Cytokinin Import Rate as a Signal for Photosynthetic Acclimation to Canopy Light Gradients 1[W][OA]

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Plants growing in dense canopies are exposed to vertical light gradients and show photosynthetic acclimation at the whole-plant level, resulting in efficient photosynthetic carbon gain. We studied the role of cytokinins transported through the transpiration stream as one of probably multiple signals for photosynthetic acclimation to light gradients using both tobacco (Nicotiana tabacum) and Arabidopsis (Arabidopsis thaliana). We show that substantial variation in leaf transpiration parallels the light gradient in tobacco canopies and experimental reduction of the transpiration rate of a leaf, independent of light, is sufficient to reduce photosynthetic capacity in both species, as well as transcript levels of the small subunit of Rubisco (rbcS) gene in Arabidopsis. Mass spectrometric analysis of xylem sap collected from intact, transpiring tobacco plants revealed that shaded leaves import less cytokinin than leaves exposed to high light. In Arabidopsis, reduced transpiration rate of a leaf in the light is associated with lower cytokinin concentrations, including the bioactive trans-zeatin and trans-zeatin riboside, as well as reduced expression of the cytokinin-responsive genes ARR7 and ARR16. External application of cytokinin to shaded leaves rescued multiple shade effects, including the S transcript levels in both species, as did locally induced cytokinin overproduction in transgenic tobacco plants. From these data, we conclude that light gradients over the foliage of a plant result in reduced cytokinin activity in shaded leaves as a consequence of reduced import through the xylem and that cytokinin is involved in the regulation of whole-plant photosynthetic acclimation to light gradients in canopies.

Photosynthetic acclimation of leaves to their local light environment in canopies contributes to increased whole-plant carbon gain and fitness (Anten, 2005; Hirose, 2005; Boonman et al., 2006). Leaves exposed to high light (HL) have greater leaf mass per unit area (LMA) and photosynthetic capacity per unit area than shaded leaves (Lambers et al., 1998). This is true both when whole plants exposed to different irradiances are compared (Evans and Poorter, 2001) and when leaves on the same plant are compared at different positions on the vertical light gradient in canopies (for review, see Grindlay, 1997; Anten et al., 2000; Kull, 2002). With increasing canopy density, lower leaves become more shaded by the foliage above them and plants acclimate by allocating less dry matter and photosynthetic capacity to the lower leaves and relatively more to the well-illuminated upper leaves. Exposure of a plant to such a light gradient or its partial shading is distinct from whole-plant shading, for instance, because partial shading accelerates senescence of the shaded leaves, whereas whole-plant shading does not (Pons and Pearcy, 1994; Weaver and Amasino, 2001).

Although the process of photosynthetic acclimation to light gradients has been extensively studied, little is known about the signaling mechanisms regulating it, except that multiple mechanisms are likely to be involved (Bailey et al., 2001; Terashima et al., 2005). Photoreceptors mediate light effects on many aspects of plant development (Sullivan and Deng, 2003) and are probably involved in photosynthetic acclimation as well. Phytochromes perceive the changes in light spectral quality that are associated with shading by foliage (Rousseaux et al., 2000; Pons and de Jong-van Berkel, 2004). However, all investigated photoreceptor mutants to date have the ability to acclimate to reduced irradiance (for review, see Terashima et al., 2005), so whether photoreceptors have a role in the response to irradiance effects remains to be elucidated. Evidence suggests that the light gradient may also be perceived indirectly through parallel changes in transpiration rates (E) of leaves and, thereby, import of compounds via the xylem (Pons and Bergkotte, 1996; Pons and Jordi, 1998; Pons et al., 2001). When E was decreased
by surrounding the leaf with humid air in a transparent leaf chamber, photosynthetic acclimation was induced even though irradiance was not reduced. Shaded leaves have decreased transpiration rates because stomata reduce their aperture in response to low light (LL; Rashke, 1975), so they receive less xylem sap than well-illuminated leaves. The differential distribution of xylem-carried compounds over the foliage might therefore provide leaves with a reliable signal pertaining to their light climate.

Among the compounds carried in the xylem sap, the hormone cytokinin is likely to have a regulatory role in photosynthetic acclimation for two reasons. First, import of root-synthesized cytokinin into the shoot depends on shoot transpiration rate (Letham et al., 1990; Aloni et al., 2005), so cytokinin distribution among leaves with different transpiration rates is likely to be regulated similarly. Second, cytokinin is known to stimulate the expression of photosynthetic enzymes like Rubisco and, more generally, the development of functional chloroplasts (Flores and Tobin, 1988; Chory et al., 1994; Kusnetsov et al., 1994). Indeed, cytokinin application to shaded leaves counteracted the decline in LMA and photosynthetic capacity in several species (Pons and Jordi, 1998; Pons et al., 2001). However, a direct demonstration that perception of and acclimation to light gradients involve differential distribution of root-borne cytokinins is lacking thus far.

In this article, we investigate the role of cytokinin as a signal for canopy light gradients using a variety of approaches in tobacco (Nicotiana tabacum) and Arabidopsis (Arabidopsis thaliana). Tobacco was used to measure density effects on \( E \) and photosynthetic acclimation in plants growing in canopies. This species was also used for the collection of xylem sap for cytokinin analysis by advanced mass spectrometry to obtain estimates of cytokinin delivery rates to leaves with different transpiration rates. Furthermore, transgenic tobacco plants were used that contain a locally inducible cytokinin biosynthesis gene. Both species showed similar photosynthetic acclimation when single leaves were shaded or treated with humid air, which could be rescued by externally applied cytokinin. By demonstrating in this manner that Arabidopsis is a suitable model for leaf level responses to light gradients, we were able to make use of the substantial knowledge of cytokinin signal transduction in this species that has been gathered in recent years (Hwang and Sheen, 2001; Kieber, 2002). In particular, cytokinin-responsive genes have been described, the type A Arabidopsis response regulators (ARRs; D’Agostino et al., 2000; Kiba et al., 2002), which provide a measure of cytokinin activity. In combination with detailed cytokinin measurements, we analyzed transcript levels of several ARRs to determine whether reduced \( E \) results in a decrease in the concentration of bioactive cytokinin and expression of cytokinin-regulated genes. Although not the only regulatory agent in the process, our results suggest that the delivery rate of cytokinins in the transpiration stream to leaves acts as a signal for photosynthetic acclimation to light gradients.

![Figure 1. Transpiration rates and photosynthetic acclimation of tobacco leaves grown in open (3.6 plants m\(^{-2}\)) and dense (35 plants m\(^{-2}\)) canopies. Shown is the distribution over canopy height of PPFD (A), \( g_s \) (B), \( E \) (C), LMA (D), \( A_{\text{max}} \) (E), and transcript levels of \( rbcS \) (F). Plants were grown for 11 weeks and measurements were taken during the last 2 weeks. \( E \) was measured as weight loss of excised leaves placed in their original position. Transcript levels were determined using real-time RT-PCR, using \( L25 \) as an internal control and expressed relative to the value of leaves at intermediate position in the open canopy. Data are means ± SE; \( n = 3 \) to 6.](https://www.plantphysiol.org/articles/143/2007/1842 FIG1.png)
midway in either stand, \( E \) was determined by \( g_{s} \). In dense-stand plants, there was a greater decline in LMA (Fig. 1D), photosynthetic capacity \( (A_{\text{max}}) \) Fig. 1E), and small subunit of Rubisco \( (rbcS) \) transcript level (Fig. 1F) from the top of the canopy downward than in open-stand plants. Differences between densities were most pronounced in midcanopy leaves. Lower leaves of the open canopy were already senescing, causing also low \( g_{s} \), \( E \), \( A_{\text{max}} \), and, to some extent, \( rbcS \) transcript levels. All of the parameters mentioned above showed significant effects of relative height, density, and the height \( \times \) density interaction when leaves in top and intermediate positions were compared or when all heights were analyzed \( (P < 0.05; \text{analysis of covariance}) \). Thus, plants had acclimated to the steeper light gradient at high-canopy density by concomitant changes in allocation of dry matter, photosynthetic capacity, and \( rbcS \) transcription, and this was accompanied by parallel changes in \( E \).

RESULTS

Photosynthetic Acclimation to Light Gradients in Tobacco Canopies

To quantify leaf transpiration rates and illustrate photosynthetic acclimation to canopy light gradients in canopies, tobacco was grown at two contrasting densities, with a much steeper vertical light gradient developing in the dense stand compared to the more open stand (Fig. 1A). Self-shading reduced irradiance incident on the lower leaves in open-stand plants, but shading was far more severe in the dense stand. Consequently, stomatal conductance \( (g_{s}) \) and \( E \) also declined more strongly from the top of the canopy downward in the dense stand compared to the open stand (Fig. 1, B and C). Because there were no apparent gradients in leaf temperature or relative air humidity in either stand, \( E \) was determined by \( g_{s} \).

Cytokinin as a Signal for Canopy Light Gradients

Table II. Effects of partial shade, experimental reduction of transpiration rate, and cytokinin on photosynthetic acclimation in tobacco leaves

| Experiment | Treatment | Leaf Dry Mass | LMA | \( A_{\text{max}} \) | \( g_{s} \) | \( g_{s} \) m \(^{-2} \) | \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \) | \( rbcS \) Transcript Relative |
|------------|-----------|---------------|-----|----------------|------|----------------|---------------------------------|--------------------------|
| 1          | HL - BA   | 1.355 ± 0.046 a | 38.5 ± 0.6 a | 19.1 ± 0.6 a | 0.711 ± 0.038 a | 0.398 ± 0.046 a | 0.509 ± 0.046 a | 0.515 ± 0.046 a |
| 2          | HL + BA   | 1.389 ± 0.032 a | 38.4 ± 0.9 a | 18.7 ± 0.5 a | 0.682 ± 0.061 a | 0.389 ± 0.032 a | 0.509 ± 0.032 a | 0.515 ± 0.032 a |
| 3          | HL + control | 0.191 ± 0.009 a | 28.7 ± 1.9 a | 17.0 ± 1.3 a | – | – | – | – |
| 4          | HL + humid | 0.138 ± 0.016 b | 21.5 ± 1.2 b | 9.1 ± 0.4 b | – | – | – | – |
| 5          | HL + dex  | 0.265 ± 0.007 b | 16.7 ± 0.6 c | – | – | – | – | – |
| 6          | HL + dex  | 0.465 ± 0.031 a | 22.5 ± 0.9 b | – | – | – | – | – |

Table I. Concentration of cytokinins detected in xylem sap collected from the midrib of a tobacco leaf exposed to HL (PPFD 200 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) or LL (PPFD 14 \( \mu \text{mol} \text{ m}^{-2} \text{s}^{-1} \)) on the same plant

| Cytokinin | Cytokinin Concentration (IF) |
|-----------|-------------------------------|
| iP        | 61 ± 30                       |
| iPR       | 56 ± 6                        |
| iPRP      | 85 ± 26                       |

Xylem sap was collected from both leaves simultaneously through a small incision in the midrib, whereas roots were pressurized with a computer-controlled pressure probe. A single leaf was shaded at the end of the light period and xylem sap was collected during a period of several hours in the following day from the shaded leaf and an adjacent leaf in HL. The rest of the plant remained in HL. Cytokinins were analyzed using micro LC-MS/MS. No Z-type or dihydrozeatin-type cytokinins were detected in significant amounts. Values are means of three independent experiments ± SE. Shading had no significant effect on cytokinin concentrations (Student’s \( t \) test).
isopentenyl (iP)-type cytokinins in low amounts in the xylem sap of shaded and control leaves (Table I). Concentrations of these cytokinins were not significantly different between the shaded and control leaves. Because cytokinin delivery rates are the product of their concentration in the xylem sap and E, it follows that shaded leaves received less iP-type cytokinins than light-exposed leaves.

Canopy light gradients can be mimicked by shading a leaf on a plant while the rest of the plant remains exposed to light (e.g. Pons and Pearcy, 1994). Tobacco leaves shaded for 10 d showed reduced dry mass, LMA, A_{max}, and rbcS transcript levels compared to leaves that were not shaded (Table II), like the bottom leaves in the dense canopy (Fig. 1). These effects were also observed when a single leaf was surrounded by humid air in a transparent leaf chamber (Table II). In this situation, the leaf remained exposed to light, but E was reduced approximately 2-fold compared to a control leaf in a chamber flushed with normal air. Thus, in tobacco, reduced E is sufficient to induce photosynthetic acclimation independent of changes in irradiance.

When cytokinin was exogenously applied to the shaded leaves, all the effects of shade were significantly counteracted (Table II). Furthermore, leaf dry mass and LMA also increased when cytokinin overproduction was locally induced in shaded leaves of transgenic TFM-isopentenyl transferase (ipt) plants (Table II). In these plants, a dexamethasone (dex)-inducible ipt gene, encoding a cytokinin biosynthesis enzyme, allows strong localized cytokinin overproduction (Böhner and Gatz, 2001). These data further support the notion that photosynthetic acclimation to shade is mediated by cytokinin through the effect of irradiance on the delivery of cytokinin via the transpiration stream.

Cytokinin Concentration and Activity in Arabidopsis Leaves

To assess the importance of cytokinin delivery rates, it is necessary to demonstrate that this affects the cytokinin concentration and action in the receiving leaf, as well as its influence on photosynthetic acclimation. Whereas tobacco is a suitable species for the analysis of cytokinin delivery and canopy density effects, quantification of cytokinin activity is better achieved in Arabidopsis because cytokinin-responsive genes have been characterized in this species. As a first step, we needed to establish that Arabidopsis can be used in the study of photosynthetic acclimation to light gradients because it is naturally not exposed to vertical light gradients like erect plants in dense vegetation are. Figure 2 shows that, also in Arabidopsis, partial shading resulted in photosynthetic acclimation and this was mimicked by humid air treatment of a leaf remaining in the light. Shading of a leaf had no effect on the leaf-to-air vapor pressure difference (VPD; Fig. 2A), but decreased g_s (Fig. 2B) and therefore E (Fig. 2C). When very low VPD was applied in the leaf chamber through flushing with humid air (Fig. 2A), a compensatory increase in g_s (Fig. 2B) was observed, but E was significantly decreased nonetheless (Fig. 2C). Both treatments resulted in significant decreases in LMA (Fig. 2D) and A_{max} (Fig. 2E), similar to the response in tobacco. Chlorophyll and total nitrogen levels per unit area were not changed as a result of shading, but were reduced due to the humid air treatment (Table III). However, a separate experiment under identical conditions revealed that nitrate accumulated in shaded leaves (14.86 ± 2.55 mmol m^{-2} and 1.61 ± 0.68 mmol m^{-2} nitrate in LL and HL leaves, respectively; n = 6) so organic nitrogen levels were reduced, as would be expected.

A continuously reduced delivery of cytokinin to shaded leaves should result in decreased cytokinin concentrations and lower expression of cytokinin-responsive genes, including those encoding photosynthetic enzymes. The total concentration of cytokinins in shaded...
Arabidopsis leaves was reduced compared to leaves that remained in the light (Table IV). In leaves remaining in the light, treatment with humid air also significantly reduced the concentration of cytokinins relative to a normal air control. Cytokinins showing significant change due to the shading treatment were not the same as those changed by the humid air treatment. The most abundant cytokinins, isopentenyl riboside monophosphate (iPRP) and trans-zeatin (Z) riboside monophosphate (ZR), did not show consistent significant effects between treatments and cis-zeatin riboside (ZR) was increased due to shading only. Nevertheless, both shading and humid air caused reductions to some extent in iPRP and trans-ZRP concentrations. Moreover, the concentration of the bioactive trans-Z and trans-ZR was reduced below or close to the detection limit due to both treatments (Table IV). The observed reduction in bioactive cytokinin concentration was accompanied by reduced transcript levels of cytokinin-responsive ARR16 and ARR7 (D’Agostino et al., 2000; Kiba et al., 2002), both when leaves were shaded and as a result of humid air treatment (Fig. 3). Reduced E alone, without a change in irradiance, was therefore sufficient to reduce cytokinin concentrations and expression of cytokinin-regulated genes. These data strongly suggest that cytokinin concentrations and activity were reduced in shaded Arabidopsis leaves as a consequence of reduced transpiration rates.

Transcript levels of the negatively light-regulated PHYTOCHROME (PHY) A gene (Canton and Quail, 1999) were also analyzed, which is itself regulated by photoreceptors PHYA and PHYB. Consistent with its known response, PHYA transcript levels were highest in LL and lowest in HL, but not affected by humid air treatment (Fig. 3). Thus, whereas the effects of partial shade may involve PHYA and PHYB signaling, photosynthetic acclimation can also be regulated independently of these photoreceptors through cytokinin. Further, transcript levels of rbcS were reduced by shading and humid air treatment. Application of cytokinin to shaded leaves strongly increased ARR transcripts (Fig. 3), confirming their cytokinin responsiveness in our experimental system. Applied cytokinin also significantly increased rbcS transcription in the shade. Adjustment of photosynthetic capacity by cytokinin involves regulation of rbcS transcription, as was also indicated by the results found for tobacco.

### Table III. Chlorophyll and nitrogen content of Arabidopsis leaves

For experimental details and statistical notation (Student’s t tests), see Figure 2.

| Treatment | Chlorophyll | Total Nitrogen |
|-----------|-------------|----------------|
| HL        | 350.60 ± 6.90 | 80.77 ± 4.38 |
| LL        | 344.45 ± 6.48 | 72.14 ± 4.28 |
| Dry       | 342.87 ± 4.10 | 86.17 ± 3.53 |
| Humid     | 237.66 ± 4.25 | 64.47 ± 1.29 |

### Table IV. Effects of partial shade and experimental reduction of transpiration rate on cytokinin concentration in Arabidopsis leaves

A single attached leaf was exposed to HL or LL or a leaf was enclosed in a transparent leaf chamber (HL) flushed with humid air or growth chamber air (dry) as a control for 3 d. The rest of the plant remained in HL and growth chamber air. Experimental conditions were as in Figure 2. Cytokinins were analyzed using micro LC-MS/MS. Data are expressed per unit fresh mass and represent means ± se; n = 3. For statistical notation of significance (Sign.; Student’s t tests), see Figure 2. †, test was not performed because one of the values was below the detection limit. ZNG, Zeatin-N9-glucoside; iPNG, isopentenyl-riboside-N9-glucoside. No dihydrozeatin-type cytokinins were detected.
3-fold higher than in the shaded leaves. Whereas humid air treatment also reduced ETR\textsubscript{\text{max}} within 3 d compared to the normal air control, the initial decline was less strong than the shade effect. After 9 d, however, humid air treatment did result in an effect of similar magnitude to shade on photosynthetic capacity (Fig. 4A). This is consistent with the decline in chlorophyll of these leaves after 7 d (Table III), which also indicated the induction of senescence. When cytokinin was applied to shaded leaves, the initial decline in ETR\textsubscript{\text{max}} could not be prevented, although ETR\textsubscript{\text{max}} of cytokinin-treated leaves remained higher between 6 and 12 d compared to controls (Fig. 4B). Furthermore, humid air treatment resulted in extensive yellowing and leaf death in most leaves by 12 d (Fig. 5). Shading for 12 d did not induce senescence, contrary to a previous report on completely darkened Arabidopsis leaves (Weaver and Amasino, 2001). Senescence induced by humid air treatment could also be rescued by applying cytokinin, as determined from the fraction of remaining green leaf area (Fig. 5). These data suggest that transpiration rate and cytokinins are more involved in the later stages of photosynthetic acclimation and senescence induced by shade, but less so in the first few days of the shade response.

**DISCUSSION**

The results presented here suggest that, in tobacco and Arabidopsis, cytokinin delivery through the xylem is dependent on \( E \). Moreover, cytokinin mediates photosynthetic acclimation to light gradients in canopies by regulation of gene expression and consequently photosynthetic capacity. Because \( E \) is controlled by the direct effect of irradiance on stomatal aperture, this

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**Figure 3.** Effects of partial shade, experimental reduction of transpiration rate, and cytokinin application on transcript levels of ARR16, ARR7, PHYA, and rbcS in Arabidopsis leaves. A single attached leaf was exposed to HL (PPFD 200 \text{\mu}mol m\textsuperscript{-2} s\textsuperscript{-1}) or LL (PPFD 14 \text{\mu}mol m\textsuperscript{-2} s\textsuperscript{-1}) or a leaf was enclosed in a transparent leaf chamber (HL) flushed with humid air or growth chamber air (dry) as a control for 4 d. VPD was as in Figure 2. Also, shaded leaves were treated with a 100 \text{\mu}M solution of the synthetic cytokinin BA or a vehicle control (\(-BA\)) at noon on day 0 and day 3. The rest of the plant remained in HL and growth chamber air. Leaves were harvested at 2 pm on day 0 (start of treatment) and day 4. Transcript levels were determined by real-time RT-PCR, normalized to 18S rRNA, and expressed relative to the value at day 0, which was set arbitrarily as 1. Values represent means ± se, \( n = 6 \). For statistical notation (Student’s \( t \) tests), see Figure 2.

**Figure 4.** Time course of effects of partial shade, experimental reduction of transpiration rate, and cytokinin application on ETR\textsubscript{\text{max}} of Arabidopsis leaves. A single attached leaf was exposed to HL (PPFD 200 \text{\mu}mol m\textsuperscript{-2} s\textsuperscript{-1}) or LL (PPFD 14 \text{\mu}mol m\textsuperscript{-2} s\textsuperscript{-1}) or a leaf was enclosed in a transparent leaf chamber (HL) flushed with humid air or growth chamber air (dry) as a control for 4 d (A). VPD was as in Figure 2. Also, shaded leaves were treated with a 200 \text{\mu}M BA or a vehicle control (\(-BA\)) at noon on day 0 and every 3 d thereafter (B). The rest of the plant remained in HL and growth chamber air. Twelve days after the start of treatment, leaves in humid air had mostly yellowed, so no measurements were taken at this time point. Values represent means ± se, \( n = 6 \).
presented results of two independent experiments with three replicates each are presented ± SE. For statistical notation (Student's t tests), see Figure 2.

simple mechanism provides plants with a reliable signal to sense a light gradient over their foliage.

Transpiration Rate and Cytokinin Delivery

We have found substantial variation in transpiration rates in the tobacco leaf canopies. In the dense stand, transpiration of intermediately positioned leaves was 87% lower than of leaves at the top of the canopy (Fig. 1C), whereas photosynthetic photon flux density (PPFD) was 71% lower (Fig. 1A). Such differences in transpiration rate are sufficient to induce photosynthetic acclimation without any change in irradiance, as demonstrated by experimental manipulation using humid air for tobacco (Table II), Arabidopsis (Fig. 2), and several other species (Pons and Bergkotte, 1996; Pons and Jordi, 1998; Pons et al., 2001). A study using Phaseolus vulgaris also showed that the decline in photosynthetic capacity of a leaf was proportional to the transpiration rate by imposing a range of vapor pressure differences in transparent leaf chambers (Pons and Bergkotte, 1996). A change in irradiance is apparently not needed to induce photosynthetic acclimation to shade. We show that photosynthetic acclimation occurs in the light due to decreased transpiration rate without a change in expression of the light-regulated PHYA gene (Fig. 3). These results are in accordance with the observation that various photoreceptor mu-

tants retain the ability to form sun and shade leaves (for review, see Terashima et al., 2005). In an extensive survey of photoreceptor mutants of the phytochrome, cryptochrome, and phototropin family, which included double and triple mutants, we also found that all of these genotypes responded normally to shading by lowering photosynthetic capacity (Boonman, 2006).

Several studies have shown that, at the whole-shoot level, transpiration rate controls the import of root-borne cytokinins (Beck and Wagner, 1994; Havelange et al., 2000; Aloni et al., 2005). Cytokinins can increase stomatal aperture (Incoll and Jewer, 1987), so potentially transpiration rates of leaves with high cytokinin delivery may be increased further through this mechanism. Cytokinin delivery is the product of their concentration in the xylem sap and sap flow rate. Parallel to the gradient in E, a gradient of cytokinin delivery rates may therefore be expected, provided there are no other factors affecting cytokinin concentration. It is known that cytokinins may be modulated along their transport pathway, for example, by movement from the xylem to parenchyma cells in the stem or petioles (Jameson et al., 1987; Singh et al., 1992).

However, our measurements showed that the concentration of cytokinins detected in the xylem sap did not differ significantly between a shaded leaf and a light-exposed leaf on the same plant (Table I). The setup employed was designed to yield a sample representative of the xylem sap actually delivered in vivo to both leaves. Measurements of cytokinins in the xylem sap of tobacco and other plant species have been reported before (Singh et al., 1992; Beck and Wagner, 1994; Emery et al., 2000; Yong et al., 2000), but usually sap was collected after excision of leaves or even whole shoots. Because the sampled leaves remained attached and an incision was made in the midrib halfway the length of the leaf, transpiration could be maintained through the many xylem vessels connecting the entire leaf area with the main vascular bundle in the petiole. Thus, the shaded leaf maintained a substantially lower transpiration rate than the light-exposed leaf. Also, any effect of retention or loading of cytokinins in the petiole would have been detected in the sampled xylem sap. Our data lead us to conclude that the delivery of cytokinins is reduced in shaded tobacco leaves proportional to the reduction in transpiration rate.

For the cytokinin delivery rate to be effective as a signal for leaves, it should control the concentration of active cytokinin at the site of action and elicit cytokinin-specific responses in proportion to the delivery rate. Many other factors may control active cytokinin concentrations, including local synthesis, breakdown, interconversions, compartmentation, and export through the phloem (Brzobohatý et al., 1994; Mok and Mok, 2001; Werner et al., 2001; Kieber, 2002). It has been shown that young leaves contain more Z-type cytokinins than older leaves, which has been ascribed to localized synthesis in young leaves (Singh et al., 1992; Nordström et al., 2004). It is not known whether the effect of canopy density involves changes in cytokinin

Figure 5. Effects of experimental reduction of transpiration rate and cytokinin application on senescence of Arabidopsis leaves. Shown are photographs of a representative leaf that was enclosed in a transparent leaf chamber (HL; PPFD 200 μmol m⁻² s⁻¹) flushed with growth chamber air (dry [A and B] or humid [C and D] air) for 12 d and treated with a 200 μmol solution BA (B and D) or a vehicle control (−BA; A and B) on day 0 and every 3 d thereafter. The rest of the plant remained in HL and growth chamber air. VPD was as in Figure 2. Scale in centimeters. E, Fraction of remaining green leaf area after 12 d. The results of two independent experiments with three replicates each are presented ± SE. For statistical notation (Student’s t tests), see Figure 2.
synthesis or any of the other mentioned processes. To specifically focus on the effect of transpiration rate on the concentration and activity of cytokinins, we chose to make use of Arabidopsis because of the availability of gene sequences encoding cytokinin-responsive genes. Our data show that the concentration of cytokinins, including the bioactive trans-Z and trans-ZR, is reduced in shaded leaves and, more importantly, in leaves in the light with reduced transpiration rate (Table IV). Not all cytokinins responded similarly between the two treatments, which may have been caused by effects of light on one of the processes mentioned above. Bioactive cytokinins did respond similarly, however. Moreover, the transcript levels of two cytokinin-responsive genes showed concomitant changes, as well as the rbcS gene (Fig. 3). The fact that this occurs independently of light strongly suggests that transpiration rate controls active cytokinin levels and regulation by cytokinin of gene expression.

Mode of Action of Cytokinin in Light Gradient Effects

Our time-course data of photosynthetic capacity (Fig. 4) showed that reduced cytokinin import cannot fully explain the effect of shading on photosynthetic acclimation. First, a reduction in transpiration rate independent of light reduced photosynthetic capacity, but not as strongly and several days later than partial shading did. The initial rapid decline in capacity induced by shade therefore does not appear to depend only on the reduction in transpiration rate and is apparently also regulated through an alternative mechanism. It should be noted, though, that humid air treatment reduced transpiration rate to a lesser extent than shading (Fig. 2C), proportional to its smaller effect on photosynthetic capacity (Fig. 2E). Second, application of cytokinins counteracted the effect of partial shading on capacity, but did not completely rescue it to the level of leaves remaining in growth light in both Arabidopsis (Fig. 4B) and tobacco (Table II). These observations suggest that, besides cytokinin, other signal transduction pathways are involved, too. Other compounds carried in the xylem sap, such as nitrate, might be delivered to leaves proportional to their transpiration rates and act as a signal. Nitrate is known to stimulate its own assimilation and incorporation into amino acids and may mimic certain cytokinin effects as well (Stitt, 1999). Our analyses showed, however, that nitrate accumulated in shaded Arabidopsis leaves, arguing against such a role at least for nitrate.

In further support of a function for cytokinin, our data on Arabidopsis leaves treated with humid air showed that a decline in cytokinin concentration and activity is associated with reductions in LMA, photosynthetic capacity (Fig. 2), and rbcS and type A ARR transcript levels (Fig. 3), and ultimately senescence (Fig. 5). Cytokinin application counteracted all these effects. Also in tobacco, application of cytokinin or induced overproduction of cytokinin in shaded leaves counteracted shade effects (Table II), as has been reported for several other species as well (Pons and Jordi, 1998; Pons et al., 2001). Cytokinin was only effective in shaded leaves and not in light-exposed leaves, again indicating that photosynthetic acclimation was caused at least in part by reduced endogenous cytokinin levels in shaded leaves. These data are consistent with the known functions of cytokinin, like stimulating the synthesis of photosynthetic enzymes (Flores and Tobin, 1988; Chory et al., 1994; Kusnetsov et al., 1994) and inhibiting senescence (Gan and Amasino, 1995; Jordi et al., 2000) and protease activity (Li et al., 2000). No other known root-born compound has properties that are so consistent with a role in photosynthetic acclimation. Based on the combination of data presented here, we propose the following model: continuous high delivery rate of cytokinins via the transpiration stream to light-exposed leaves contributes to the maintenance of a large photosynthetic apparatus, whereas low cytokinin delivery to shaded leaves leads to reduced synthesis of photosynthetic proteins, but also stimulates their breakdown and accelerates senescence.

CONCLUSION

The delivery of xylem-carried cytokinins to shaded leaves of plants growing in dense canopies is reduced compared to light-exposed leaves as a result of their lower transpiration rates. Both shading and reduction of E in light-exposed leaves are sufficient to reduce photosynthetic capacity, which is associated with consistent reduction in the concentration of bioactive cytokinins and cytokinin activity. From these data, we conclude that the delivery of cytokinins is involved in the regulation of whole-plant photosynthetic acclimation to light gradients in canopies.

MATERIALS AND METHODS

Tobacco Density Experiment

Tobacco (Nicotiana tabacum L. cv Wisconsin 38) was grown in a greenhouse under natural daylight during the spring. Seeds were sown on potting soil mixed with sand (3:1 [v/v]), and after 6 weeks plants were transferred to 3-L pots filled with a mixture of soil, sand, and clay (16:1:1; by volume), supplemented with 7 g of slow-release complete fertilizer (Osmocote mini plus, release time 3–4 months; Scotts) and 7 g of 17% CaMg carbonate (Vitasol BV) per pot. An open stand with 3.6 plants m\(^{-2}\) and a dense stand with 35 plants m\(^{-2}\) were established. To reduce border effects in the dense stand, the outer plants were transgenic P\(_{\text{KOR}}\)-ipt tobacco that retained their lower leaves (Gan and Amasino, 1995) and only plants inside the stands were used for measurements. The pots were placed on irrigation mats that were watered daily. After a further 4 weeks, the distribution of PPFD, \(\phi_s\), and \(E\) in the stands was measured. PPFD in the dense canopy was measured on a horizontal plane in six positions at 10-cm-height increments using a line sensor (AccuPAR Ceptometer PAR-80; Decagon) and directly above the canopy using a quantum sensor (LI-185A; LI-COR), 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. Also, leaves were sampled from three different heights, snap frozen in liquid nitrogen, and stored at ~80°C until analysis of \(rbcS\) transcript levels. Three biological replicates for each height in each stand were obtained by combining sampled leaves from a total of 12 plants in the dense stand and six
in the open stand. Finally, 1 week later, when plants were 11 weeks old, six randomly selected plants per stand were transported to the laboratory for measurement of $A_{\text{max}}$ on leaves at different heights, after which they were destructively harvested and analyzed for the distribution of leaf area and dry mass. Leaf area was measured using a leaf area meter (LI-3100; LI-COR) and dry mass after oven drying at 70°C for at least 24 h.

**Tobacco Xylem Sap**

Tobacco (cv SNN) seeds were germinated in moist sand in a climate-controlled growth room. After 3 weeks, seedlings were transferred to an aeroponics system and grown for a further 5 weeks as described previously (Herdel et al., 2001). Growth conditions were 12/12-h photoperiod, PPFD 200 $\mu$mol m$^{-2}$ s$^{-1}$ at plant level (HL), 25°C, and 60% relative air humidity. A single plant was then transferred to a computer-controlled root pressure chamber (Schurr and Schulze, 1995). To simulate canopy light gradients, one almost fully expanded leaf was shaded (LL), whereas an adjacent control leaf remained in the light, as did the rest of the plant. Shading was applied by enclosing a 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 a plastic foam so gas exchange and leaf expansion were not hindered by the treatment. The upper paper sheet had fine gray print on the downward-facing side. Shading reduced PPFD to 7% of the HL control and was spectrally neutral, as determined with a spectroradiometer (LI-1800; LI-COR). Shading of the leaf started at the end of the light period. The following day, xylem sap was collected from both leaves simultaneously over a period of several hours through a small cut in the midrib approximately halfway the length the leaves. Pressure was applied to the roots to allow sap flow from both cuts, whereas $O_2$ and CO$_2$ partial pressures of the air inside the pressure chamber were maintained at ambient levels (Schurr and Schulze, 1995). The xylem sap was collected in a vial kept on ice, containing 1 mM Na$_2$MoO$_4$ to inhibit phosphatase activity (Dieleman et al., 1997), and subsequently stored at $-80^\circ$C until cytokinin analysis. The total volume of xylem sap collected was approximately 2 mL, representing only a marginal fraction of the estimated total amount of xylem sap collected from both leaves simultaneously over a period of several hours.

**Partial Shade, Humidity Treatment, and Manipulation of Cytokinin Levels**

Tobacco and Arabidopsis (Arabidopsis thaliana) 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 as a PPFD of 200 $\mu$mol m$^{-2}$ s$^{-1}$, except during the humidity experiments with tobacco when PPFD was 300 $\mu$mol m$^{-2}$ s$^{-1}$. Growth chamber PPFD is designated as HL. Tobacco was grown in 1.5-L pots on a mixture of soil and perlite (1:1, v/v) containing 3 g L$^{-1}$ slow-release complete fertilizer (Osmocote mini plus, release time 3–4 months; Scotts); 0.5 g L$^{-1}$ micronutrient fertilizer (MicroMins; Scotts Europe BV), 2 g L$^{-1}$ 12% CaMg carbonate, and 5 mL L$^{-1}$ 75 mM KH$_2$PO$_4$. With this nutrient dose, nitrogen was mildly limiting, but all other nutrients were sufficiently available as tests revealed. Surplus nitrogen availability may inhibit the response to partial shading, which involves nitrogen reallocation. Pots were placed in a tub covered with a glass plate for a period of 10 d and then placed on an irrigation mat that was automatically watered daily. Tobacco cv SNN was used for the humidity experiments and the fourth true leaf counted from below was used. Tobacco cv Wisconsin 38 was used in the experiment with partial shade and cytokinin application and the sixth leaf was used. Plants of both cultivars responded similarly to partial shade and were 30 d old when treatments started.

Arabidopsis accession Columbia-0 seeds were stratified for 3 d at 4°C on moist filter paper in sealed petri dishes and subsequently sown on a mixture of soil and perlite (1:1, v/v) in 220-mL plastic pots containing 150 g of slow-release complete fertilizer (Osmocote mini plus, release time 3–4 months; Scotts Europe BV), 150 mg of micronutrient fertilizer (MicroMins; Scotts Europe BV), 600 mg of 17% (w/w) CaMg carbonate (Vitasol BV), and 1.4 mL of 125 mM KH$_2$PO$_4$ per pot. Again, tests showed that nitrogen was mildly limiting under these conditions. Total nitrogen was determined on homogenized dry material with an elemental analyzer (Carlo Erba; model EA NA 1100) and the results were used for a reagentization study (Scotts Europe BV, 1975). Homogeneous plants were selected based on dimensions of the treated leaf as measured with a caliper. In the experiments, the eighth true leaf from below was used, which was still expanding. Plant age was 34 to 35 d after sowing when treatments started. Throughout the experiments, leaves adjacent to the treated leaf were gently bent away to prevent the treated leaf from being shaded.

Partial shade was applied to both species using layers of paper as described above. Transpiration rate of an attached leaf was reduced independent of light by enclosing the leaf in a transparent leaf chamber flushed with humid air, as described earlier (Pons and Bergkotte, 1996; Pons et al., 2001). Pressurized air was humidified in a Perspex vessel filled with water and had a dew point of 20°C before entering the leaf chamber, as measured with a humidity sensor (Humicap HMI). As a control, leaf chambers were flushed with growth chamber air (designated dry). Leaf-to-air VPD was calculated based on the dew point of air entering the leaf chamber and leaf temperatures and was 150 and 1,500 Pa for humid and dry treatments of tobacco leaves, respectively. We designed and built leaf chambers to suit the small leaf size of Arabidopsis. Internal dimensions were 70 mm long, 15 mm wide, and 4 mm high. Airflow rate was controlled by one flowmeter (Brooks Instrument) for each leaf chamber and maintained at 50 mL s$^{-1}$ (tobacco) or 26 mL s$^{-1}$ (Arabidopsis). Air fans were directed at the treated leaves to prevent the buildup of leaf temperature gradients. PPFD below the leaf chamber lid was only marginally lower (8%) compared to HL. Neither the shade mitten nor the Perspex leaf chamber lids affected the red to far-red ratio of the light, as determined by spectroradiometer (LI-1800; LI-COR). Leaf temperatures were measured in situ on the abaxial surface with a thin type K thermocouple (0.12 mm) and maintained between 19.9°C and 22.5°C in tobacco and between 19.9°C and 20.6°C in Arabidopsis. In the Arabidopsis leaf chamber, airflow passing by the leaf sides was blocked by applying small layers of foam inside the leaf chamber, forcing most of the air over both leaf surfaces at a fast rate. This reduced boundary layer resistance and cooled the leaves in humid air, thus compensating for the extra evaporative cooling.

The synthetic cytokinin benzyl adenine (BA) was dissolved in dimethyl sulfoxide (DMSO) and diluted in water to the indicated concentration, with a final DMSO concentration of 0.1% (v/v) and 0.02% (v/v) Silwet L77. The BA solution or the same solution without BA as a vehicle control was applied with a brush to both sides of the leaf.

For cytokinin and gene transcript level analyses, leaves were immediately frozen in liquid nitrogen and stored at $-80^\circ$C until analysis. Leaf area was measured using a leaf area meter (LI-3100; LI-COR) and dry mass after oven drying at 70°C for at least 24 h.

**Induction of Cytokinin Production in Shaded Leaves**

Transgenic TFM-Irp tobacco cv SNN (Böhner et al., 1999; Böhner and Gatz, 2001) seeds were surface sterilized and germinated in sterile containers on Murashige and Skoog medium containing 0.8% (w/v) plant agar and 2% (w/v) Suc, and placed on a growth chamber under the same conditions as described above for tobacco. After 3 weeks, one leaf was cut off from each seedling and transferred aseptically to fresh medium containing 30 $\mu$M dex to induce cytokinin overproduction. If a clear cytokinin phenotype (callus formation, greening, and shoot formation) developed in the leaf on dex medium and the corresponding plant appeared as wild type, it was used for the experiments. Three weeks later, the selected plants were carefully treated and raised in the growth chamber as described above. The eighth true leaf counted from below was shaded for 8 d and treated three times with 30 $\mu$M dex and 0.02% (v/v) Silwet or a vehicle control.

**Gas Exchange, Chlorophyll Fluorescence, and Leaf Yellowing**

A portable gas-exchange meter (LI-6400; LI-COR) was used to measure $g_c$. In the tobacco density experiment, two positions per leaf were measured on three leaves per plant on six plants in each tobacco stand. Average leaf $T$ in the measuring chamber was 25°C and VPD was 1 kPa. Measurements were taken under sunny conditions when $g_c$ readings had stabilized and were always completed within 60 s, which was well before $g_c$ was affected by enclosing the leaf in the measuring chamber. In the same experiment, $E$ was measured as weight loss in situ per unit time and leaf area, also under sunny conditions. It was measured on six different plants in each stand and on three leaves per plant. Leaves were cut off, immediately weighed, put back in the original position and using orientation, and weighed again after 2 min for open canopy leaves and upper leaves in the dense canopy, or 6 min for lower leaves in the dense canopy. Cut leaves were held in position in the canopies by means of metal frames clamped on the stem. Separate measurements using a gas-exchange system showed that, after the leaf was cut, $E$ remained stable for
longer periods than those chosen for incubation (Supplemental Table S1). Therefore, stomatal closure did not influence the measurements. Leaf area was measured afterward.

During \( g_s \) measurements on Arabidopsis leaves in the growth chamber, average leaf temperature in the measuring chamber was 20.7°C and VPD was approximately 1.3 kPa. Efforts were made to reduce the accumulation of \( CO_2 \) in the growth room as much as possible during the measurements by using a mouthpiece connected to a column containing \( CO_2 \)-absorbing material (Sodasorb; Grace). Measurement of \( g_s \) on a leaf commenced directly after the shade mitten or leaf chamber, if present, was removed and was again completed within 60 s. The leaf area enclosed in the chamber was measured. For the calculation of \( E \) in situ, boundary layer conductance \( (g_s) \) was assumed to be negligible, so \( g_s \) was assumed to be equal to the total leaf conductance for water vapor. For comparison of \( E \) between humid and dry air treatments, this assumption is valid because any boundary layer effect should be small and comparable between treatments because of the high airflow rates used. Between the HL and LL treatment, \( g_s \) was probably higher, but again comparable between treatments. \( E \) was calculated as \( E = \frac{q_s \cdot (w_1 - w_2)}{w_1 - \bar{w}} \), where \( w_1 \) and \( \bar{w} \) are the molar fraction (mol m \(^{-4} \)) of water vapor in leaves and in the surrounding air, respectively. For tobacco, \( E \) was calculated in the same manner, but using a separately determined relationship between VPD and \( g_s \).

In both species, \( A_{max} \) was measured in the laboratory using a gas-exchange measuring system with leaf chambers with a 69- \(	imes\) 67-mm window that was described previously (Pons and Welschen, 2002). An infrared gas analyzer (LI-6262; LI-COR) was used to measure \( CO_2 \) and water partial pressure. Leaf absorbance \( (\text{A}_a) \) was calculated as a function of leaf chlorophyll content \( (\text{Chl}; \text{a}) \) of water vapor in leaves and in the surrounding air, respectively. For tobacco, \( E \) was calculated in the same manner, but using a separately determined relationship between VPD and \( g_s \).

ETR \(_{\text{max}} \) of Arabidopsis leaves was measured in the growth room using a portable chlorophyll fluorometer (Mini-PAM; Waltz). Steady-state chlorophyll fluorescence \( (F_s) \) was measured under saturating PPFD \((600-1,000 \mu\text{mol}\ m^{-2}\ s^{-1}, \text{depending on expected capacity}) \) and maximal fluorescence \( (F_{\text{m}}) \) in a saturating light pulse \((\text{approximately 5 mmol}\ \text{m}^{-2}\ \text{s}^{-1}) \). Light intensity was provided by a halogen lamp. An air fan prevented any increase in leaf temperature. Photophysical yield \( (\text{Y}) \) was determined as \( \frac{(F_s - F_{\text{m}})}{F_{\text{m}}} \) (Genty et al., 1989). The distance from the measuring head to the leaf surface was 8 mm. PPFD at the leaf surface was measured with a light sensor (LI-185A; LI-COR). Leaves were preinduced at a PPFD of approximately 600 \( \mu\text{mol}\ m^{-2}\ s^{-1} \) 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 determination with a spectrophotometer after extraction in dimethyl formamide (Inskeep and Bloom, 1985). Leaf absorbance \( (\text{a}) \) was calculated as a function of leaf chlorophyll content \( (\text{Chl}; \mu\text{mol}\ m^{-2}) \) following Evans (1993): \( \text{a} = \frac{\text{Chl}}{\text{Chl} + 76} \). ETR \(_{\text{max}} \) was then calculated as \( \text{ETR}_{\text{max}} = \text{a} \times \frac{0.35 \times \text{PPFD}}{\text{Y}} \) (Genty et al., 1989).

Chlorophyll \( a \) and \( b \) were measured in tobacco leaves induced by humid air treatment, the leaves were photographed and the fraction of yellow and green leaf area was determined using a custom-designed program in the software package KS400 (version 3.0; Carl Zeiss Vision).

Cytokinin Analysis with Micro Liquid Chromatography-Tandem Mass Spectrometry

Cytokinins were analyzed as previously described (Corbesier et al., 2003), with some modifications. Frozen leaf samples were ground in liquid nitrogen, transferred into Bielski solution (Bielski, 1964), and extracted overnight at \( 20^\circ \C \). For each cytokinin compound determined, 10 pmol \( \text{H}_2\text{dihydrozeatin}, \text{H}_2\text{dihydrozeatin riboside, H}_2\text{dihydrozeatin 9-glucoside, H}_2\text{dihydrozeatin riboside S-monophosphate, H}_4\text{N}_6\text{N}_3\text{N}_2\text{N}_4-(\text{Ac})\text{isopentenyl} \text{adensosine, H}_4\text{N}_6\text{N}_3\text{N}_2\text{N}_4-(\text{Ac})\text{isopentenyldenyadenosine, H}_4\text{N}_6\text{N}_3\text{N}_2\text{N}_4-(\text{Ac})\text{isopentenyldenyl} \text{glucoside, and H}_4\text{N}_6\text{N}_3\text{N}_2\text{N}_4-(\text{Ac})\text{isopentenyldenyl} \text{AMP (OLCHEMIM Ltd.) were added as internal standards. Cytokinins were purified by a combination of solid-phase and immunoaffinity chromatography using a broad-spectrum anti-isopenoid cytokinin immunoaffinity column (OLCHEMIM) as described (Redig et al., 1996). O-Glucosides were not collected. Xylem sap samples did not require extensive purification and were directly dissolved in water, after which standards were added. Cytokinins were quantified by micro liquid chromatography (LC) positive electrospray tandem mass spectrometry (MS/MS) in multiple reactant monitoring mode (Prinsen et al., 1998). The chromatograms obtained were processed using Masslynx software (Micromass).

Real-Time Reverse Transcription-PCR Analysis

Total RNA was extracted from 100 mg fresh leaf material with the RNaseasy mini kit (Qiagen) and treated with DNase (DNA-free; Ambion) to remove genomic DNA. RNA was electrophoresed on 1% agarose gel to confirm integrity. cDNA was synthesized from 2 \( \mu\text{g} \) of RNA with 100 units of SuperScriptIII RNase H \( ^{-}\) reverse transcriptase (Invitrogen) and 100 ng of random hexamers in a 20- \( \mu\text{l} \) reaction. Quantitative real-time reverse transcription (RT)-PCR was performed in a 25- \( \mu\text{l} \) reaction containing 12.5 \( \mu\text{L} \) of 2 \( \times \) SYBR Green Superscript (Bio-Rad), 100 pmol of the forward and reverse primer, and 10 ng of cDNA, except 0.1 ng of cDNA was used for Arabidopsis 18S rRNA. A Bio-Rad MyiQ single-color real-time PCR detection system (Bio-Rad) was used with the following thermal profile for all genes: 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 62°C, and 60 s at 72°C. Melting curves were obtained after each PCR and confirmed the amplification of single products. Primer sequences (5\text{-}3\text{') for tobacco genes were ribosomal L25 (GenBank accession no. L18908) forward, ATTTGCGACATCACGGTCGA; L25 reverse, GGCAACGTCCTACACATCCTCA; rbcS (X02353) forward, TGCCGACCAAATTAACCA- GA; and rbcS reverse, AAAGCGAGAACCCTACCA. Primers for Arabidopsis genes were 18S rRNA (GenBank accession no. X16077) forward, AAAGCCTC-TACCAGATCCAAACG; 18S rRNA reverse, ACTCGAAAGAGCCCGGTATT; ARR7 (At1g19050) forward, GCCGGTGGAGATTTGACTGTTA; ARR7-reverse, AGCC- CTTTGGCTCCATCTCA; ARR16 (At2g40670) forward, TACGGAGGTCCTTGGTCGCTT; ARR16 reverse, AACCCGAAATCTAACACTTCCGTCA; PHYA (At1g09570) forward, AAAGCCCTTGGATGGATGGT; PHYA reverse, CTT- TGGCGACGACGTGGTATGAAT; PHYB (At5g07900) forward, ACCCGCAAGCG- TAACAAGCACATG; and rks reverse, GTGCAAACGCAACAGGGAAC. Primers for the \( \text{ARK} \) genes were designed around an intron to confirm absence of genomic DNA. PCR products were resolved on 1% agarose gel, which showed single products of expected size in each case. All primers were designed with primer3 software (http://frodo.wi.mit.edu/primer3/primer3_code.html).

Statistical Analysis

Analysis of covariance was used to study effects of stand density and relative height on the distribution of various parameters in the tobacco canopies. Two-way ANOVA with Tukey’s honestly significant difference test as a posthoc test and Student’s \( t \) tests were used in the other experiments. Data were log-transformed when it improved homogeneity of variance.

Supplemental Data

The following materials are available in the online version of this article. Supplemental Table S1. \( E \) and \( g_s \) of tobacco leaves before and after cutting from the plant.

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