Journal of Experimental Botany, Vol. 60, No. 4, pp. 1179–1190, 2009
doi:10.1093/jxb/ern364 Advance Access publication 24 February, 2009
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Redundant roles of photoreceptors and cytokinins in regulating photosynthetic acclimation to canopy density

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Received 10 December 2008; Accepted 22 December 2008

Abstract

The regulation of photosynthetic acclimation to canopy density was investigated in tobacco canopies and in tobacco and *Arabidopsis* plants with part of their foliage experimentally shaded. Both species acclimated to canopy light gradients and partial shading by allocating photosynthetic capacity to leaves in high light and adjusting chloroplast organization to the local light conditions. An investigation was carried out to determine whether signalling mediated by photoreceptors, sugars, cytokinin, and nitrate is involved in and necessary for proper photosynthetic acclimation. No evidence was found for a role for sugars, or for nitrate. The distribution of cytokinins in tobacco stands of contrasting density could be explained in part by irradiance-dependent delivery of cytokinins through the transpiration stream. Functional studies using a comprehensive selection of *Arabidopsis* mutants and transgenics showed that normal wild-type responses to partial shading were retained when signalling mediated by photoreceptors or cytokinins was disrupted. This indicates that these pathways probably operate in a redundant manner. However, the reduction of the chlorophyll *a*/*b* ratio in response to local shade was completely absent in the *Arabidopsis* Ws-2 accession mutated in *PHYTOCHROME D* and in the triple *phyAphyCphyD* mutant. Moreover, cytokinin receptor mutants also showed a reduced response, suggesting a previously unrecognized function of phyD and cytokinins.

Key words: *Arabidopsis* mutants, cytokinin, environmental signalling, photoreceptors, photosynthetic acclimation, tobacco.

Introduction

An important ecophysiological question has been how plants perceive the light gradient imposed upon them by the proximity of neighbouring plants. Crowding leads to severe shading, especially of lower canopy layers, while upper leaves remain fully exposed to sunlight (Monsi and Saeki, 1953), inducing adaptive photosynthetic acclimation responses at the whole-plant, leaf, and chloroplast level (Niinemets, 2007). Mainly, such partial shading reduces photosynthetic capacity and hence nitrogen per unit area. Furthermore, it accelerates senescence of the shaded, oldest leaves, accompanied by reallocation of resources for the photosynthetic machinery from lower to upper leaves (Grindlay, 1997; Ono *et al.*, 2001; Hirose, 2005; Terashima *et al.*, 2005; Niinemets, 2007). Within the chloroplasts, shading leads to enhanced allocation of resources to light harvesting at the expense of photosynthetic capacity (Evans, 1993; Pons and Pearcy, 1994). This suite of traits is an example of phenotypic plasticity in response to neighbour proximity and was shown to be beneficial in terms of photosynthetic carbon gain by using model calculations (Grindlay, 1997; Anten *et al.*, 2000; Pons and Anten, 2004; Hirose, 2005) and recently by using transgenic plants with delayed senescence (Boonman *et al.*, 2006).

The perception of neighbouring plants and regulation of photosynthetic acclimation were shown to depend on photoreceptors and xylem-translocated cytokinin, and

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various other mechanisms were postulated (reviewed by Ono *et al.*, 2001; Kull, 2002; Terashima *et al.*, 2005). Photoreceptors perceive the quantity and spectral quality of light and regulate photosynthetic gene expression (Terzaghi and Cashmore, 1995; Chen *et al.*, 2004). The phytochrome photoreceptors were shown to regulate senescence induced by the low red to far-red ratio of light (R:FR) at the bottom of dense sunflower canopies (Rousseaux *et al.*, 1996, 2000). Reduced irradiance without a change in spectral composition of the light is sufficient to induce photosynthetic shade acclimation, with little additional effect of a change in R:FR (Pons and de Jong-van Berkel, 2004). All photoreceptor mutants studied to date showed normal acclimation to such spectrally neutral shade in the same way as the wild types (Walters *et al.*, 1999; Weaver and Amasino, 2001), indicating they are not of major importance, or at least not the only players. Photoreceptors are characterized by partially redundant functions of different gene family members, possibly masking any phenotype when single mutations are studied (Franklin *et al.*, 2003*b*; Chen *et al.*, 2004). To date, a comprehensive study including higher order mutants encompassing all known members of the phytochrome, cryptochrome, and phototropin photoreceptor families is lacking.

Light may also be perceived independently of photoreceptors. For example, the redox state of components of the electron transport chain is used as a signal for light acclimation (Huner *et al.*, 1998; Pfannschmidt *et al.*, 1999). Another alternative pathway to regulate shade acclimations involves the xylem-transported phytohormone cytokinin (Pons and Bergkotte, 1996; Pons and Jordi, 1998; Pons *et al.*, 2001; Boonman and Pons, 2007; Boonman *et al.*, 2007). Measurements showed that cytokinin delivery to shaded leaves is decreased as a consequence of reduced stomatal conductance and consequently transpiration rates, resulting in a canopy density signal that regulates, at least in part, photosynthetic capacity and senescence (Boonman *et al.*, 2007). Other xylem-transported compounds can also be considered potential signals, including nitrate, which is known to have signalling properties (Crawford, 1995; Stitt, 1999). Finally, foliar sugar concentrations are modulated in response to irradiance, and sugars repress photosynthetic gene expression (Sheen, 1990; Koch, 1996). It has therefore been proposed that they are involved in signalling in dense leaf canopies as well (Ono *et al.*, 2001; Kull, 2002).

While it is clear that multiple signal transduction pathways are involved in canopy density perception, the relative importance of these routes for the regulation of acclimation at the level of whole plants or chloroplasts is not known. Two complementary approaches were used to clarify further the role of these various mechanisms. First, soluble sugars, nitrate, and a wide range of cytokinins were measured as putative signals along canopy height in tobacco stands of contrasting density. Vertical profiles of these compounds in tobacco have been reported before, but only in plants grown alone and for a limited number of cytokinins (Masclaux *et al.*, 2000; Nordström *et al.*, 2004). Whole-plant level acclimation to canopy density has been found previously in those tobacco stands, as demonstrated by a steeper decline in photosynthetic capacity from the top of the canopy downwards at the higher canopy density (Boonman *et al.*, 2007). Here, the question was asked of whether the distribution of putative signals was consistent with a role in acclimation at both the whole-plant and chloroplast level. Secondly, acclimation was measured in artificially shaded tobacco and *Arabidopsis* leaves on plants of which the rest remained in high light, and found that this treatment mimics the responses of shaded leaves in a real canopy. This allowed the use of a large number of *Arabidopsis* mutants defective in photoreceptor, cytokinin, and sugar-mediated signalling. Double and higher order mutants corresponding to all known cytokinin receptors and all known photoreceptors, sugar-insensitive or hypersensitive mutants, and genotypes with constitutively lower or higher cytokinin content were included. The question was then asked of whether any of these pathways are necessary for proper acclimation to partial shading as found in dense canopies.

Materials and methods

Plant material and growth conditions

The density experiment using tobacco (*Nicotiana tabacum* L.; cultivar Wisconsin 38) has been described elsewhere (Boonman *et al.*, 2007). Briefly, an open stand (3.6 plants m^{-2}) and a dense stand (35 plants m^{-2}) were established in a greenhouse, grown for 11 weeks, and measurements were taken during the last 2 weeks. Leaves at three heights from at least three replicate individuals were harvested 70 d after sowing and immediately frozen in liquid nitrogen for biochemical analyses. In each stand, non-senescent leaves were taken from positions exposed to maximal, intermediate, or minimal irradiance. One week later, plants were moved to the laboratory for gas exchange and chlorophyll measurements. Tobacco and *Arabidopsis* plants for the partial shade experiments were grown in a climate-controlled growth chamber with a 20 °C light/16 °C dark cycle, a 16 h light period for tobacco, and a 9 h light period (short days) for *Arabidopsis* at a photosynthetic photon flux density (PPFD) of 200 mmol m^{-2} s^{-1}, as described by Boonman *et al.* (2007).

Arabidopsis thaliana accession Columbia-0 (Col-0) was used for sugar and nitrate analysis, and mutants and transgenics were in the Col-0 background unless stated otherwise. The following cytokinin receptor mutants which show reduced cytokinin sensitivity were studied: *ahk2-2tk*, *ahk3-3*, the double mutant *ahk2-2tkahk3-3* (Higuchi *et al.*, 2004), and the *cre1-2* line with a T-DNA insertion in the *CRE1* gene (Inoue *et al.*, 2001), as well as *cre1-1* in the Landsberg *erecta* (L*er*-0) background. The pleiotropic mutant *amp1-1* that has constitutively elevated cytokinin levels (Chaudhury *et al.*, 1993) was used together with transgenic 35S::CKX1, 35S::CKX2, 35S::CKX3, and 35S::CKX4 plants that have constitutively reduced

cytokinin levels, through overexpression of cytokinin oxidase genes (Werner *et al.*, 2003). The following mutants with altered sugar sensitivity were used: *ctr1-1*, defective in a central component of the ethylene signal transduction pathway and known to be sucrose insensitive (Kieber *et al.*, 1993; León and Sheen, 2003), and the ethylene-insensitive and, by implication, sugar-hypersensitive mutants (León and Sheen, 2003) *ein4-1* (Roman *et al.*, 1995) and *etr1-1* (Bleecker *et al.*, 1988). In the L*er*-0 background, the glucose-insensitive mutant *gin2-1* (Jang *et al.*, 1997) and the photoreceptor mutants *phyAphyB* (Reed *et al.*, 1994), *cry1cry2*, which was a cross of the *fha1* and *hy4-1* mutant alleles (Ahmad *et al.*, 1998; Weston *et al.*, 2000), and *hy2*, defective in phytochrome chromophore synthesis (Koornneef *et al.*, 1980; Parks and Quail, 1991), were used. The Wassilewskija (Ws-2) accession naturally harbours the *phyD* mutation (Aukerman *et al.*, 1997). Also in the Ws background was *phyAphyCphyD* (Franklin *et al.*, 2003*a*). Finally, *phot1phot2* (Kinoshita *et al.*, 2001) had a combined L*er*/Ws background.

Partial shade treatment

To simulate light gradients as they occur in dense leaf canopies, single attached leaves were shaded for 6–7 d, while the rest of the plant remained in normal light. Previous work demonstrated that this treatment mimics changes induced by vertical light gradients in dense canopies (Boonman *et al.*, 2007). Spectrally neutral shading was applied by enclosing the leaf in a mitten made of two paper sheets on the upper side and coarse mosquito mesh on the lower side. The sides were held apart by black foam so leaf expansion was not hindered by the treatment. The upper paper sheet had a fine grey print on the downward-facing side, resulting in a reduction of PPFD by 93% to ~14 μmol m^{-2} s^{-1}. Control leaves on different plants remained in growth light. The eighth true leaf counted from below which was still expanding was used in most cases, or a lower leaf number for those mutants that formed fewer leaves. In *amp1-1* and *ctr1-1*, an expanding and exposed leaf was selected because leaf number could not be determined.

Humidity treatment

Transpiration rates of individual *Arabidopsis* leaves were reduced independently of irradiance by enclosing the leaf in a transparent cuvette flushed with humid air for 6 d as described by Boonman *et al.* (2007). Incident PPFD remained at 200 μmol m^{-2} s^{-1} and leaf temperature was equal to that of control leaves, which were enclosed in a cuvette flushed with growth chamber air.

Light quantity and light quality measurements in tobacco stands

PPFD in the dense tobacco canopy was measured on a horizontal plane in six positions at 10 cm height increments using a line sensor (AccuPAR Ceptometer PAR-80, Decagon, Pullman, WA, USA) and directly above the canopy using a quantum sensor (LI-185A; Li-Cor, Lincoln, NE, USA), to obtain relative PPFD. In the open stand, PPFD was measured using the LI-185A quantum sensor on the upper surface of leaves at various positions on six individual plants and also directly above the plants. R:FR gradients were measured with a line sensor with three R-sensitive sensors alternating with three FR-sensitive sensors. The R sensors were composed of a Hamamatsu G1118 GaAsP photodiode with a Schott RG-645 optical filter resulting in a peak sensitivity of ~660 nm. The FR sensors consisted of a G1738 GaAsP photodiode with a Schott RG-9 optical filter resulting in a peak sensitivity of ~730 nm. The sensors were calibrated against a spectroradiometer (LI-1800, LI-COR).

Leaf analyses

Photosynthetic capacity was measured as the light- and CO_2-saturated rate of net photosynthesis (A_{max}) based on gas exchange, or as the light-saturated rate of electron transport based on chlorophyll fluorescence (ETR_{max}). In both species, A_{max} was measured in the laboratory using a gas-exchange measuring system with leaf chambers with a 69×67 mm window that was described previously (Pons and Welschen, 2002). An infrared gas analyser (LI-6262, Li-Cor) was used to measure CO_2 and H_2O partial pressure. Leaf temperature was kept at 25 °C, VPD was ~1 kPa, CO_2 partial pressure of the air entering the leaf chambers was 110 Pa, and PPFD was 1200 μmol m^{-2} s^{-1}, which saturated photosynthesis. The leaf area enclosed in the chamber was measured. Net rates of photosynthesis were calculated according to von Caemmerer and Farquhar (1981). ETR_{max} of *Arabidopsis* leaves was measured in the growth room using a portable chlorophyll fluorometer with attached leaf-clip holder (Mini-PAM, Waltz, Effeltrich, Germany) that kept the leaf at 8 mm from the fibreoptic probe. Steady-state chlorophyll fluorescence (F') was measured under saturating PPFD (600–1000 μmol m^{-2} s^{-1} for shaded and control leaves, respectively) using a halogen lamp which was assessed with a quantum sensor (LI-185A, Li-Cor). Maximal fluorescence (F_m') was measured with a saturating light pulse of ~5 mmol m^{-2} s^{-1}. An air fan prevented any increase in leaf temperature. Photochemical yield (Y) was calculated as $Y=(F'-F_m')/F_m'$ (Genty *et al.*, 1989). Leaves were pre-induced at a PPFD of ~600 μmol m^{-2} s^{-1} for at least 10 min and were placed in the measuring position for 2 min before readings were taken. A fresh 1 cm^2 sample was taken for chlorophyll analysis with a spectrophotometer after extraction in dimethylformamide (Inskeep and Bloom, 1985). Leaf absorbance (α) was calculated as a function of leaf chlorophyll content (Chl, μmol m^{-2}) following Evans (1993): $\alpha=Chl/(Chl+76)$. ETR_{max} was then calculated as: $ETR_{max}=\alpha\times0.5\times PPFD\times Y$ (Genty *et al.*, 1989). Acclimation at the chloroplast level was analysed by measuring the following parameters, A_{max}/Chl or ETR_{max}/Chl, calculated using the parameters described above, and the Chl *a*/*b* ratio. For soluble sugar analysis, leaf material was freeze-dried and ground, and

extracted twice in 80% ethanol at 80 °C during 30 min. The extract was centrifuged and purified according to Bligh and Dyer (1959). Soluble sugars were determined in the supernatant with a spectrophotometer with anthrone as a colour reagent, and using glucose as a standard (Yemm and Willis, 1954). Nitrate was analysed in homogenized, dry material using salicylic acid as a reagent (Cataldo *et al.*, 1975).

Cytokinin analysis with micro LC-MS/MS

Cytokinins were analysed as previously described (Corbesier *et al.*, 2003; Boonman *et al.*, 2007). Frozen leaf samples were ground in liquid nitrogen, transferred into Bieleski solution (Bieleski, 1964), and extracted overnight at –20 °C. For each cytokinin compound determined, 10 pmol of $^{2}[H_3]$dihydrozeatin, $^{2}[H_3]$dihydrozeatin riboside, $^{2}[H_3]$dihydrozeatin 9-glucoside, $^{2}[H_3]$dihydrozeatin riboside 5′-monophosphate, $^{2}[H_6]$*N*6-(Δ^{2}isopentenyl)adenine, $^{2}[H_6]$*N*6-(Δ^{2}isopentenyl)adenosine, $^{2}[H_6]$*N*6-(Δ^{2}isopentenyl) adenine glucoside, and $^{2}[H_6]$*N*6-(Δ^{2}isopentenyl)adenosine 5′-monophosphate (OLCHEMIM Ltd, Olomouc, Czech Republic) were added as internal standards. Cytokinins were purified by a combination of solid-phase and immunoaffinity chromatography using a broad-spectrum anti-isoprenoid cytokinin immunoaffinity column (OLCHEMIM) as described (Redig *et al.*, 1996). *O*-Glucosides were not collected. Cytokinins were quantified by micro-liquid chromatography-positive electrospray-tandem mass spectrometry (micro-LC MS/MS) in multiple reactant monitoring mode (Prinsen *et al.*, 1998). The chromatograms obtained were processed using Masslynx software (Micromass, Manchester, UK).

Statistical analyses

Analysis of covariance (ANCOVA) was used to study the effects of stand density and relative height on the distribution of various parameters in the tobacco canopies. The effects of partial shade were analysed with a Student's *t*-test. High light control levels of ETR_{max} and Chl *a*/*b* of mutants were compared with those of their respective wild types using a one-way analysis of variance (ANOVA) followed by Tukey's *b*-test. Data were log-transformed when it improved homogeneity.

Results

Effects of canopy density on tobacco

Tobacco plants growing in a dense stand were exposed to a much steeper gradient in irradiance (Fig. 1A) and R:FR (Fig. 1B) when compared with the open stand. Besides these primary signals, canopy density also affected the distribution of soluble sugars (Fig. 1C), nitrate (Fig. 1D), and cytokinins (Table 1). Soluble sugars accumulated to much higher levels in open stand plants at all canopy positions (Fig. 1C), whereas nitrate accumulated much more in dense stand plants and was only present at low concentrations in the open stand at all positions (Fig. 1D). In the dense stand, only upper leaves had significant sugar concentrations, and intermediate and lower leaves exposed to deep canopy shade contained almost no sugar (Fig. 1C). In contrast, nitrate accumulated in the lower leaves in dense stand plants, opposite to the light gradient (Fig. 1D). Eleven different cytokinins were detected using micro-LC MS/MS (Table 1). In general, the dominant cytokinins were *cis*-zeatin riboside monophosphate (ZRP) and isopentenyl adenosine monophosphate (iPRP), and concentrations were highest in the upper leaves and declined towards the bottom in both stands. High canopy density reduced the concentration of *cis*-ZRP in upper leaves by ~50%. *Trans*-Z-type cytokinins were only detected in upper leaves in both stands, and dihydrozeatin (DHZ)-type cytokinins were below or close to the detection limit in all samples. For one of the cytokinins, isopentenyl adenosine (iPR), a significant height by density interaction was observed when only upper and intermediate leaves were considered

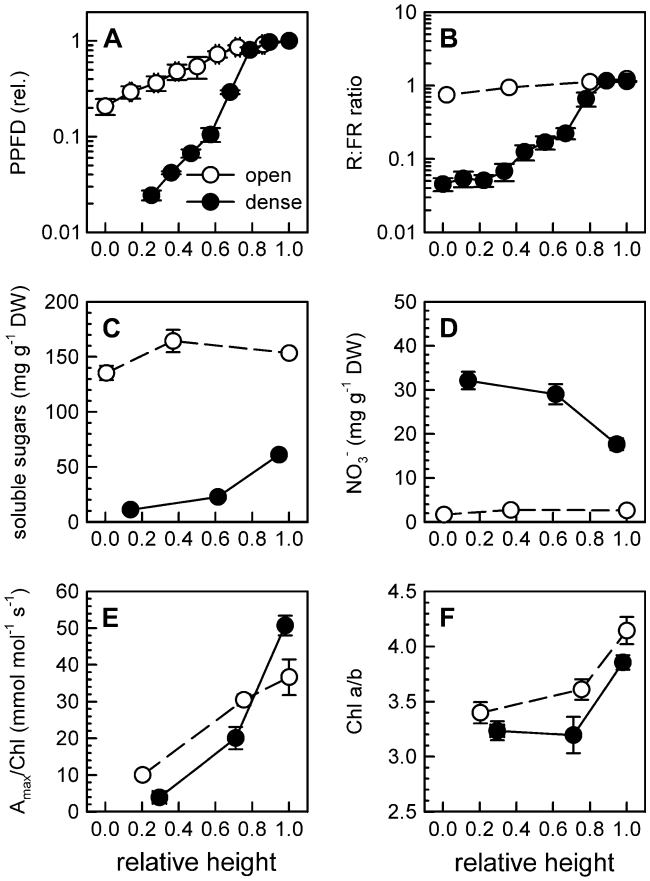

Fig. 1. Canopy density effects on putative signal parameters and photosynthetic acclimation in tobacco. Shown are PPFD (A), R: FR (B), soluble sugars (C), and nitrate (D), photosynthetic capacity per unit chlorophyll (A_{max}/Chl) (E) and the chlorophyll *a*/*b* ratio (Chl a/b) (F) measured in open (3.6 plants m^{-2}) and dense (35 plants m^{-2}) tobacco canopies at three heights representative for maximal, intermediate, and minimal irradiance in each stand. Data are means±SE, n=6–12. Note the log-scale on the *y*-axis in A and B.

Table 1. Canopy density effects on the cytokinin distribution in tobacco

Values represent means±SE, n=3.

	Cytokinin (pmol g^{-1} fresh weight)					
	Dense canopy			Open canopy		
	Low*	Intermediate	High	Low	Intermediate	High
cis-Z	0.282±0.114	0.246±0.009	0.217±0.040	0.390±0.117	0.386±0.139	0.392±0.096
cis-ZR	0.198±0.079	0.156±0.028	0.176±0.014	0.146±0.028	0.177±0.079	0.205±0.007
cis-ZRP	0.631±0.095	1.315±0.143	3.140±1.181	0.908±0.094	1.763±0.679	7.661±1.361
trans-Z	nd	nd	0.129±0.056	nd	nd	0.166±0.047
trans-ZR	nd	nd	0.081±0.016	nd	nd	nd
trans-ZRP	nd	nd	1.430±0.566	nd	nd	1.279±0.143
DHZ	nd	nd	nd	nd	nd	0.067±0.028
DHZRP	nd	0.028±0.008	0.242±0.097	nd	nd	0.570±0.095
iP	0.342±0.037	0.333±0.051	0.266±0.028	0.212±0.015	0.261±0.019	0.369±0.102
iPR	0.227±0.035	0.236±0.019	0.632±0.059	0.179±0.008	0.284±0.046	0.409±0.020
iPRP	1.063±0.227	2.777±0.115	11.39±2.275	1.376±0.286	2.844±1.067	12.04±0.682

DHZ, dihydrozeatin; DHZRP, dihydrozeatin riboside monophosphate; iP, isopentenyl adenine; iPR, isopentenyl adenosine; iPRP, isopentenyl adenosine monophosphate; nd, not detected; Z, zeatin; ZR, zeatin riboside; ZRP, zeatin riboside monophosphate.

* Positions correspond to relative heights in Fig. 1C, D.

(P <0.05; ANCOVA), that was consistent with the irradiance gradient. That is, iPR concentrations were reduced more strongly from the top of the canopy downwards in the dense stand than in the open stand (Table 1).

Chloroplast acclimation to canopy density was evident in the tobacco stands because A_{max}/Chl (Fig. 1E) and to a lesser extent Chl *a*/*b* (Fig. 1F) both declined more strongly from the top of the canopy downwards in the dense stand as compared with the open stand. Both parameters showed significant height by density interactions when only the upper and intermediate leaves were considered (P <0.05; ANCOVA).

Partial shading of tobacco and Arabidopsis *plants*

In order to obtain a model system that could easily be used in the lab, partial shade was applied to mimic the effects of canopy light gradients. When a single leaf was shaded (93% spectrally neutral shade) on tobacco or *Arabidopsis* plants remaining in the light, a decline in soluble sugar concentration (Fig. 2A, B) and accumulation of nitrate (Fig. 2C, D) were again observed. This treatment also induced acclimation within chloroplasts, as indicated by the reduction in A_{max}/Chl (Fig. 2E, F) and Chl *a*/*b* (Fig. 2G, H). Furthermore, in both species, A_{max} was reduced in the shaded leaves (Fig. 2I, J), as was described previously for a similar experiment using *Arabidopsis* (Boonman *et al.*, 2007). Thus, photosynthetic acclimation at the whole-plant and chloroplast level as observed in tobacco canopies was mimicked by the partial shade treatment in both tobacco and *Arabidopsis*.

Arabidopsis *mutants and transgenics*

In order to study the role of signalling mediated by cytokinin, photoreceptors, or sugars, the partial shade treatment was applied to a wide range of *Arabidopsis* mutants and transgenics defective in signalling mediated by these pathways. In all genotypes tested, ETR_{max} was reduced in shaded leaves the same way as in the wild types (Fig. 3A). These data suggest that there is apparently a large degree of redundancy in the regulatory mechanisms controlling photosynthetic capacity in response to irradiance. Photoreceptor mutants in particular did show variation with respect to ETR_{max} in control leaves, but ETR_{max} in low light was remarkably similar between genotypes. ETR_{max} was reduced by 64% in Col-0 and 62% in L*er* (Table 2). Several mutants and transgenics had a significantly lower ETR_{max} in high light controls than their respective wild types (P <0.05, ANOVA) and these also showed the smallest relative reductions upon partial shading (Table 2): *phyD* (–46%), *phyAphyCphyD* (–53%), *cry1cry2* (–49%), and *etr1-1* (–44%). ETR_{max}/Chl, a parameter pertaining to chloroplast-level acclimation, was also significantly reduced in all genotypes (Supplementary Fig. S1A available at *JXB* online), as well as Chl *a*/*b* in most cases (Fig. 3B). ETR_{max}/Chl was reduced to a rather similar level in all genotypes, and the reduction of ETR_{max}/Chl correlated well with the reduction of ETR_{max}. Chl *a*/*b* was reduced by 6.4% in Col-0 and 9.6% in L*er* (Table 2). Relatively large decreases in Chl *a*/*b* were observed in *cry1cry2* and *etr1-1*, as well as *ein4* (all ~15%). Chl *a*/*b* of *hy2* in high light was exceptionally high (6.22±0.198), as was found previously for the similar *hy1-1* mutant (Walters *et al.*, 1999), but normally reduced in response to local shade. However, Chl *a*/*b* did not change significantly in response to partial shade in the triple photoreceptor mutant *phyAphyCphyD* and the *phyD* mutant (Fig. 3B). Also, only small reductions were observed in the cytokinin receptor mutants *ahk2-2tk* (–2.7%), *ahk3-3* (–3.4%), and

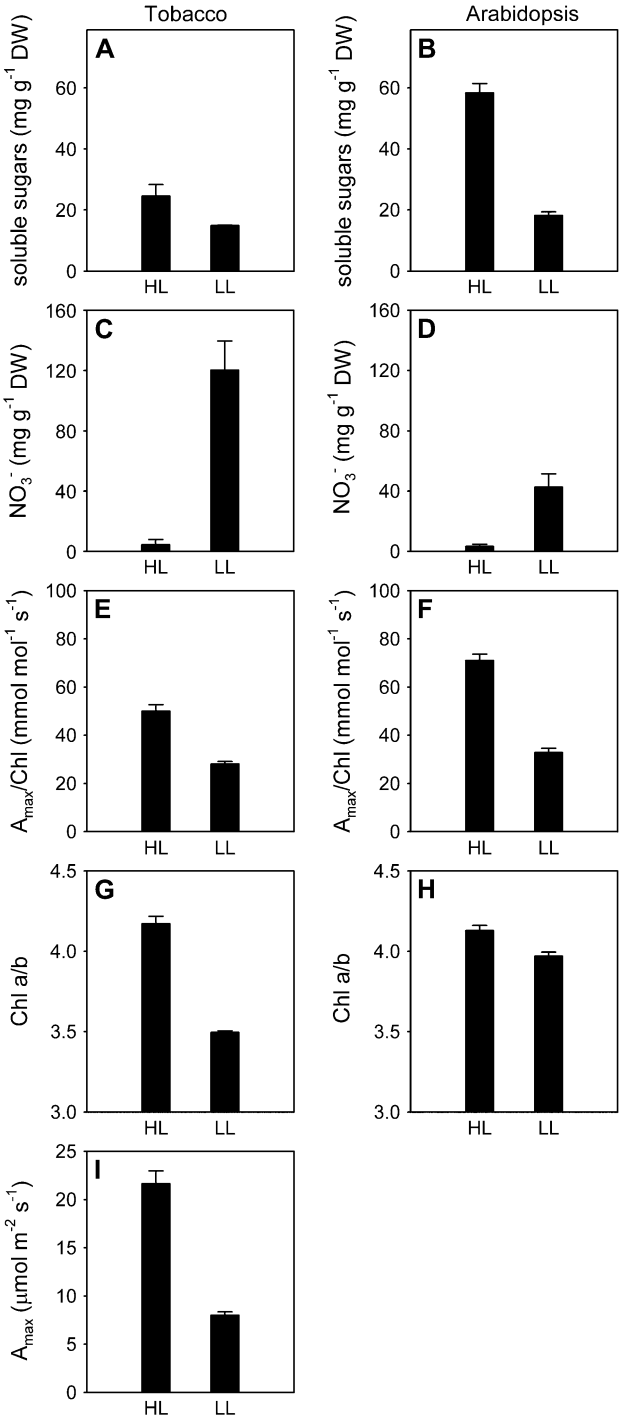

Fig. 2. Effects of shading a single leaf of a plant on putative signals and photosynthetic acclimation in tobacco (A, C, E, G, H, I) and *Arabidopsis* (B, D, F, H, J). Shown are soluble sugars (A, B), nitrate (C, D), photosynthetic capacity per unit chlorophyll (A_{max}/Chl) (E, F), chlorophyll *a/b* ratio (Chl *a/b*) (G, H), and photosynthetic capacity per unit area (A_{max}) (I). One attached leaf was shaded (PPFD ~14 μmol m^{-2} s^{-1}; low light, LL) for 5–6 d and compared with a leaf on a different plant that remained in the light (PPFD ~200 μmol m^{-2} s^{-1}; high light, HL). Data are means±SE, n=2–6. *, P <0.05 (Student's t-test).

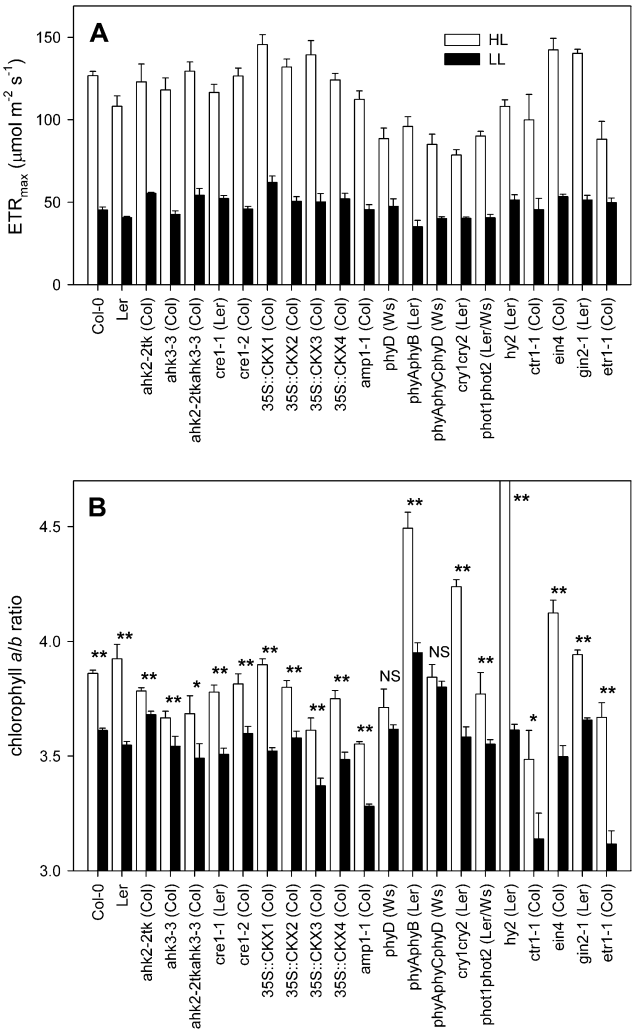

Fig. 3. Effects of shading a single leaf of a plant on photosynthetic acclimation in *Arabidopsis* mutants and transgenics disrupted in signalling mediated by cytokinin, photoreceptors, or sugars. Shown are the light-saturated rate of electron transport per unit area (ETR_{max}) (A) and chlorophyll *a/b* ratio (Chl *a/b*) (B). One attached leaf was shaded (PPFD ~14 μmol m^{-2} s^{-1}; low light, LL) for 6 d and compared with a leaf on a different plant that remained in the light (PPFD ~200 μmol m^{-2} s^{-1}; high light, HL). The Chl *a/b* of *hy2* in HL was 6.22±0.198. Shading induced significant (P <0.05; Student's t-test) decreases in ETR_{max} in all genotypes tested. Data are means±SE, n=6. In B, NS, not significant; *, P < 0.1; **, P <0.05 (Student's t-test).

ahk2-2tkahk3-3 (–5.3%) (Table 2). These findings identify a new role for phytochromes and cytokinins in the response of Chl *a/b* to light availability.

Chloroplast-level acclimation controlled by transpiration rate in Arabidopsis

Because of the finding that cytokinin receptor mutants showed less responsiveness to partial shade in terms of Chl *a/b*, chloroplast-level acclimation in *Arabidopsis* in response to manipulation of the transpiration rate independently of light was analyzed. This humid air treatment was previously

Table 2. Decease in ETR_{max} and CHL a/b in shaded leaves (low light; LL) relative to leaves remaining in high light (HL). Single attached leaves were shaded of Arabidopsis mutants and transgenics, and their respective wild types

For further description see Fig. 3

Genotype	Decrease in LL relative to HL (%)	
	ETR_{max}	Chl *a/b*
Col-0	64.3	6.4
L*er*	62.4	9.6
ahk2-2tk (Col)	54.9	2.7
ahk3-3 (Col)	64.0	3.4
cre1-1 (L*er*)	55.2	7.2
cre1-2 (Col)	63.8	5.6
ahk2-2tkahk3-3 (Col)	58.2	5.3
35S::CKX1 (Col)	57.4	9.7
35S::CKX2 (Col)	61.7	5.8
35S::CKX3 (Col)	64.1	6.7
35S::CKX4 (Col)	58.1	7.1
amp1-1 (Col)	59.5	7.7
Ws/*phyD*	46.4	2.6
phyAphyB (L*er*)	63.5	12.1
phyAphyCphyD (L*er*)	52.8	1.1
cry1/cry2 (L*er*)	48.9	15.5
phot1phot2 (L*er*/Ws)	55.0	5.8
hy2 (L*er*)	52.4	41.9
ctr1-1 (Col)	54.5	9.9
ein4 (Col)	62.5	15.2
gin2-1 (L*er*)	63.4	7.2
etr1-1 (Col)	43.5	15.0

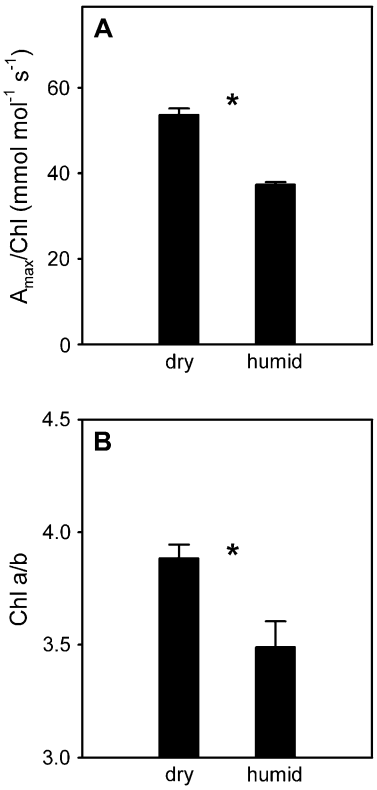

Fig. 4. Effects of experimental reduction of the transpiration rate on chloroplast-level acclimation in *Arabidopsis*. One attached leaf was placed in a cuvette flushed with humid air or with growth chamber air (dry) as a control for 6 d. Shown are photosynthetic capacity per unit chlorophyll (A_{max}/Chl) (A) and chlorophyll *a/b* ratio (Chl *a/b*) (B). Data are means±SE, n=6. *P <0.05; (Student's *t*-test).

shown to reduce cytokinin content and activity (Boonman *et al.*, 2007). Indeed, A_{max}/Chl (Fig. 4A) and Chl *a/b* (Fig. 4B), parameters associated with chloroplast-level acclimation, were reduced in response to humid air.

Discussion

No evidence for the regulation of canopy density acclimation by sugars or nitrate

The proximity of neighbouring vegetation is primarily associated with reductions in irradiance and R:FR in the lower canopy layers (Fig. 1A, B). In this study, an investigation was carried out to determine to what extent light signals and various alternative signals are required for the photosynthetic acclimation shown by plants in response to the proximity of neighbours. The sugar and nitrate data on tobacco and *Arabidopsis* argue against a role for these compounds. High sugar concentrations repress the expression of photosynthetic genes while, conversely, low sugar concentrations induce their expression (Sheen, 1990; Koch, 1996; Yu, 1999; Smeekens, 2000). In the tobacco canopies as well as in the partially shaded plants, the highest sugar concentrations were found in the control leaves in high light (Figs 1, 2) that had the highest photosynthetic capacity (Boonman *et al.*, 2007), and levels were reduced in the shade, opposite to what would be expected. Sugar levels were also found to be decreased in shaded sunflower leaves (Ono *et al.*, 2001). Hence, sugar accumulation probably reflects photosynthetic activity in a leaf canopy. There is a possibility though that individual sugars show different responses to partial shade than shown by the bulk soluble sugar level, and this may have an impact on sugar signalling.

Nitrate stimulates its own assimilation (Crawford, 1995; Stitt, 1999) and may thus contribute to the synthesis of photosynthetic enzymes and other proteins, as well as provide a nitrogen source. However, the upper leaves in the tobacco canopy and the control leaves in the partial shading experiments that had the highest photosynthetic capacity (Boonman *et al.*, 2007) showed the lowest nitrate concentrations, while nitrate accumulated to high levels in shaded leaves (Figs 1, 2). These findings are also opposite to what would be expected if nitrate signalling played a role in regulating photosynthetic capacity. The lower photosynthetic capacity of shaded leaves does not appear to be the result of limited nitrogen supply, since the highest nitrate concentrations were found there. It is possible that nitrate delivery rates, rather than foliar concentrations, function in

the regulation of photosynthetic capacity distribution in leaf canopies. It is known that nitrate in shaded leaves is mostly localized in the vacuole and not metabolically active (Aslam *et al.*, 1976; Granstedt and Huffaker, 1982), but further studies are required to unravel any role for nitrate.

The mutants *ctr1-1*, *ein4*, *etr1-1*, and *gin2-1* with altered sugar responsiveness retained the capacity for photosynthetic acclimation to partial shade in hte same way as the wild types (Fig. 3). Even though a role cannot be ruled out, these data also do not provide evidence in support of an important role for sugar signalling. There were quantitative differences from the wild types though: both *ein4* and *etr1-1*, which are insensitive to the plant hormone ethylene and are sugar hypersensitive, showed a stronger reduction of Chl *a*/*b* upon shading than the Col-0 background, which may be related to either of these signals. Furthermore, *etr1-1* had a lower ETR_{max} and Chl *a*/*b* in high light than the wild type. These findings are in agreement with previous reports showing that *Arabidopsis etr1-1* had a lower rate of photosynthesis per unit leaf area (Tholen *et al.*, 2004), while ethylene-insensitive tobacco was also shown to have a lower investment of foliar nitrogen in electron transport and Rubisco, and a lower Chl *a*/*b* ratio as the result of sugar hypersensitivity (Tholen *et al.*, 2007).

Regulation by photoreceptors of allocation of photosynthetic capacity

Previous studies have demonstrated that several *PHYTOCHROME* and *CRYPTOCHROME* mutants showed wild-type acclimation to shading or darkening (Walters *et al.*, 1999; Weaver and Amasino, 2001). Here, it has been shown that also in double and triple photoreceptor mutants including *PHOTOTROPIN* mutants, a normal wild-type reduction of photosynthetic capacity occurred when part of the foliage was shaded (Fig. 3A). Although the change in capacity in response to shade still occurred in all photoreceptor mutants, there was a reduction in capacity in light-exposed leaves relative to the wild types (Fig. 3A), and chlorophyll contents were lower (Supplementary Fig. S1B at *JXB* online). This probably reflects the essential role of the photoreceptors in green leaf development (Sullivan and Deng, 2003). Studies in which R:FR was manipulated have shown that phytochrome photoreceptors are involved in the induction of leaf senescence in dense canopies (Rousseaux *et al.*, 1996; Rousseaux *et al.*, 2000; Pons and de Jong-van Berkel, 2004). Whether Cry or Phot photoreceptors are involved is not known at present, although low blue light perceived by these photoreceptors can be used by plants as a neighbour proximity signal in morphological shade avoidance (Ballaré *et al.*, 1991; Pierik *et al.*, 2004). A possible explanation for these findings is that photoreceptors operate in a functionally redundant manner, such that double or triple photoreceptor mutants are still able to regulate photosynthetic capacity in response to partial shade. Moreover, these results emphasize that there are alternative signalling pathways that may act independently of photoreceptors.

Regulation by cytokinins of allocation of photosynthetic capacity

A forceful indication that plants perceive irradiance also independently of photoreceptors was provided by experiments in which the transpiration rate was reduced without a reduction in irradiance (Pons and Bergkotte, 1996). In this treatment, the leaf was surrounded by humid air in a transparent cuvette, which proved to be sufficient to reduce photosynthetic capacity. Shading is accompanied by reduced stomatal conductance and leaf transpiration rates in a canopy (Boonman *et al.*, 2007). Root-borne cytokinins carried in the transpiration stream are therefore delivered more to light-exposed leaves than to shaded leaves, and regulate photosynthetic capacity accordingly (Pons *et al.*, 2001; Boonman *et al.*, 2007). Also at the whole-plant level, the transpiration rate controls the cytokinin transport rate from roots to shoots (Aloni *et al.*, 2005). In experiments where part of the foliage of *Arabidopsis* plants was shaded, it was shown that a reduced transpiration rate decreased the concentration and activity of cytokinins, while applied cytokinins partially rescued the shade-induced decline in photosynthetic capacity (Boonman *et al.*, 2007). The mutant data presented here (Fig. 3A) suggest that, similar to photoreceptor-mediated signalling, cytokinin signalling is not absolutely required for the regulation of photosynthetic capacity. The evidence outlined above does, however, support a role for cytokinins in canopy density acclimations, but as one of multiple, redundantly operating mechanisms.

Cytokinin distribution in tobacco canopies

The distribution of cytokinins in the tobacco canopies is the result of import through the xylem (see above), as well as a multitude of other factors, including local synthesis (Singh *et al.*, 1992; Nordström *et al.*, 2004) and possibly breakdown (Werner *et al.*, 2001) and transport through the phloem (Hoad, 1995). Cytokinin concentrations in both stands were highest in the upper leaves (Table 1), which can be explained by their higher transpiration rates. Furthermore, it has been shown that Z-type cytokinins are synthesized particularly in young, upper leaves in tobacco (Singh *et al.*, 1992; Nordström *et al.*, 2004), which contributes to the high ZRP concentrations there. Possibly there is also synthesis of iP-type cytokinins in upper leaves, but this has not been explored. The surprisingly large quantity of *cis*-ZRP in upper leaves in the open stand may be the result of transport from dormant buds through the phloem, as well as transport from the roots through the xylem, which has been observed in other species (Vonk and Davelaar, 1981; Mader *et al.*, 2003). Notably, transgenic plants with reduced *cis*-ZRP were chlorotic (Miyawaki *et al.*, 2006). In accordance, it was found that upper leaves in the open stand had a higher *cis*-ZRP concentration (Table 1), as well as higher chlorophyll contents per unit area, than upper leaves in the dense stand (673.1 μmol Chl m^{-2} and 399.4 μmol Chl m^{-2}, respectively).

Canopy density also had a significant effect on one of the active cytokinins, iPR, that was qualitatively correlated to the light gradient, i.e. iPR concentrations were reduced more strongly from upper canopy leaves downwards at high stand density compared with low density. The significant height by density effect was found when considering only the upper and intermediate leaves, consistent with the previous finding that A_{max} and transcript levels of the small subunit of Rubisco also changed most prominently in mid-canopy leaves (Boonman *et al.*, 2007). The oldest, lower leaves were already starting to senesce and therefore showed less effect of canopy density in this experiment. Interestingly, iPR was one of three cytokinins that could be detected in the xylem sap collected from intact, transpiring plants, with the others being iP and iPRP (Boonman *et al.*, 2007). The canopy density effect on iPR can therefore be explained by the gradient in transpiration rate, and hence cytokinin delivery, that is ultimately controlled by the light gradient. Accordingly, a previous study on bean plants grown at contrasting densities demonstrated that in lower canopy layers, cytokinin concentrations were more reduced in the dense stand compared with the open stand (Pons *et al.*, 2001). Combined with the known stimulation of photosynthetic capacity by cytokinins (Chory *et al.*, 1994), these data support the model that cytokinin import controlled by transpiration rates provides leaves with a canopy density signal that regulates photosynthetic acclimation to the light gradient (Boonman and Pons, 2007).

Regulation of chloroplast-level acclimation by phytochromes and cytokinins

Chl *a*/*b* showed no change in response to partial shading in the *phyAphyCphyD* mutant and in the *phyD* mutant, and reduced effects compared with the wild type were observed in the cytokinin receptor mutants *ahk2-2tkahk3-3*, *ahk2-2tk*, and *ahk3-3* (Fig 3B). However, another parameter pertaining to chloroplast-level acclimation, ETR_{max}/Chl, was reduced in these mutants in the same way as in the wild types (Supplementary Fig. S1A at *JXB* online). The lower Chl *a*/*b* normally observed in shaded leaves reflects the greater abundance of light-harvesting complex II (LHCII) that contains Chl *b*, relative to core Chl of photosystem II (PSII) and the chlorophyll associated with PSI which is only Chl *a* (Evans and Seemann, 1989; Anderson *et al.*, 1995). The results suggest that cytokinins, phyD, and possibly phyA and phyC, are involved in the regulation of the abundance of LHCII. It should be noted that the Ws-2 accession was used, which has the *phyD* mutation, but also many other polymorphisms compared with Col-0 and L*er*, so the possibility that other genes also affect photosynthetic acclimation of this accession cannot be excluded.

The possibility that cytokinins are involved is exciting since experimental reduction of transpiration rates was sufficient to induce chloroplast-level acclimation in *Arabidopsis* (Fig. 4) and a range of other species (Pons and Bergkotte, 1996; Pons and Jordi, 1998; Pons *et al.*, 2001). Furthermore, *ahk3-3*, *ahk2-2tkahk3-3*, and 35S::CKX3 had a reduced Chl *a*/*b* in high light compared with the wild type (Fig. 3B), which suggests that cytokinin has an impact on this aspect of chloroplast organization during development. It shows that Chl *a*/*b* is not only under control of the local light environment, but is also affected systemically, as is the case with the allocation of photosynthetic capacity in canopies. The effect of the local transpiration rate on Chl *a*/*b* further suggests that it is mediated by the action of cytokinins carried in the transpiration stream according to the model described above.

Conclusions

The regulation of photosynthetic acclimation to canopy density at the whole-plant level involves photoreceptors and xylem-transported cytokinins operating in a redundant manner, because neither mechanism can fully explain the distribution of photosynthetic capacity in response to partial shade. No evidence was found for a role for sugars or nitrate, although their involvement could not be excluded completely. A possible role for phyD and cytokinin in regulating Chl *a*/*b* as part of the acclimation at the chloroplast level has been identified. The distribution of cytokinins in tobacco leaf canopies could be explained in part by the effect of irradiance on delivery of cytokinins via the transpiration stream. This mechanism is involved in acclimation at both the whole-plant and chloroplast levels.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. ETR_{max} per unit chlorophyll (A) and chlorophyll level per unit area (B) of *Arabidopsis* mutants and transgenics.

Acknowledgements

We gratefully acknowledge Yvonne de Jong-van Berkel and Sevgi Öden for skilled technical assistance. We thank two anonymous reviewers for their helpful suggestions.

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