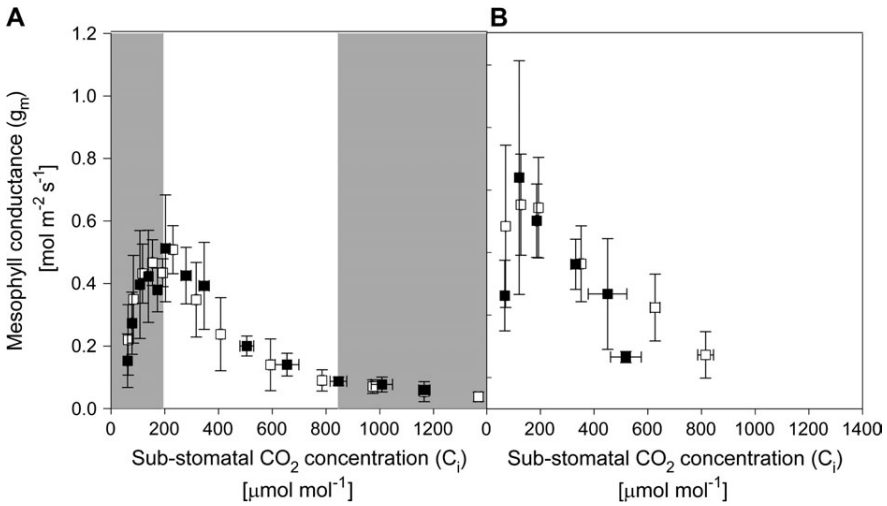

Fig. 2. Response of mesophyll conductance to substomatal CO_2 concentration estimated by using two independent methods: (A) Variable J method according to Harley *et al.* (1992). Values are means ±SD of 12 replicates; the unshaded region indicates g_m data with a dC_c/dA_N between 10 and 50, which are reliable according to Harley *et al.* (1992). (B) Isotopic method according to Evans *et al.* (1986). Values are means ±SD of six replicates. Open symbols represent controls while closed symbols represent ABA-treated plants.

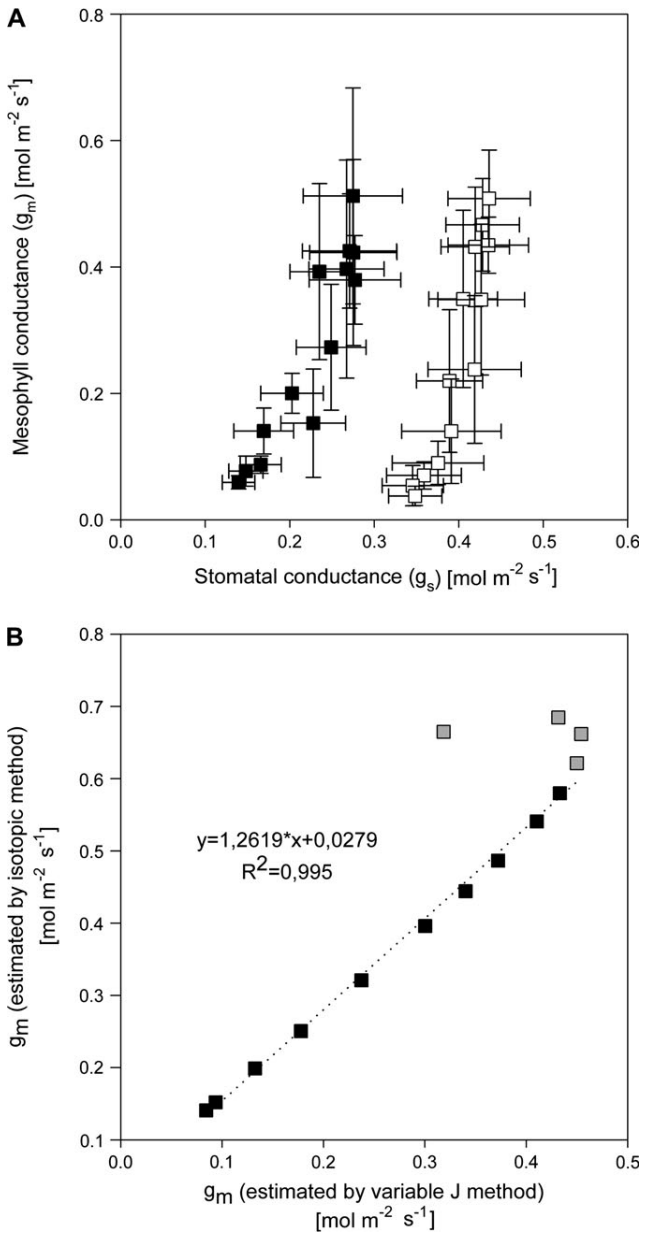

Fig. 3. (A) Correlation between mesophyll conductance (g_m) and stomatal conductance (g_s) in control (open symbols) and ABA-treated plants (closed symbols). (B) Correlation between mesophyll conductance (g_m) estimated by two independent methods: the variable *J* method (Harley *et al.*, 1992) and the isotopic method (Evans *et al.*, 1986) for control and ABA-treated plants in the range of substomatal CO_2 concentration of 100–800 μmol mol^{-1}. Grey symbols represent values of g_m that did not satisfy Harley *et al.*'s criterion of reliability (cf. Fig. 2).

the precise value for discrimination by Rubisco (Pons *et al.*, 2009). Nevertheless, despite some disagreement in the absolute values, the comparison of these two methods revealed a very high correlation (R^2=0.99), except for g_m estimations at C_i <200 μmol mol^{-1} (Fig. 3). These data are situated out of the region where dC_c/dA_N was between 10 and 50 and thus did not satisfy the criterion of reliability defined by Harley *et al.* (1992).

Therefore, the present results largely confirm, using two independent methods, that g_m is truly responsive to CO_2 in the short term (Flexas *et al.*, 2007*a*; Hassiotou *et al.*, 2009; Yin *et al.*, 2009), even for a species, wheat, in which the absence of response has been described (Tazoe *et al.*, 2009). The similarity of the patterns of response of both g_s and g_m to C_i could indicate that these two major CO_2 transport-limiting factors are tightly coupled. However, the reduction of g_s in ABA-treated plants did not have an effect on g_m, which resulted in different slopes for the g_s–g_m relationship, indicating some degree of independence of these two variables. Therefore, both g_s and g_m seem to respond similarly to variations in C_i, but the link between the two variables is flexible.

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Acclimation of Norway spruce photosynthetic apparatus to the combined effect of high irradiance and temperature

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Xanthophyll cycle

ABSTRACT

Diurnal courses of photosynthetic gas exchange parameters, chlorophyll *a* fluorescence characteristics and the de-epoxidation state of the xanthophyll cycle pigments (DEPS) were measured during the gradual acclimation of 4-year-old Norway spruce seedlings to different photosynthetic photon flux density (PPFD) and air temperature (T_{air}) regimes, simulating cloudy days with moderate T_{air} (LI, maximum PPFD 300 μmol m^{-2} s^{-1}, T_{air} range 15–25 °C), sunny days with moderate T_{air} (HI, maximum PPFD 1000 μmol m^{-2} s^{-1}, T_{air} range 15–25 °C) and hot sunny days (HI-HT, maximum PPFD 1000 μmol m^{-2} s^{-1}, T_{air} range 20–35 °C). The plants were acclimated inside a growth chamber and each acclimation regime lasted for 13 d. Acclimation to HI conditions led to a strong depression of the net CO_2 assimilation rates (A_N), particularly during noon and afternoon periods. Exposure to the HI-HT regime led to a further decrease of A_N even during the morning period. Insufficient stomatal conductance was found to be the main reason for depressed A_N under HI and HI-HT conditions. Only slight changes of the maximum photosystem II (PSII) photochemical efficiency (F_V/F_M), in the range of 0.78–0.82, supported the resistance of the Norway spruce photosynthetic apparatus against PSII photoinhibition during acclimation to both HI and HI-HT conditions. The HI plants showed increased content of xanthophyll cycle pigments (VAZ) and enhanced efficiency of thermal energy dissipation within PSII (D) that closely correlated with the increased DEPS. In contrast, acclimation to the HI-HT regime resulted in a slight reduction of VAZ content and significantly diminished D and DEPS values during the entire day in comparison with HI plants. These results indicate a minor role of the xanthophyll cycle-mediated thermal dissipation in PSII photoprotection under elevated temperatures. The different contributions of the thermal dissipation and non-assimilatory electron transport pathways in PSII photoprotection during acclimation of the Norway spruce photosynthetic apparatus to excess irradiance and heat stresses are discussed.

Introduction

Under natural conditions, the assimilatory apparatus is exposed to environmental conditions (high solar irradiance, elevated air temperature, and drought) that often result in a midday depression of photosynthetic CO_2 assimilation (Faria et al., 1996; Muraoka et al., 2000; Špunda et al., 2005). The depression of net CO_2 assimilation usually ceases during the afternoon and the two distinct maxima of the CO_2 assimilation rate occur in the morning and afternoon. Under more severe stress conditions, photosynthetic activity increases with irradiance only in the morning and the depression of CO_2 assimilation persists until the late afternoon (González-Rodríguez et al., 2002; Franco et al., 2007). The main physiological processes responsible for the midday depression are stomatal closure and/or photosystem II (PSII) photoinhibition (Muraoka et al., 2000).

In sunny conditions, insufficient soil moisture and a high vapor potential deficit may lead to a decrease of stomatal conductance (G_S) to CO_2 diffusion (Panek and Goldstein, 2001; Urban et al., 2007) followed by decreases of CO_2 concentrations at both the intercellular (C_i) and chloroplast levels, causing the CO_2 uptake to decline. In addition, Bota et al. (2004) concluded that the impairment of Rubisco activity and ribulose-1,5-bisphosphate

Abbreviations: A_N, net CO_2 assimilation rate; Car *x*+*c*, total carotenoids; Chl, chlorophyll; *D*, efficiency of thermal energy dissipation; DEPS, de-epoxidation state of the xanthophyll cycle pigments; ETR, photosystem II electron transport rate; F_V/F_M, maximal photochemical efficiency of photosystem II; G_S, stomatal conductance; HI, high irradiance acclimation regime; HI-HT, high irradiance and high air temperature acclimation regime; LI, low irradiance acclimation regime; P, actual photochemical efficiency of photosystem II; PPFD, photosynthetic photon flux density; PSII, photosystem II; VAZ, pool of xanthophyll cycle pigments (violaxanthin+antheraxanthin+zeaxanthin)

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content limit photosynthesis under severe drought stress. As a result, an assimilation optimum, given by the synergic influence of the photosynthetic photon flux density (PPFD) and other environmental factors, has been observed (Muraoka et al., 2000).

PSII photoinhibition can be of a dynamic and chronic nature (Werner et al., 2002). The first involves a rapidly reversible down-regulation of PSII photochemical efficiency observed at midday regulated by thermal dissipation of absorbed excitation energy. Chronic PSII photoinhibition is associated with slowly reversible energy dissipating mechanisms, repair processes or with permanent damage to the photosynthetic apparatus, and is reflected by a sustainable decrease in pre-dawn maximal PSII photochemical efficiency (maximal photochemical efficiency of photosystem II – F_V/F_M) (Werner et al., 2002; Williams et al., 2003).

Thus, thermal dissipation is one of the crucial photoprotective mechanisms preventing photo-oxidative damage to the photosynthetic apparatus under stressful conditions during midday (Iio et al., 2004; Franco et al., 2007). The major part of non-radiative dissipation occurs within light-harvesting complexes of PSII, thereby reducing the over-excitation of the PSII reaction centers. Under conditions of low C_i due to stomatal closure at midday, a high degree of thermal dissipation is needed to achieve a balance between the electron flow and reduction potential for carboxylation and oxygenation. The mechanism of thermal dissipation is associated with the reversible conversion of violaxanthin to antheraxanthin and zeaxanthin in the xanthophyll cycle (Štroch et al., 2004; Horton et al., 2008). The thermal dissipation efficiency and de-epoxidation state of the xanthophyll cycle pigments (DEPS) in sun-exposed leaves exhibit a similar pattern of the daily course on clear summer days. They increase gradually with the rising solar irradiance in the morning, reach their maxima around midday, and then decrease in the afternoon (Schindler and Lichtenthaler, 1996; Demmig-Adams et al., 1999). The other typical feature of the xanthophyll cycle activity in plants under environmental stress conditions is an overnight retention of de-epoxidized xanthophylls, usually associated with sustained reduction of F_V/F_M (Williams et al., 2003; García-Plazaola et al., 2008).

During midday, low C_i and elevated temperature enhance the affinity of Rubisco for O_2. As a consequence, the photorespiration rate increases. An enhanced contribution of photorespiratory CO_2 production to the total electron flow is considered another important photoprotective mechanism preventing an over-reduction of the photosynthetic electron transport chain (Valentini et al., 1995; Muraoka et al., 2000; Franco and Lüttge, 2002).

An elevated temperature during the midday period can also exert direct negative effects on photosynthetic CO_2 assimilation of high-temperature-sensitive species. In particular, the thermal lability of Rubisco activase can lead to a Rubisco activity reduction at temperatures normally considered as optimal (Salvucci and Crafts-Brandner, 2004; Sharkey, 2005). The temperature optimum of the electron transport rate is usually higher than that of CO_2 assimilation (Yamori et al., 2008). However, at super-optimum temperatures, the inhibition of linear electron transport occurs at the expense of cyclic electron transport pathways that lead to stimulation of ΔpH-dependent thermal dissipation (Kramer et al., 2004; Sage and Kubien, 2007). Thus, usually both enhanced photorespiration and thermal dissipation contribute to PSII photoprotection under combined exposure to high irradiance and temperature (Franco et al., 2007).

Thermal acclimation abilities differ considerably among species (Sage and Kubien, 2007) and even among congeneric plants originating in different latitudes and/or altitudes (Atkin et al., 2006; Weston and Bauerle, 2007). Systematic studies on acclimation to increased temperature (particularly to the periods of extremely high temperatures) in coniferous species, representing dominant tree species of boreal and temperate forests, are relatively scarce. Recently, Way and Sage (2008a, b) documented that the development of black spruces at a constantly elevated growth temperature (30/22 °C day/night temperature) led to a reduction of net photosynthetic rates at their growth temperature in comparison with plants acclimated to a 22/16 °C temperature regime. To the best of our knowledge, there has been no comprehensive study on the adaptability of the Norway spruce assimilatory apparatus to combined high irradiance and high temperature conditions.

In the present study Norway spruce seedlings were exposed to defined conditions simulating daily courses of microclimatic parameters during cloudy and sunny days with moderate temperatures and during hot sunny days. With regard to sensitivity of the spruce photosynthetic apparatus to high growth temperature (Way and Sage, 2008a), we expected that high temperature would be a synergic stress factor resulting in a persistent depression of photosynthetic CO_2 assimilation during the prevailing part of the photoperiod. The aims of this study were (1) to analyze the acclimation ability of the assimilatory apparatus of Norway spruce to elevated irradiance and temperature during simulated hot sunny days, and (2) to test the hypothesis that combined exposure of spruce seedlings to elevated irradiance and temperature would result in an increased demand on photoprotective processes, such as a xanthophyll cycle-dependent thermal energy dissipation and photorespiration.

Materials and methods

Plant material and experimental design

Four-year-old seedlings of Norway spruce (*Picea abies* [L.] Karst.) were gradually acclimated to three consecutive environmental conditions inside a growth chamber (HB 1014, Bioline-Heraeus, Germany). The duration of each acclimation regime was 13 d. The daily courses of microclimatic parameters for the individual acclimation regimes were adjusted as shown in Fig. 1. The seedlings were initially acclimated to low photosynthetic photon flux density (PPFD) together with moderate air temperature (T_{air}) (low irradiance acclimation regime (LI regime); maximum PPFD at "midday" 300 μmol m^{-2} s^{-1}, T_{air} in the range 15–25 °C). Then, PPFD was increased (maximum PPFD 1000 μmol m^{-2} s^{-1}), whereas the daily course of T_{air} remained unchanged (high irradiance acclimation regime – HI regime). Finally, only T_{air} over the entire day was increased (high irradiance and high air temperature acclimation regime (HI-HT regime); T_{air} range 20–35 °C). The plants were sufficiently watered and the daily course of relative air humidity was adjusted in the range of 50–65% for all regimes to avoid drought stress. Due to horizontal heterogeneity of illumination inside the growth chamber, the incident PPFDs on the measured shoots varied up to 10% in comparison with the estimated average PPFDs, which were 10, 100, 160, 230 and 300 μmol m^{-2} s^{-1} for LI plants and 25, 310, 590, 850 and 1000 μmol m^{-2} s^{-1} for HI plants (Fig. 1). All measurements were carried out on current needles and shoots from the two uppermost whorls of the crown, where the incident PPFD was at the required level (see Fig. 1).

Gas-exchange measurements

Measurements of the steady-state net CO_2 assimilation rate (A_N) and stomatal conductance (G_S) were carried out on attached shoots under the given acclimation conditions (PPFD, T_{air} and CO_2

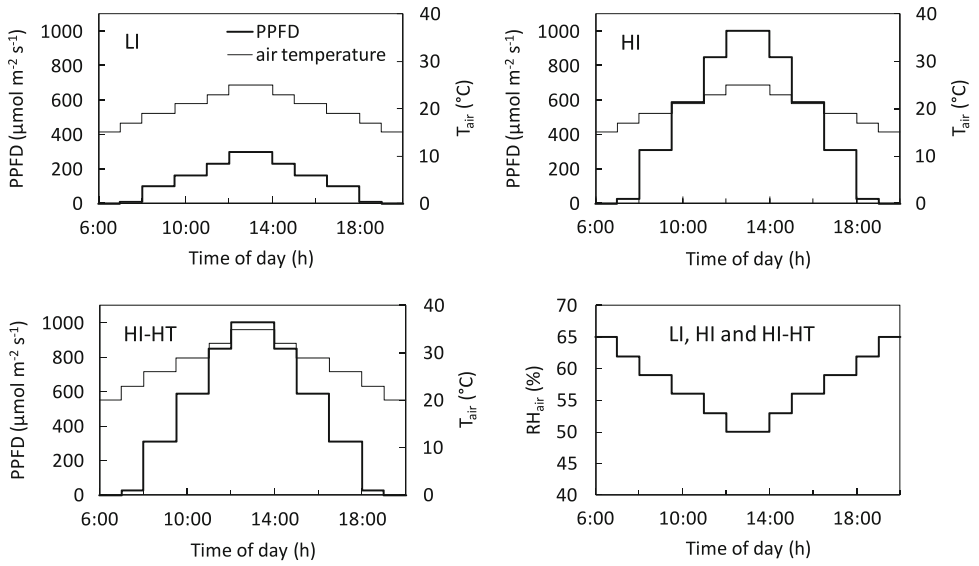

Fig. 1. Diurnal courses of photosynthetically active photon flux density (PPFD), air temperature (T_{air}) and relative air humidity (RH_{air}) for LI, HI and HI-HT acclimation regimes in a growth chamber. PPFD values represent PPFD incident on spruce needles used for measurements and sampling. LI, low PPFD with moderate T_{air}; HI, high PPFD with moderate T_{air}; HI-HT, high PPFD with high T_{air}.

concentration) inside the growth chamber using an open gas-exchange system CIRAS-2 equipped with PLC5 (C) Conifer Leaf Cuvette (PP Systems, UK). The measurements were carried out 20 min after the change in PPFD and T_{air} and 5–10 min after placing the shoot inside the leaf cuvette once the steady-state was reached. A_N and G_S were determined per shoot projection area, estimated using a flat bed scanner and *Cernota* software developed by Kalina and Slovák (2004).

Chlorophyll a fluorescence measurements

Chlorophyll *a* (Chl *a*) fluorescence was measured on detached needles using a pulse amplitude-modulated fluorometer (PAM 101/103, Heinz Walz, Effeltrich, Germany) equipped with the emitter-detector unit 101-ED. The maximal photochemical efficiency of PSII, $F_V/F_M=(F_M-F_0)/F_M$ was determined by the end of the night period, i.e. after 12 h of darkness. F_0 and F_M are the minimal and maximal fluorescence levels under dark-adaptation, respectively. The needles taken for the measurements of the daily courses of Chl *a* fluorescence parameters were detached from the shoots after at least a 20-min exposure to the given acclimation conditions in the growth chamber. Then, the needles were immediately illuminated by a KL 1500 halogen lamp (Schott, Mainz, Germany) with the actinic light corresponding to the actual acclimation PPFDs (i.e. 100, 160 and 300 µmol m^{-2} s^{-1} for LI plants and 310, 590 and 1000 µmol m^{-2} s^{-1} for HI plants). At the steady-state of fluorescence (F) the maximum fluorescence ($F_{M'}$) was determined. The actinic light was subsequently switched off and the lowest fluorescence level during 5 s darkness following illumination was regarded as $F_{0'}$-level. For F_M and $F_{M'}$ determinations, the saturating "white light" pulses of 0.8 s duration and incident PPFD of approximately 5000 µmol m^{-2} s^{-1} were applied using another KL 1500 halogen lamp. After the measurements, the needles were immediately frozen at the temperature of liquid nitrogen (77 K) for chromatographic analysis of xanthophyll cycle activity. Diurnal courses of the following fluorescence parameters were determined: the fraction of absorbed excitation energy utilized in PSII photochemistry (actual photochemical efficiency of photosystem II–P), $P=(F_{M'}-F)/F_{M'}$ and dissipated thermally (efficiency of thermal energy dissipation – D), $D=1-(F_{M'}-F_{0'})/F_{M'}$ (Demmig-Adams et al., 1996), PSII electron transport rate ETR=$P\times$ incident PPFD $\times$ 0.8 $\times$ 0.5 (Genty et al., 1989), where 0.8 is the assumed needle absorptance and 0.5 is a factor that assumes equal distribution of excitation energy between PSI and PSII (Krall and Edwards, 1992).

Photosynthetic pigment analysis

The needles sampled at the end of the night period, i.e. after 12 h of darkness, were used for the determination of the ratios of Chl *a* to Chl *b* (Chl *a*/*b*) and of total chlorophylls to total carotenoids (Chl *a*+*b*/Car *x*+*c*), and for determination of the content of the pool of xanthophyll cycle pigments (violaxanthin+antheraxanthin+zeaxanthin; VAZ). Chl *a*/*b* and Chl *a*+*b*/Car *x*+*c* were estimated spectrophotometrically (UV/VIS 550, Unicam, England) from pigment extracts in 80% acetone with a small amount of $MgCO_3$ according to Lichtenthaler (1987). The VAZ content expressed per projected needle area was estimated via gradient reversed-phase high-performance liquid chromatography (TSP Analytical, USA) according to Färber and Jahns (1998) with a minor modification (Kurasová et al., 2003). The details have been described in our previous work (Štroch et al., 2008a). To examine the daily courses of the xanthophyll cycle activity, the de-epoxidation state of the xanthophyll cycle pigments expressed as the ratio of de-epoxidized xanthophylls (*Z*+*A*) to VAZ pool (DEPS) was determined using the samples from fluorescence measurements.

Statistical analysis

Statistical differences between the means were determined using a two-sample *F*-test for variances followed by a Student's *t*-test at three levels of significance (0.001, 0.01 and 0.05). Based on the results of the *F*-test, the *t*-test, assuming either equal or unequal variances, was used.

Results and discussion

Gas-exchange characteristics: acclimation responses and daily courses

The net CO_2 assimilation rate (A_N) in low irradiance acclimation regime (LI)-acclimated spruce plants showed a slight decrease at the maximal PPFD at midday (around 300 μmol m^{-2} s^{-1}) and lower values (by 22–42%) in the afternoon in comparison with the morning values at the corresponding PPFDs (Fig. 2A). The first day under high irradiance acclimation regime (HI) conditions was characterized by a significant increase of A_N ($P < 0.01$) in the early morning and a higher depression at midday compared to LI plants (Fig. 2C). The inhibition of A_N during midday and afternoon was markedly enhanced after a 13-d acclimation to the HI regime (Fig. 2A). A_N at midday was reduced by 61% compared to the morning A_N maximum, which was reached around PPFD of 600 μmol m^{-2} s^{-1}. Compared to LI spruce, A_N values after HI acclimation were also significantly lower in the morning hours ($P < 0.01$ at 10:15 data point, $P < 0.001$ at 11:30 data point), despite an incident PPFD that was more than three-fold higher. Thus, light use efficiency (determined as A_N/PPFD for the individual PPFDs) after HI acclimation was markedly reduced during a day (by about 70–90%). High irradiance and high air temperature acclimation regime (HI-HT) treatment for the next 13 d resulted in a further significant A_N depression in the morning ($P < 0.05$ at 10:15 data point, $P < 0.001$ at 11:30 data point) and persistently low A_N values during midday and afternoon (Fig. 2A). The progressive inhibition of A_N in plants acclimated to HI and combined HI-HT conditions was accompanied by a decreased G_S (Fig. 2B and D). Moreover, the diurnal changes of G_S were qualitatively similar to those of the A_N for plants acclimated to all of the regimes. The same A_N–G_S dependence for all acclimation regimes is shown in Fig. 3.

The aim of this study was to assess the role of photosynthetic and photoprotective processes adjustments during acclimation to high irradiance and the combined action of high irradiance and elevated temperature. As the acclimation of photosynthetic activity depends considerably on the sink for assimilates (Adams et al., 2002; Öquist and Huner, 2003), the experiments were performed during late autumn, when the sink strength was greatly reduced. Thus, we attempted to induce the conditions that should lead to a minimum CO_2 assimilation adjustment and, consequently, to considerably enhanced demands on photoprotective processes, in order to explore their limits.

In agreement with our previous findings (Štroch et al., 2008a) and general hypothesis on the acclimation strategy of conifers (Öquist and Huner, 2003; Demmig-Adams and Adams, 2006), A_N was reduced after HI acclimation, more noticeably during the noon and afternoon periods (Fig. 2A). The severity of midday depression of A_N was documented by the 90% reduction of light use efficiency at the maximum PPFD period in comparison with LI plants. Moreover, acclimation to HI-HT regime led to a further reduction of the CO_2 assimilation rate over the entire day (Fig. 2A). The main difference in the daily A_N course in comparison with HI-treated plants was the pronounced decline of A_N during the morning period, already at a PPFD around 850 μmol m^{-2} s^{-1} and 32 °C. As T_{air} was 9 °C higher for the HI-HT regime in comparison with the HI regime during that period of the simulated day (Fig. 1), this supports the inability of the conifers of the temperate and boreal forests to adjust their photosynthetic capacity to elevated temperatures (Way and Sage, 2008a, b). It has been shown that the thermal lability of Rubisco activase can lead

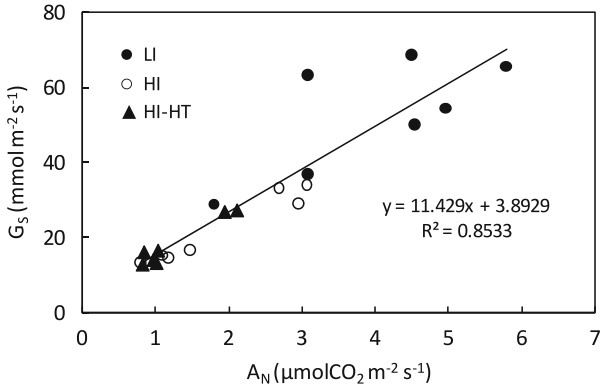

Fig. 3. Net CO_2 assimilation rate (A_N) in relation to stomatal conductance (G_S) for the Norway spruce acclimated to LI, HI and HI-HT conditions. Means from six samples obtained from measurements of daily courses of A_N and G_S are presented. Data were taken from Fig. 2A and B. Linear regression line with the coefficient of determination (R^2) is shown.

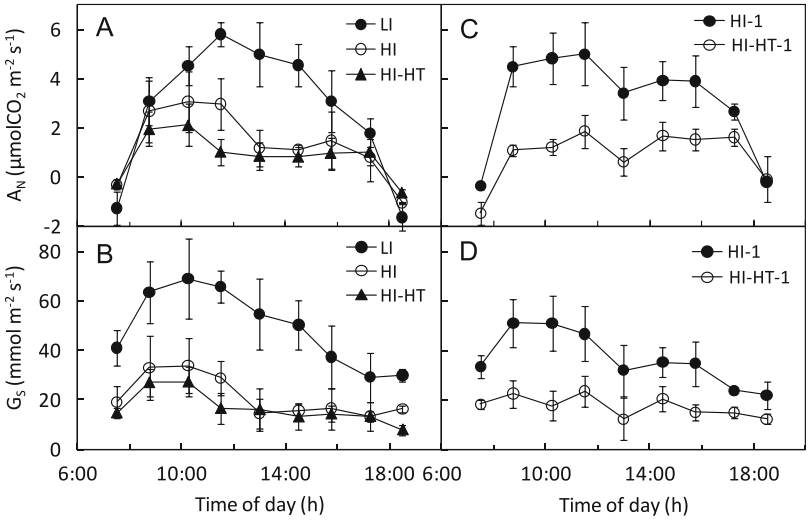

Fig. 2. Diurnal courses of (A, C) net CO_2 assimilation rate (A_N) and (B, D) stomatal conductance (G_S). The measurements were carried out on the attached Norway spruce shoots with current-year needles (A, B) at the end of the particular acclimation treatment (13th day of acclimation to LI, HI and HI-HT conditions) and (C, D) on the first day following transition from LI to HI acclimation regime (HI-1) and from HI to HI-HT regime (HI-HT-1). $n = 6 \pm$ S.D.

to the reduction of Rubisco activity under moderate high-temperature stress (Salvucci and Crafts-Brandner, 2004). However, the A_N–G_S relationship was not altered for seedlings exposed to combined HI and HT stress (Fig. 3), indicating insufficient stomatal opening as the main reason for reduced A_N.

Amiard et al. (2005) demonstrated that the acclimation of photosynthetic capacity in fully expanded, mature leaves to the light environment depends, among other factors, on the mechanism loading assimilates into the phloem. Although both apoplastic and symplastic loaders exhibited an increase of photosynthetic capacity of plants transferred from a LI to HI environment, it was less pronounced in symplastic loaders. A less efficient symplastic pathway of phloem loading leads to accumulation of non-structural carbohydrates in leaves that may result in a down-regulation of photosynthesis (Körner et al., 1995). In Norway spruce, phloem loading was suggested to proceed primarily via the symplastic pathway (Blechschmidt-Schneider, 1990; Blechschmidt-Schneider et al., 1997). Thus, the phloem transport capacity may represent another important factor leading to the down-regulation observed for A_N in HI-acclimated plants. However, the sensitivity of phloem loading processes to elevated temperatures does not seem to contribute significantly to the differences in the A_N diurnal course observed for HI-HT-acclimated shoots in comparison with HI plants.

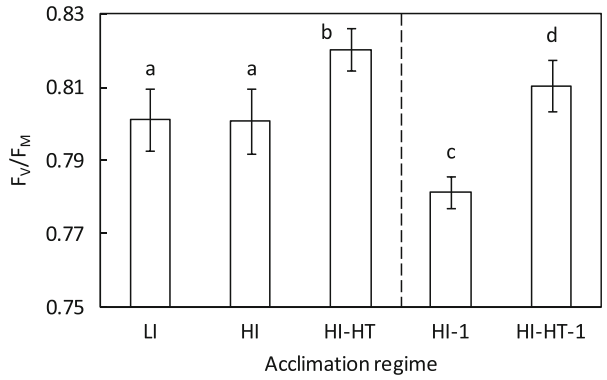

Fig. 4. Maximal photochemical efficiency of photosystem II (F_V/F_M) for the Norway spruce needles during acclimation to HI and HI-HT conditions. F_V/F_M was determined by the end of the night period, i.e. after 11–12 h of dark-adaptation on the 13th day of acclimation to the given treatment (LI, HI, HI-HT) and after the first day under HI and HI-HT conditions (HI-1, HI-HT-1). Data followed by the same letter indicate non-significant differences ($P > 0.05$; Student's t-test). n=6–10 ± S.D.

Changes of PSII functional state upon acclimation to HI and HI-HT regime

The pronounced inhibition of A_N observed after the acclimation to both HI and HI-HT conditions was not accompanied by PSII photoinhibition, estimated by the maximal PSII photochemical efficiency (F_V/F_M; Fig. 4) that was determined by the end of the night period, i.e. after 12 h of darkness. After the first day under HI conditions, F_V/F_M decreased from 0.801, observed in LI-acclimated plants, to 0.781. F_V/F_M then recovered, and after acclimation to the HI regime, it reached the initial state observed prior to the HI treatment. Transient PSII down-regulation is a typical response to elevated acclimation irradiance (Parker and Mohammed, 2000; Štroch et al., 2008b). The decline of F_V/F_M was observed despite high A_N values during a day (Fig. 1C). It should be noted that the recorded chlorophyll *a* (Chl *a*) fluorescence emission originates from a fraction of chloroplasts at the upper HI-exposed side of needles, whereas A_N reflects photosynthetic activity of the total pool of chloroplasts. It seems probable that only the photosynthetic apparatus of the chloroplasts near the upper epidermis layer was photoinhibited, while the chloroplasts in the lower mesophyll cells remained photosynthetically active. The importance of this methodological aspect in the interpretation of the mutual relation between gas-exchange and Chl fluorescence

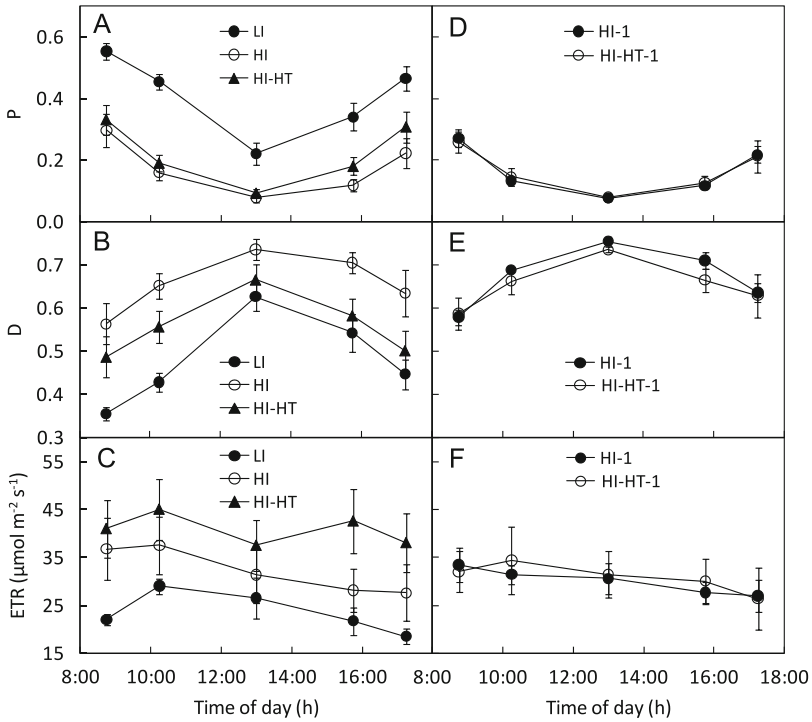

Fig. 5. Diurnal courses of (A, D) the fraction of absorbed excitation energy utilized in photosystem II photochemistry (*P*), (B, E) dissipated thermally (*D*) and (C, F) photosystem II electron transport rate (ETR). The measurements were carried out (A–C) at the end of the particular acclimation treatment (13th day of acclimation to LI, HI and HI-HT conditions) and (D–F) on the first day after transition from LI to HI acclimation regime (HI-1) and from HI to HI-HT regime (HI-HT-1). n=6–10 ± S.D.

measurements was documented in Schindler and Lichtenthaler (1996).

Surprisingly, exposure of spruce plants to elevated T_{air} led to a significant increase ($P < 0.001$) in F_V/F_M, reaching a value of 0.820 after the acclimation to the HI-HT regime. This clearly shows that, in our case, an increased acclimation temperature did not represent an additional stress factor to HI itself, with respect to the PSII functional state.

Since permanent PSII photoinhibition was not observed under any of the acclimation conditions, efficient regulation of the utilization of excess absorbed light energy in photochemical and/or non-photochemical de-excitation processes had to be ensured. Following the approach of Demmig-Adams et al. (1996), the allocation of light energy absorbed in PSII to photosynthetic electron transport and thermal energy dissipation was evaluated by the fluorescence parameters *P* and *D*, respectively. For LI-acclimated plants, PSII photochemical efficiency (*P*) markedly decreased with increasing PPFD in the morning (Fig. 5A). At maximal PPFD, *P* declined to 0.222. In the afternoon, *P* values were significantly lower ($P < 0.001$) compared to the corresponding morning values at the same PPFDs. The first day under HI conditions was characterized by a marked decline of the fraction of absorbed light energy utilized in PSII photochemistry throughout the whole day (Fig. 5D). The same diurnal pattern of *P* remained after HI acclimation and during the first day under the HI-HT regime, but acclimation to the HI-HT regime resulted in an increase of *P*, mainly in the afternoon. As a result, in contrast to the LI and HI acclimation regimes, the daily course of *P* after HI-HT treatment showed a symmetrical pattern, i.e. nearly the same *P* values at the corresponding PPFDs during the morning and afternoon periods.

We observed an asymmetrical daily course of the efficiency of thermal dissipation (*D*) for LI-acclimated plants with significantly higher ($P < 0.001$) *D* values during the afternoon, an immediate increase of *D* under HI conditions to values that persisted for the entire HI acclimation period, and a clear decrease of *D* during the entire day after HI-HT acclimation (Fig. 5B and E). For HI-HT-acclimated plants, we found a symmetrical daily course of *D*, with its afternoon values close to *D* in LI-acclimated plants, even though incident PPFD was more than three times higher under HI-HT conditions. Thus, daily courses of the utilization of absorbed light energy in PSII confirmed a positive effect of elevated acclimation temperature on the capacity of PSII photochemical de-excitation, resulting in a decreased demand on thermal energy dissipation. This result is quite surprising, as the pronounced reduction of CO_2 assimilation at high irradiances and elevated temperatures is usually accompanied by an increased efficiency of thermal dissipation (Franco et al., 2007; Weston and Bauerle, 2007).

After the acclimation to the HI-HT regime, the relative PSII electron transport rate (ETR; Fig. 5C and F) increased compared to previous LI and HI regimes, despite the lowest A_N (Fig. 2A). This indicates an enhanced contribution of non-assimilatory electron transport pathways to the total electron flux. We hypothesize that the enhancement of non-assimilatory pathways was the dominating protective process leading to the optimal function of PSII, and that it might lower the demand for thermal energy dissipation in HI-HT needles in comparison with HI needles (Fig. 5B).

Response of pigment composition and xanthophyll cycle activity to HI and HI-HT treatment

The response of pigment composition in spruce plants upon acclimation to the HI regime corresponds to a typical reaction of plants exposed to elevated acclimation irradiance (Kurasová et al., 2002). The Chl *a*/*b* ratio has often been used as an indirect indicator of the size of PSII light-harvesting complexes (LHCII) (Lichtenthaler and Babani, 2004). The increase of Chl *a*/*b* by 6.1% after HI acclimation indicates a moderate LHCII reduction (Fig. 6A) in comparison with other published data (e.g. Lichtenthaler and Babani, 2004). The further increase of Chl *a*/*b* (by 3.7%) was observed after acclimation to HI-HT conditions. An enhanced need for carotenoid-mediated photoprotection under HI treatment was documented by the slight decrease of the Chl *a*+*b*/Car *x*+*c* ratio after a 13-d exposure to HI (by 7.5%, Fig. 6B). However, acclimation to the HI-HT regime did not lead to a continued decrease of Chl *a*+*b*/Car *x*+*c*. As noted above, the utilization of absorbed light energy within PSII was optimized under HI-HT treatment, resulting in the maximum F_V/F_M values (Fig. 4). Thus, there was no need for plants to invest in an enhancement of photoprotective de-excitation pathways mediated by carotenoids. The decrease of Chl *a*+*b*/Car *x*+*c* in HI- and HI-HT-acclimated plants was observed due to increased Car *x*+*c* content per needle area (by 12% and 13%, respectively; data not shown). Chl *a*+*b* content tended to increase under HI and HI-HT conditions,

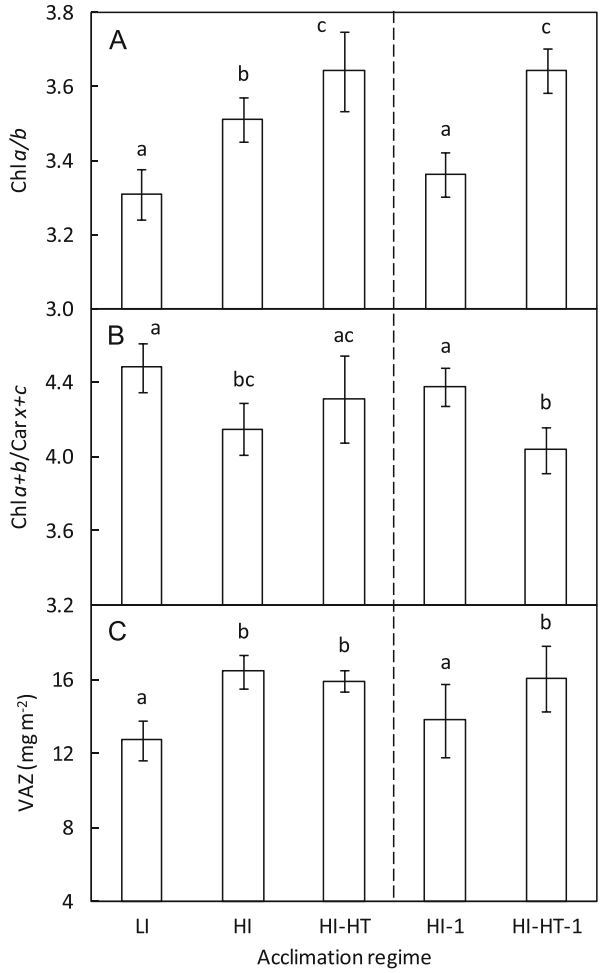

Fig. 6. (A) Ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b*), (B) ratio of total chlorophylls to total carotenoids (Chl *a*+*b*/Car *x*+*c*) and (C) the content of xanthophyll cycle pigments (VAZ) per needle area for the particular acclimation regimes. Needles were sampled by the end of the night period, i.e. after 12 h of darkness on the 13th day of acclimation to the given treatment (LI, HI, HI-HT) and after the first day under HI and HI-HT conditions (HI-1, HI-HT-1). Data followed by the same letter indicate non-significant differences ($P > 0.05$; Student's *t*-test). $n = 6–10 \pm$ S.D.

although this increase was non-significant ($P > 0.05$; data not shown).

The importance of a xanthophyll cycle-dependent thermal dissipation of absorbed light energy as a photoprotective mechanism in Norway spruce under excess irradiance was reported in our previous studies (Kurasová et al., 2003; Štroch et al., 2008a). Therefore, we monitored changes in the content of xanthophyll cycle pigments (VAZ) after exposure to HI and HI-HT conditions (Fig. 6C) and diurnal courses of the de-epoxidation state of xanthophyll cycle pigments, expressed as the ratio of de-epoxidized xanthophylls ($Z+A$) to VAZ pool (DEPS, Fig. 7). In agreement with other studies (Demmig-Adams, 1998; Kurasová et al., 2002; Lichtenthaler, 2007), the reduction of LHCII after HI acclimation was typically accompanied by an increase of the VAZ content (by 29%, Fig. 6C). On the contrary, HI-HT-acclimated plants showed a slightly, but non-significantly ($P > 0.05$) lower VAZ level compared to HI-acclimated plants.

DEPS in LI-acclimated plants increased in the morning from 28% in the darkness to 67% at the maximal PPFD (Fig. 7A). At the end of the night period, no Z was detected; only A contributed to persistent de-epoxidation (data not shown). As with D (Fig. 5B), DEPS gradually decreased in the afternoon but remained at higher values compared to the morning values corresponding to the same PPFDs. The response of DEPS to HI and HI-HT conditions matched the response of D. DEPS of plants after a 13-d exposure to HI conditions increased during the day and reached a maximal value of 86%. This high DEPS value supports previous results showing very efficient V de-epoxidation in the photosynthetic apparatus of Norway spruce compared to other plant species (Kurasová et al., 2003). DEPS decreased after HI-HT acclimation, mainly in the afternoon, resulting in a symmetrical pattern of the daily course. The close relationship between D and DEPS was observed to be independent of the acclimation regime (Fig. 8). Thus, xanthophyll cycle-dependent thermal energy dissipation was a dominant component of D, and higher T_{air} did not induce any change in the relation between thermal dissipation and xanthophyll de-epoxidation. DEPS determined in darkness at the end of a night period after HI-HT acclimation showed the lowest value (22%). Considering the well-known fact that the retention of de-epoxidized xanthophylls in darkness is usually associated with a F_V/F_M depression (Williams et al., 2003; Štroch et al., 2008a), the efficient epoxidation of $Z+A$ back to V in HI-HT-acclimated plants may be the reason for the highest F_V/F_M value.

Conclusions

We expected that a gradual acclimation of the Norway spruce seedlings to high irradiance and to elevated temperatures would result in a progressive inhibition of A_N and in a gradually increasing demand on photoprotective processes, such as non-assimilatory electron transport pathways and thermal dissipation mediated by xanthophyll cycle pigments. Indeed, the strongest depression of A_N during the entire day was observed after acclimation of the seedlings to simulated hot sunny days (HI-HT regime, Fig. 2A), confirming a very low capacity of evergreen conifers from the cold regions to adjust their temperature optimum for photosynthesis (Way and Sage, 2008a, b). On the contrary, neither acclimation to HI nor to HI-HT regimes led to permanent PSII photoinhibition and, surprisingly, the HI-HT-acclimated spruces showed the highest quantum yield of PSII photochemistry (F_V/F_M, Fig. 4), indicating that efficient photoprotective processes maintained the optimum PSII function, even during exposure to excess irradiance and heat stresses.

In agreement with previous studies (Franco and Lüttge, 2002; Franco et al., 2007), the enhancements of non-assimilatory

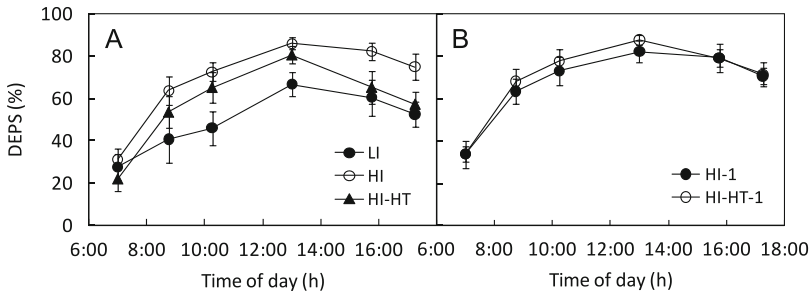

Fig. 7. Diurnal courses of the de-epoxidation state of the xanthophyll cycle pigments [DEPS=$(Z+A)/(V+A+Z)$], determined (A) at the end of the particular acclimation treatment (13th day of acclimation to LI, HI and HI-HT conditions) and (B) on the first day following transition from LI to HI acclimation regime (HI-1) and from HI to HI-HT regime (HI-HT-1). The first data points correspond to the samples collected after 12 h of the dark period. DEPS in darkness for HI-1 and HI-HT-1 was determined after the first day under HI and HI-HT conditions, respectively. n=6–10 ± S.D.

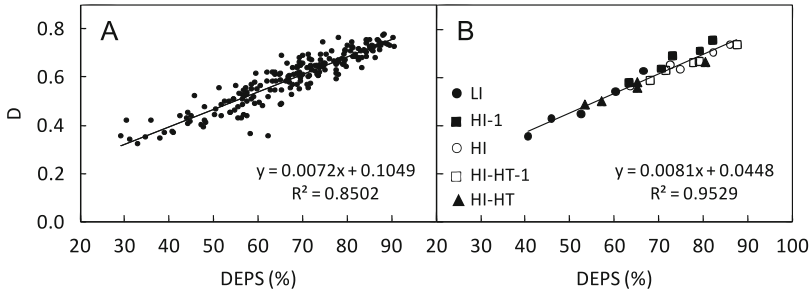

Fig. 8. Efficiency of thermal energy dissipation (D) in relation to the de-epoxidation state of the xanthophyll cycle pigments [DEPS=$(Z+A)/(V+A+Z)$]. Data obtained from measurements of daily courses of D and DEPS for all treatments (LI, HI-1, HI, HI-HT-1, HI-HT) are presented as (A) data for all the individual samples (n=200) and (B) means from 6 to 10 samples (data were taken from Figs. 5B and E and 7A and B). Linear regression lines with the coefficients of determination (R^2) are shown.

electron transport and xanthophyll cycle-dependent thermal dissipation of excess absorbed light were engaged in the efficient PSII photoprotection in HI-acclimated seedlings (Fig. 5B and C). However, after acclimation to the HI-HT regime, both a slight decrease of the content of xanthophyll cycle pigments (Fig. 6C) and a lower de-epoxidation state of xanthophylls, particularly in the afternoon period (Fig. 7A), corresponded to a diminished photoprotective role of heat dissipation of absorbed light (Fig. 5B). To the best of our knowledge, this is the first report that strong depression of A_N in evergreen conifers acclimated to high irradiances and super-optimum temperatures was compensated exclusively by the enhancement of non-assimilatory electron transport that reduced the demand on the xanthophyll cycle-mediated thermal dissipation of absorbed light energy.

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Low concentration of abscisic acid enhances mesophyll conductance to CO_2

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Abstract

Stomatal conductance (g_s) and mesophyll conductance (g_m) are two key components of diffusive limitations of photosynthesis since they restrict CO_2 flux from the leaf surface to the substomatal cavities (C_i) and from there to the sites of carboxylation (C_c). The responses of g_s and mainly of g_m to different concentrations (10 and 20 M) of abscisic acid (ABA) at three CO_2 concentrations were estimated using coupled gas-exchange and variable electron transport rate measurements. In contrast to g_s, which decreased in all ABA-treated plants, the response of g_m to ABA was concentration dependent. g_m was enhanced at lower ABA concentration, and *vice versa*. After ABA application, we observed a considerable reduction in g_s which in the end resulted in a significant decrease in C_i. In spite of this, C_c values were as high as in the controls due to the enhancement in g_m in 10 M ABA-treated plants. As a result, a low concentration of ABA could positively affect carbon gain and transpiration efficiency since the rate of CO_2 assimilation was unaffected. This effect seems to be less pronounced at sub-ambient CO_2 concentration.

Introduction

Carbon dioxide diffusion from the ambient atmosphere to the chloroplast stroma is crucial for plants as photosynthesis is highly limited by CO_2 availability at the sites of carboxylation in chloroplasts (C_c) (Evans *et al*., 1986; Evans *et al*., 2004; Evans and vonCaemmerer, 1996). Stomatal (g_s) and mesophyll (g_m) conductances significantly restrict CO_2 flux from the leaf surface to sub-stomatal cavities and further on to the sites of carboxylation. Therefore, g_s and g_m represent significant diffusional limitations of photosynthesis (Flexas *et al*., 2008; Warren, 2008a)

The functional proximity of g_s and g_m raises the question of their mutual co-regulation. The response patterns of g_s and g_m to most variables are similar, thus the g_s/g_m relationship is usually proportional. Only a slight difference in the slopes of the linear increase of g_s/g_m has been observed between soil and atmospheric water deficits treatments (Perez-Martin *et al.*, 2009; Warren, 2008b). A greater independence of g_s and g_m has been observed with regard to their temperature responses. In the range of 20-35°C, a significant reduction in g_s was not accompanied by a change in g_m (Yamori *et al.*, 2006). Similarly, the response of g_s to CO_2 concentration was found to be substantial while the g_m response was only moderate (Tazoe *et al.*, 2011) or, on the other hand, more pronounced than in g_s (Flexas *et al.*, 2007b; Vrabl *et al.*, 2009). Moreover, the significant decline of g_s induced by the application of abscisic acid (ABA) was not accompanied by any reduction of g_m (Vrabl *et al.*, 2009). Few studies show g_s and g_m responding in opposing directions to any given treatment. (Pons and Welschen, 2003) reported that, during midday depression of photosynthesis at 28-33°C, an initial increase of g_m was accompanied by a simultaneous decrease of g_s, and recently (Scafaro *et al.*, 2011) showed opposing temperature response patterns of g_s and g_m in the range of 18-42°C in three *Oryza* species.

The effect of ABA on g_s is well known; on the other hand, the sensitivity of g_m to ABA treatment has not been investigated sufficiently. ABA possesses the ability to trigger stomatal closure and affect g_s, therefore it could be a useful tool for evaluating the link between g_s and g_m. To date, few studies describing the effect of ABA on g_m have been published, presenting contradictory results. (Flexas *et al.*, 2006a) showed a reduction of g_m after ABA addition in soybean and tobacco, while (Vrabl *et al.*, 2009) found no effect in sunflower, using an exogenous ABA concentration that was five times lower. In canopy-scale measurements,(Schaufele *et al.*, 2011) showed that g_m decreases in the presence of elevated ABA, especially at higher ambient CO_2 concentrations (C_a), while at low CO_2 concentrations the effect of ABA was less significant.

In the present study, the effect of ABA, applied exogenously at low concentrations without introducing drought stress, on hydroponically grown plants of *Helianthus annuus* was investigated. The aim of the work was to evaluate the effect of this treatment on g_m and examine the relationship between g_s and g_m.

Materials and methods

Plant material and growth conditions

Plants of *Helianthus annuus* were cultivated from seeds in 0.5 L pots filled with perlite in a growth chamber (Sanyo, Gallenkamp, UK). Photoperiod length was 16h, day/night temperatures were 22/18°C, and relative humidity was 70%. After 21 days, plants were transferred to 3 L pots filled with ceramsite and grown hydroponically. Photosynthetic photon flux density (*PPFD*) was held constant during the day (400 μmol m^{-2} s^{-1} at the top of the canopy). All plants were watered every 2–3 days with a commercial nutrient solution (Kristalon Start, NU3 BV Vlaardingen, Netherlands).

Two months after sowing, two-thirds of the plants were treated with abscisic acid (ABA; Sigma Aldrich, Seelze, Germany) – the first-third by 10 M ABA and the second-third by 20 M ABA. A solution of 0.1 M ABA in 1 mL methanol was prepared and the appropriate amount added to the nutrient solution to reach final concentrations of 20 or 10 μM ABA.

Gas exchange and chlorophyll fluorescence measurements

Measurements were made on young fully expanded leaves 3 days after ABA addition. Light respiration rate (R_d) and the apparent CO_2 compensation point (C_c^*) were determined simultaneously using the method of (Laisk, 1977). A set of five A_N/C_i (assimilation rate/sub-stomatal CO_2 concentration) curves was constructed with *PPFD* ranging from 50 to 500 μmol photons m^{-2} s^{-1}. The CO_2 concentrations ranged from 30 to 250 μmol CO_2 mol^{-1} air as only the linear parts of the A_N/C_i curves were of interest. The different *PPFDs* were chosen following preliminary trials to ensure a large difference between the slopes of individual A_N/C_i curves (Warren, 2008c). The intersection points of the A_N/C_i curves at different *PPFDs* represent C_c^* (*x*-axis) and R_d (*y*-axis). Following (Warren, 2006), C_c^* was used as a proxy for the chloroplastic photocompensation point (Γ^*). All these measurements were performed with an open gas-exchange system (Li-6400, Li-Cor Inc., Lincoln, NE, USA) equipped with a 6 cm^2 broadleaf chamber and an integrated light source (Li-6400-02B; Li-Cor Inc.).

The photochemical efficiency of photosystem II (Φ_{PSII}) was estimated from steady state fluorescence (F_s) and maximal fluorescence (F_m') during a light-saturating pulse according to (Genty *et al*., 1989) as:

$$\Phi_{PSII} = (F_m' - F_s)/F_m' \quad (1)$$

The rate of linear electron transport (J_f) is related to Φ_{PSII} as follows:

$$J_f = \Phi_{PSII} * PPFD * \alpha * \beta \quad (2)$$

where α is the total leaf absorptance and β represents the partitioning of absorbed quanta between photosystems II and I, which is assumed to be 0.5 for C_3 plants (Ogren and Evans, 1993). However, fluorescence estimates of J_f are not strictly related to electron transport because the product of $\alpha \times \beta$ can vary. Therefore, an empirical relationship between J_f and J_{CO2} was determined under non-photorespiratory conditions (1% O_2) in which J_{CO2} – representing the electron transport rate calculated from gas exchange – is assumed to be wholly related to gross photosynthesis ($J_{CO2} = 4(A_N+R_d)$). Subsequently, fluorescence data measured at 21% O_2 were corrected in accordance with the calibration relationship of J_{CO2}/J_f obtained at 1% O_2 (Fig. 1). Moreover, the linear shape of that relationship shows constant and uniform electron transport rates across different treatments (CO_2 concentration, ABA addition). Fluorescence measurements were made using the Li-6400 with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc.)

Mesophyll conductance (g_m) was determined at three different CO_2 concentrations (146, 389 and 687 μmol mol^{-1}) from simultaneous measurements of gas exchange and fluorescence measurements on two or three light-adapted leaves of four 20 µM ABA-treated and four control plants and on one leaf of three 10 M ABA-treated plants. Photosynthesis was induced with a CO_2 concentration outside the leaf (C_a) of 389 µmol mol^{-1} and *PPFD* of 1500 µmol m^{-2} s^{-1}, based on previous measurements of light response curves that proved these values to be saturating. The amount of blue light was set to 10% to maximize stomatal aperture. Leaf temperature was kept close to 23°C and leaf-to-air vapor pressure deficit between 0.7 and 1.3 kPa during all measurements. The experiment was performed right after the steady state

was reached, i.e. 20-30 minutes after clamping the leaf into the leaf chamber. Gas exchange and chlorophyll fluorescence were first measured at C_a of 389 μmol mol^{-1}, then C_a was decreased to 146 μmol mol^{-1}, and after that returned to 389 μmol mol^{-1} to restore the original A_N value. Thereafter, C_a was increased to 687 μmol mol^{-1}. The time lag between consecutive measurements at different C_a was 15–20 min.

Possible leakages into and out of the cuvette for the range of CO_2 concentrations used were determined according to (Flexas *et al*., 2007a). The original estimates of A_N were corrected accordingly.

Estimation of g_m by gas exchange and chlorophyll fluorescence measurements

Mesophyll conductance was estimated by the variable *J* method according to (Harley *et al*., 1992), which allows assessing g_m at different CO_2 concentrations and is based on simultaneous measurements of gas exchange and chlorophyll *a* fluorescence. The method allows estimation of g_m from the rate of electron transport (J_f) and CO_2 concentration at the site of Rubisco (C_c), the latter being equal to $C_i - A_N/g_m$ based on the first Fick's law. Thus, g_m can be calculated as:

$$g_m = \frac{A_N}{C_i - \frac{\Gamma^*\left[J_f + 8(A_N + R_d)\right]}{J_f - 4(A_N + R_d)}} \qquad (3)$$

where R_d and Γ^* were determined according to (Laisk, 1977) (see above), and A_N and C_i were taken from gas exchange measurements.

Statistical analysis

Because our data did not fulfill the assumptions for classic one way analysis of variance (ANOVA) we used its non-parametric equivalence, the Kruskal-Wallis one-way analysis of variance with multiple comparisons (p-values, 2-tailed) to analyze differences between controls, 20 μM ABA-treated and 10 μM ABA-treated plants. All tests were performed using the program Statistica (StatSoft, Inc.).

Results

Measurements under non-photorespiratory conditions revealed a strong positive relationship ($R^2 > 0.97$) between electron transport rate calculated from gas exchange measurements (J_{CO2}) and electron transport rate calculated from fluorescence measurements (J_f) at different CO_2 concentrations in controls and ABA-treated plants (Fig. 1). This indicates a constant non-assimilatory electron flow across the various CO_2 concentration and ABA treatments. The average slope of the relationship between J_{CO2} and J_f was 1.40 with no significant difference between treatments, which is in approximate accordance with previously published results (Warren, 2008b). The results of the linear regression for J_f/J_{CO2} were used to "calibrate" the electron transport rate measured at photorespiratory conditions.

Using the method of (Laisk, 1977) and (Warren, 2006), no differences in C_c^* between controls and ABA-treated plants were found. C_c^* averaged around 33 μmol mol^{-1} in all treatments. R_d values ranged from 0.44 to 0.48 μmol CO_2 m^{-2} s^{-1} without significant differences between treatments.

Stomatal conductance (g_s) decreased with increasing CO_2 concentration in all plants. Exogenous addition of abscisic acid allowed us to introduce a diffusional limitation to photosynthesis by closing stomata and therefore markedly decreasing g_s. In plants treated with 20 or 10 μM ABA, we observed significantly lower g_s than in controls at all three CO_2 concentrations. The effect of ABA on g_s was more pronounced at higher CO_2 (Fig. 2 A). Under such conditions, net photosynthetic rate (A_N) is usually expected to be lower as well because CO_2 availability is restricted (Nobel, 2009). This was true for plants treated with 20 μM ABA at 389 and 687 μmol CO_2 mol^{-1}, whose g_s values differed significantly from the controls ($p = 0.001$). However, for plants treated with 10 μM ABA no significant decrease of A_N was recorded at these CO_2 concentrations (Fig. 2 B).

Values of mesophyll conductance (g_m) found in sunflower are in accordance with previously observed estimates for herbs (Warren, 2008a; Flexas *et al.*, 2008). Interestingly, at C_a of 389 μmol mol^{-1}, g_m was found to be significantly higher ($p = 0.05$) in plants treated with 10 μM ABA than in controls, but not so in plants treated with 20 μM ABA (Fig. 2 C). At C_a of 687 μmol mol^{-1}, a similar effect was apparent, albeit not significant. Therefore, the response of g_m to the lower ABA concentration was opposite to that of g_s except at low C_a where g_m was ABA-insensitive (see Fig. 2 A, C).

Since CO_2 flux through stomata was restricted in ABA-plants, substomatal CO_2 concentration (C_i) was substantially lower at all CO_2 concentrations compared with controls (see Fig. 3, for example at C_a 389 μmol mol^{-1}). Therefore, the drawdown between C_a and C_i was significantly higher in ABA-plants and ranged from 118 to 145 μmol mol^{-1}, in contrast with controls where the drawdown was 71 μmol mol^{-1}. On the other hand, the difference between C_i and C_c was significantly smaller in plants treated with 10 μM ABA (80 μmol mol^{-1}) than in controls (152 μmol mol^{-1}) and plants treated with 20 μM ABA (135 μmol mol^{-1}) (Fig. 3). This observation goes hand in hand with the enhanced g_m, i.e. enhanced CO_2 flux from intercellular air spaces to chloroplastic stroma. The total drawdown of CO_2 concentration from C_a to C_c did not differ between controls and 10 μM ABA-treated plants; however it was significantly higher in 20 μM ABA-treated plants (Fig. 3).

At all CO_2 concentrations, the C_i/C_a ratio was higher in controls than in ABA-treated plants. The C_i/C_a ratio ranged between 0.81 and 0.86 in controls and between 0.56 and 0.80 in ABA-treated plants. The C_c/C_a ratio was highest in controls (0.36 – 0.62), lowest in 20 M ABA-treated plants (0.22 – 0.55), and intermediate or similar to the controls in 10 M ABA-treated plants (0.31 – 0.61).

Discussion

The application of ABA introduced a substantial diffusion limitation for photosynthesis since g_s was reduced at all CO_2 concentrations and both ABA concentrations. The reduction of g_s in plants treated with 20 μM ABA was accompanied by a slight non-significant decrease in A_N, but g_m remained almost unchanged. These findings are in accordance with those of Vrabl *et al*. (2009). However, after application of 10 μM ABA, g_m was enhanced. The increase of g_m reduced the drawdown of CO_2 concentration from substomatal cavities into the chloroplasts and, consequently, C_c of plants treated with 10 μM ABA was similar to that of controls (Fig. 3, inset). As a result, the rate of photosynthesis remained unchanged despite CO_2 flux across stomata being restricted at all CO_2 concentrations. Contrary to these results, (Flexas *et al*., 2006a) found a decrease of g_s, g_m and A_N in *Arabidopsis thaliana* using 100 μM ABA, and Vrabl *et al*. (2009) observed unchanged g_m and A_N despite decreased g_s in *sunflower* using 20 μM ABA. This comparison indicates that the pattern of responses of g_s and g_m to ABA is

concentration-dependent, and the compensating responses of g_s and g_m occur only at low ABA concentration. As a result, the rate of CO_2 assimilation may not change or even increase while water loss through stomata decreases. This would lead to an enhancement of water use efficiency without any depression of photosynthesis. Hence, it can be hypothesized that under conditions of moderate water shortage, low concentrations of ABA could positively affect carbon gain. The reduction of C_i due to low g_s and high g_m may also promote stomatal opening, with a consequent increase of C_i, which may lead to oscillations in photosynthesis (Santrucek *et al.*, 2003).

When taking measurements with ABA-treated plants, some errors in C_i estimation can be introduced due to patchy stomatal closure (Laisk, 1983; Meyer and Genty, 1998; Pospisilova and Santrucek, 1994). However, although the reduction in g_s was substantial, g_s values in ABA-treated plants were still high (min. around 0.2 mol m^{-2} s^{-1} in sunflower). Moreover, (Flexas *et al.*, 2006a) showed that exogenous ABA did not induce patchy stomatal closure even when g_s dropped to much lower values (0.03 mol m^{-2} s^{-1}) in other herbaceous species. In addition, the close similarity in the A_N/C_i curvature observed at high and low water pressure deficits found here (data not shown) has been taken as an indication for the absence of patchy stomatal closure in ABA-treated plants (Grassi and Magnani, 2005).

The results presented here suggest a possible involvement of ABA in the regulation of g_m. ABA could be involved in controlling a part of g_m that is a protein-facilitated process (Bernacchi *et al.*, 2002). Aquaporins were shown to be closely associated with CO_2 transport within the mesophyll (Flexas *et al.*, 2006b; Hanba *et al.*, 2004), and (Wan *et al.*, 2004) demonstrated enhancement of aquaporin activity for water transport by ABA; thus it could be hypothesized that regulation of g_m by ABA could be either directly or indirectly connected with aquaporins.

Direct regulation could occur if ABA enhances the permeability of aquaporins for CO_2 as well as for water. If that was the case, CO_2 could permeate through the central or side pore of the AQP tetramer (Wang *et al.*, 2007). On the other hand, indirect regulation might be mediated by enhancement of water flux through AQP, thus reducing the thickness of unstirred layers on the surface of the membrane that represent a barrier for CO_2 diffusion (Missner *et al.*, 2008). In long-term experiments, ABA was also found to increase gene expression and protein content of most PIP isoforms (plasma membrane intrinsic proteins) AQPs are

composed of. In short-term experiments, ABA was hypothesized to affect AQP gating in root cells (Hose *et al*., 2000).

Studies showing natural abundance of endogenous ABA would be valuable, since the effective endogenous concentration need not mirror the changes in ABA concentration in the root medium. Moreover, experimentally applied ABA may produce artificially high effective concentrations in stomatal guard cells compared with control plants or plants exposed to mild water stress (Hartung and Slovik, 1991). For instance, the endogenous ABA concentration was observed to be 3.405 nmol/g f.w. in plants exogenously treated with 10μM ABA, whereas the concentration was only 0.189 nmol/g fw. in controls (unpublished results; see also (Guerfel *et al*., 2009). Hence, such a high concentration of endogenous ABA could trigger a stronger stress response than usually observed for plants experiencing water shortage. High ABA concentrations may lead to different, more complex responses that finally reduce the rate of CO_2 carboxylation, and consequently g_m, and *vice versa*. On the other hand, when ABA acts at low concentration mimicking moderate stress conditions, plants may "prefer" to balance the transient reduction in carbon gain caused by partial stomatal closure with an enhancement of g_m to keep the photosynthetic rate unchanged.

There is a major question about ABA sensing in plants and what receptors are responsible for the response to low ABA concentration that finally leads to enhanced g_m. It seems that different physiological responses triggered by ABA are based on different types of ABA-sensing receptors (McCourt and Creelman, 2008; Wang and Zhang, 2008). For instance, the ABA-binding protein *GCR2* is responsible for regulation of stomatal aperture and conductance (McCourt and Creelman, 2008). On the other hand, (Wan *et al*., 2004) speculated that ABA could bind directly to aquaporins and enhance their activity for water transport. If CO_2 flux is mediated by aquaporins as well, it could eventually enhance g_m. However , to reveal that mechanism requires further study.

Conclusion

The assessment of g_m at three CO_2 concentrations in the absence and presence of abscisic acid (10 and 20 M) revealed that g_m response to ABA is concentration dependent. g_m was enhanced at low ABA concentration, and *vice versa*. Due to the

enhancement in g_m, CO_2 concentration at sites of carboxylation in chloroplasts (C_c) in 10 M ABA-treated plants did not differ from controls although C_i was significantly less through g_s reduction after ABA application. Therefore, the photosynthetic rate was unaffected. Hence, it can be hypothesized that, under conditions of moderate water shortage, low concentrations of ABA could positively affect carbon gain and transpiration efficiency. This effect seems to be less pronounced at sub-ambient CO_2 concentrations.

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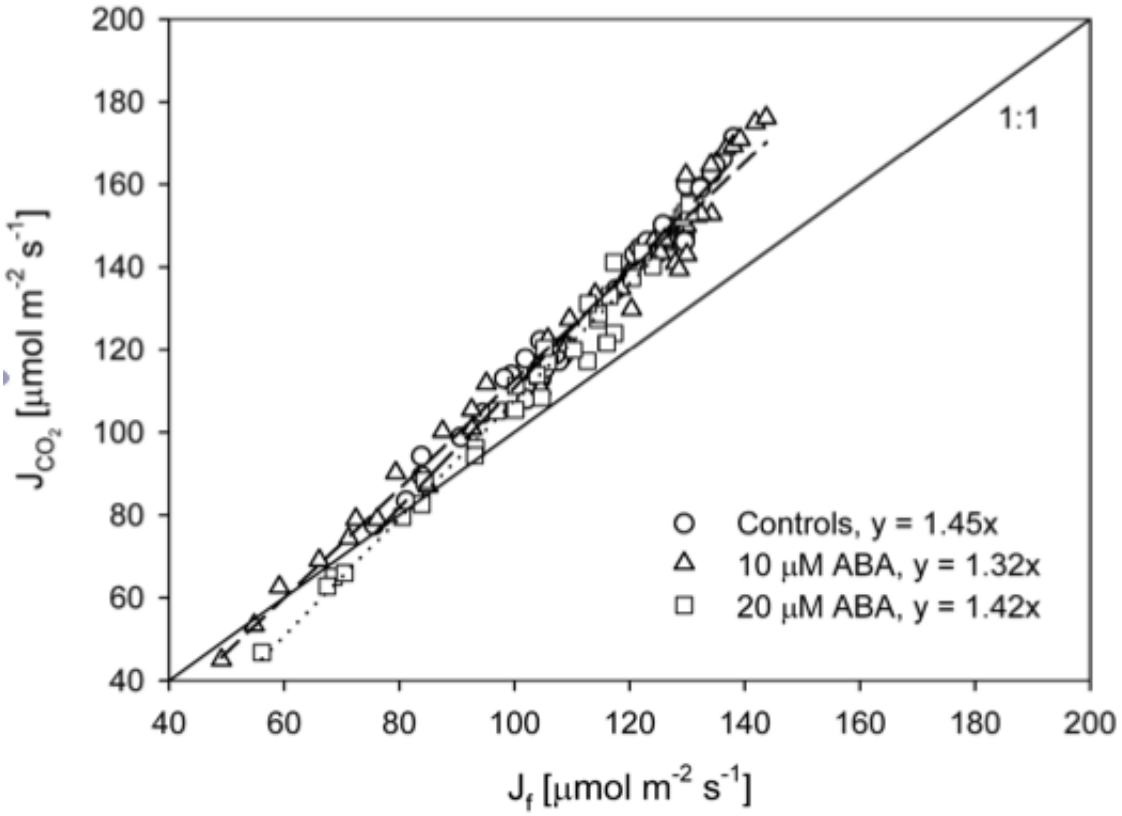

Figure 1: Relationship between rates of electron transport estimated from chlorophyll fluorescence (Jf) and from gas exchange measurements (JCO_2), obtained by varying CO_2 concentration under non-photorespiratory conditions in an atmosphere containing less than 1% O_2, in controls (circles) and plants treated with 10 μM ABA (triangles) or 20 μM ABA (squares). The linear regression equations are shown. A strong positive relationship was observed in all treatments ($R^2 > 0.97$).

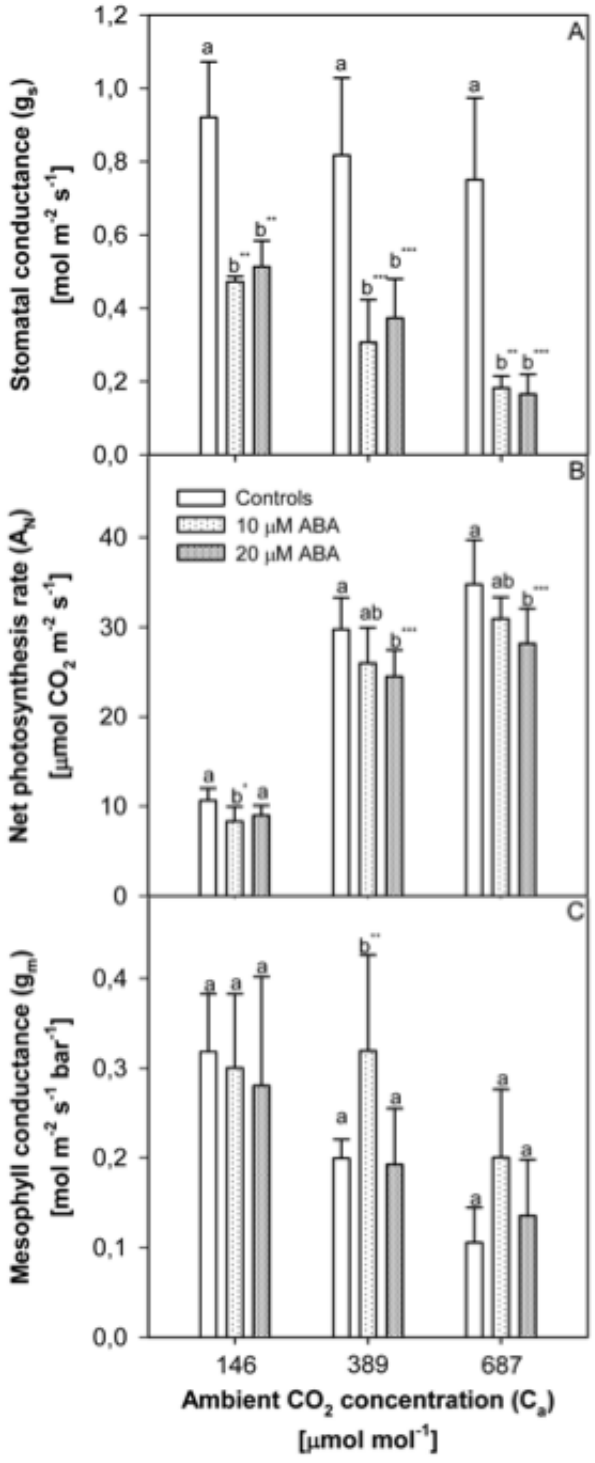

Figure 2: Response of (A) stomatal conductance, (B) rate of net photosynthesis and (C) mesophyll conductance in Helianthus annuus to three CO_2 concentrations (146, 389, 687 μmol mol^{-1}) in controls and plants treated with 10 or 20 μM ABA. Values are means +/- SD of 9 (controls), 3 (10 μM ABA) and 8 (20 μM ABA) replicates, respectively. $*$p = 0.05, $***$p = 0.01.

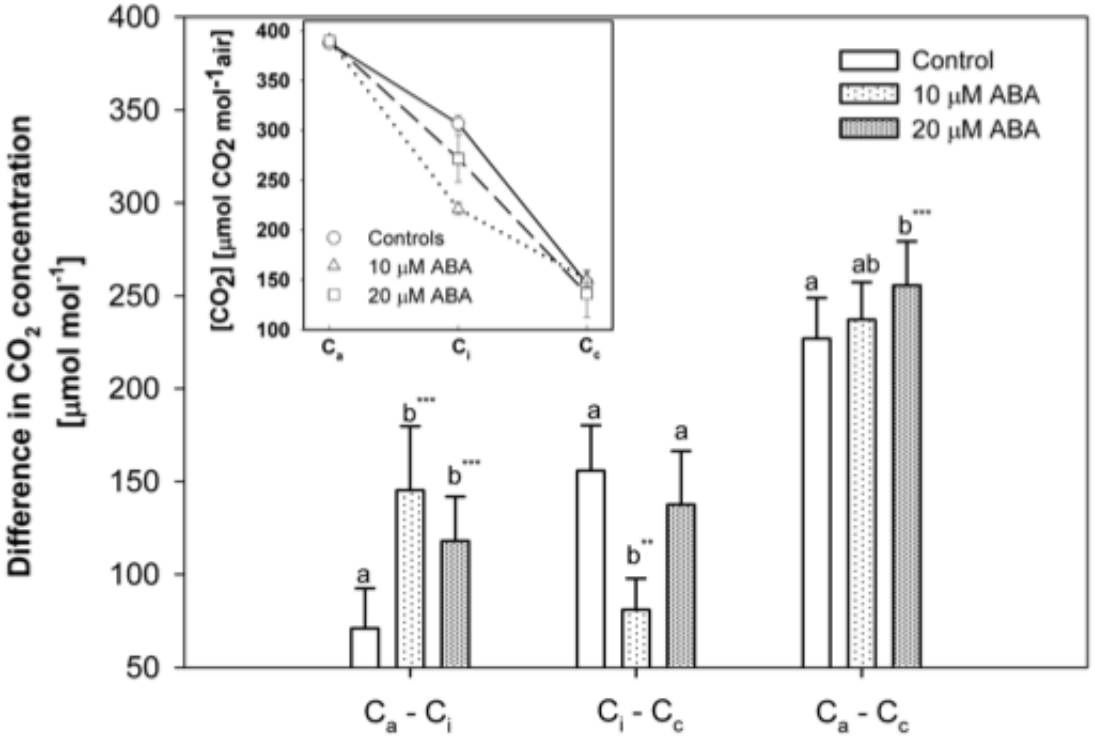

Figure 3: Differences in CO_2 concentration between ambient air (C_a) and sub- stomatal cavities (C_i); between C_i and chloroplast stroma (C_c); and overall difference between C_a and C_c at C_a of 389 μmol mol^{-1}in controls (while columns) and plants treated with 10 μM ABA (sparsely dotted columns) or 20 μM ABA (densely dotted columns). The inset shows the drop from C_a to C_i and from there to C_c in controls (circles) and plants treated with 10 μM ABA (triangles) or 20 μM ABA (squares). Values are means +/- SD of 9 (controls), 3 (10 μm ABA) and 8 (20 μM ABA) replicates, respectively. $**p = 0.05$, $***p = 0.01$.

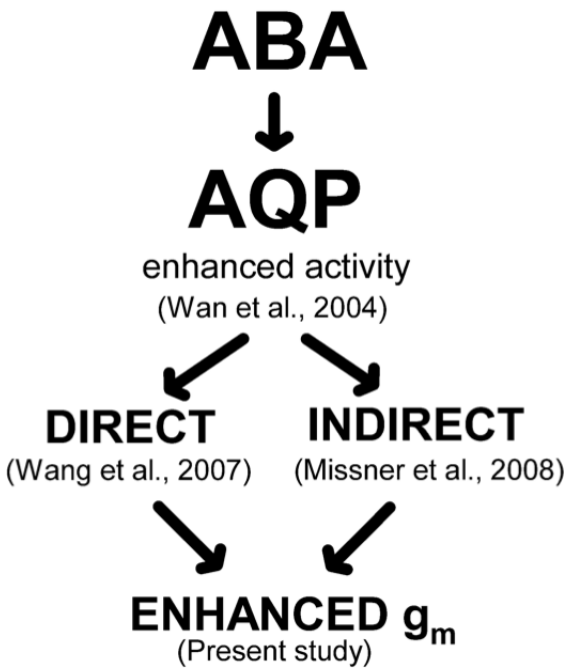

Figure 4: Schematic illustration of possible processes behind the regulation of g_m by ABA.

Curriculum Vitae

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9/2004 – 5/2006: Master degree in Biophysics, Faculty of Science, University of Ostrava.
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TEACHING

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Jitka Neuwirthová (2012): Effect of light quality on diffusional limitations of photosynthesis (Bachelor Thesis)

PUBLICATIONS

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GRANTS

D. Vrábl: Diffusion of CO_2 within the leaf mesophyll: The role of aquaporin proteins (GAAV KJB601410917, 2009-2011)
D. Vrábl: Mechanism of CO_2 diffusion from leaf mesophyll into chloroplast (GAJU 020/2008/P, 2009)

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GAČR P501/12/1261: Carbon dioxide signaling in leaf gas exchange and development (2012-2014)
GAČR 13-28093S: Impact of temperature and photosynthetically active radiation on dynamics of regulation of photosystem II function in higher plants. (2013-2016)
GAČR 206/08/0787: Regulation of Stomatal Morphogenesis by the Conditions of the Environment and Physiological Processes in the Leaf (2008-2011)
MZE QH91192: Comparison of methods of plant physiology and molecular biology for evaluation of wheat and barley sensitivity to drought and selection of water stress tolerant genotypes (2009-2011)
GAAV IAA601410505: Patterns of isotopic composition of plant organs - a marker of photosynthetic activity, environmental conditions and leaf anatomy (2005-2008)

OTHER

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