

Guard cells in albino leaf patches do not respond to photosynthetically active radiation, but are sensitive to blue light, CO₂ and abscisic acid

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ABSTRACT

Stomatal openings can be stimulated by light through two signalling pathways. The first pathway is blue light specific and involves phototropins, while the second pathway mediates a response to photosynthetically active radiation (PAR). This second pathway was studied with the use of albino *Vicia faba* plants and variegated leaves of *Chlorophytum comosum*. Treatment of *V. faba* with norflurazon (Nf) inhibits the synthesis of carotenoids and leads to albino leaves with guard cells that lack functional green chloroplasts. Guard cells in albino leaf patches of *C. comosum*, however, do contain photosynthetically active chloroplasts. Stomata in albino leaf patches of both plants did not respond to red light, although blue light could still induce stomatal opening. This shows that the response to PAR is not functioning in albino leaf patches, even though guard cells of *C. comosum* harbour chloroplasts. Stomata of Nf-treated plants still responded to CO₂ and abscisic acid (ABA). The size of Nf-treated guard cells was increased, but impalement studies with double-barrelled microelectrodes revealed no changes in ion-transport properties at the plasma membrane of guard cells. Blue light could hyperpolarize albino guard cells by triggering outward currents with peak values of 37 pA in albino plants and 51 pA in green control cells. Because of the inhibition of carotenoid biosynthesis, Nf-treated *V. faba* plants contained only 4% of the ABA content found in green control plants. The ABA dose dependence of anion channel activation in guard cells was shifted in these plants, causing a reduced response to 10 μM ABA. These data show that despite the dramatic changes in physiology caused by Nf, the gross responsiveness of guard cells to blue light, CO₂ and ABA remains unaltered. Stomata in albino leaf patches, however, do not respond to PAR, but require photosynthetically active mesophyll cells for this response.

Key-words: anion channel; H⁺-ATPase; norflurazon; PAR-response; plasma membrane; stomata.

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INTRODUCTION

Leaves of land plants are covered by waxy cuticle on their epidermis, which prevents excessive loss of water. Within the epidermis, stomata form small adjustable pores, which allow gas exchange between the leaf interior and the atmosphere (Assmann & Shimazaki 1999; Hetherington 2001; Roelfsema & Hedrich 2005). The stomata allow the uptake of CO₂ required for photosynthesis and to this end, C₃ and C₄ plants open their stomata in the light. This response is triggered through at least two independent light signalling pathways (Assmann & Shimazaki 1999; Roelfsema & Hedrich 2005). One pathway has a specific responsiveness to blue light, while the other pathway is stimulated by photosynthetically active radiation (PAR). As suggested by the name, the PAR response is linked to the photosynthetic activity in the leaf. This response is inhibited by 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (DCMU), which specifically inhibits photosystem II (PSII) (Sharkey & Raschke 1981; Schwartz & Zeiger 1984). The mechanism through which photosynthesis is linked to stomatal movement, however, is still under debate (Messinger, Buckley & Mott 2006).

Several studies have suggested a role for the guard cell chloroplast in the response of the stomata to PAR. Stomatal opening is stimulated by red light in isolated epidermal strips, which are devoid of mesophyll cells, and this response is inhibited by DCMU (Olsen *et al.* 2002). Isolated guard cells are thus most likely responsive to red light through the activity of their chloroplasts. This hypothesis is supported by a patch clamp study that showed activation of plasma membrane H⁺-ATPases by red light (Serrano, Zeiger & Hagiwara 1988). However, this result could not be confirmed in a later study using the same technique (Taylor & Assmann 2001). Single guard cells in intact plants studied with microelectrodes revealed that red light inhibits plasma membrane anion channels (Roelfsema *et al.* 2002). However, this response was only observed by a red light projected on a large area of the leaf including mesophyll cells, but not by a small beam illuminating only a small area with the guard cell recorded. The large beam of red light, not the small beam, induced a change in the intercellular

CO₂ concentration. These observations led to the hypothesis that the guard cell did not respond to red light directly, but rather to lowered intercellular CO₂ concentration induced by PAR. Here, this hypothesis is tested with albino *Vicia faba* plants and variegated leaves of *Chlorophytum comosum*.

Albino plants can be raised through the application of carotenoid synthesis inhibitors like norflurazon (Nf). The herbicide Nf inhibits the conversion of geranylgeranyl diphosphate (GGPP) to carotenoids in the methylerythritol 4-phosphate (MEP) pathway (Sandmann, Linden & Boger 1989; Breitenbach, Zhu & Sandmann 2001). Just as in carotenoid-deficient mutants, Nf-inhibition of carotenoid synthesis causes photooxidation of chlorophyll and leads to albino plants (Henson 1984). In these albino plants, plastidogenesis is also destroyed and causes decreased transcription numbers of nuclear-encoded chloroplast genes (Oelmüller & Mohr 1986; Strand *et al.* 2003; McCormac & Terry 2004a). In addition, Nf affects the transcription rates of a large number of other genes encoding proteins that are not transported to chloroplasts (McCormac & Terry 2004b). The latter changes in gene transcription, to some extent, may be due to low levels of the stress hormone abscisic acid (ABA), because it is derived from the carotenoids 9-cis-violaxathin and 9'-cis-neoxathin (Tian, Della-Penna & Zeevaart 2004). Nf therefore also leads to ABA deficiency (Henson 1984).

Albino plants have played an important role in plant biology, helping dissect photosensory systems. In the 1950s, the Galston laboratory used carotenoid-deficient coleoptiles to demonstrate that phototropic curvature does not depend on carotenoids (Bandurski & Galston 1951; Asomaning & Galston 1961). Experiments with Nf-treated maize coleoptiles led to essentially the same conclusion (Vierstra & Poff 1981). In albino *Chenopodium rubrum* plants, blue light can still induce flowering, but these plants have lost sensitivity to red light (Jabben & Deitzer 1979; Bavrina *et al.* 2002). A similar situation was found for light-induced stomatal opening, because the stomata of Nf-treated wheat plants open in blue light (Karlsson, Höglund & Klockare 1983), but do not respond to red light (Karlsson 1986). Likewise, stomata in the white areas of variegated *Hedera helix* leaves did not show a response to red light (Aphalo & Sanchez 1986). These data provide evidence for the role of photosynthetically active mesophyll cells in the PAR response of stomata; however, alternative explanations may apply, such as a general loss of stomatal sensitivity to stimuli acting in green leaves.

Stomata in albino and variegated leaves were tested for responses to several stimuli affecting stomatal movement. Guard cells in Nf-treated albino *V. faba* plants were larger than in green controls, which may be due to a 20× lower level of ABA in these plants. Despite the difference in size, the K⁺-conductances of albino guard cells were not altered. Furthermore, the stomata of Nf plants still responded to CO₂, blue light and ABA, although the ABA concentration dependence of anion channel activation was altered. Stomata in albino leaf patches of Nf-treated *V. faba* plants as

well as *C. comosum* were not responsive to red light, even though the guard cells of *C. comosum* contained functional chloroplasts. This indicates that in intact leaves, mesophyll cells influence guard cells through changes in the intercellular CO₂ concentration, which underlies the stomatal response to PAR.

MATERIALS AND METHODS

Plant growth and gas exchange measurements

Vicia faba (L.) plants (Französische Weisskeimige, Gebag, Hannover, Germany) and *Chlorophytum comosum* (Thumb.) Jacques were grown in a greenhouse. Albino *V. faba* plants were obtained by watering germinated seeds with 100 µM Nf two times a week (Zorial Rapid 80; Syngenta, Basel, Switzerland). Sections of pre-darkened *V. faba* or *C. comosum* leaves were enclosed in a sandwich-type cuvette (2.1 cm diameter) with windows on the upper and lower side. Where indicated, the green area of *C. comosum* leaves was covered with black tape. The abaxial side of the leaves was directed to the upper side of the cuvette and exposed to a gas stream of 0.5 L min⁻¹. Relative humidity was 46%, and the temperature was 24 °C. Transpiration and CO₂ uptake were measured with the infrared gas analysis technique (Binos 1; Heraeus, Hanau, Germany). Experiments were started at 8 AM.

Impalement of guard cells in intact plants

Three to four-week-old-plants were placed next to an inverse microscope (IM 35; Carl Zeiss, Göttingen, Germany). The adaxial side of a leaf was mounted on a plexiglas holder in the focal plane, using double-sided adhesive tape. In this experimental configuration, the abaxial side was facing downwards and was accessible for impalement with double-barreled microelectrodes. The leaves were illuminated with red light from a microscope lamp (12 V/60 W, Zeiss), passed through a long-pass glass filter (610 nm edge wavelength) and focussed on the adaxial side of the leaf at an area 2 cm in diameter, with a photon flux density of 500 µmol m⁻² s⁻¹. Before impalement, stomata were selected for a small aperture (~3 µm), which minimizes the loss of cytoplasm during impalement of guard cells. A small degree of stomatal opening was obtained by passing air over the leaf (0.4 L min⁻¹) via a split (14 × 2 mm). The air was CO₂ free with Nf-treated plants and contained 700 µL l⁻¹ CO₂ with green controls. Blue light was obtained with a band pass filter transmitting wavelengths of 360–500 nm and projected via the objective at the abaxial side of the leaf. The area illuminated with blue light was 0.43 mm in diameter, and the photon flux density was 200 µmol m⁻² s⁻¹. The radiation was measured with a Li-Cor light metre (quantum sensor, LI-190; Li-Cor, Lincoln, NE, USA). The reference electrode, a 50 mM KCl and 1.5% agarose salt bridge, connected to an AgCl/Cl half cell, was inserted into the plexiglas holder on the adaxial side of the leaf. The surface potential across the cuticle was measured

with blunt single-barreled electrodes ($R_{\text{tip}} < 20 \text{ M}\Omega$) and did not differ between Nf and control plants; the average surface potential was 14 mV (Nf-treated leaves, $\text{SD} = 10$, $n = 8$; control leaves, $\text{SD} = 11$, $n = 9$).

The guard cells were impaled using a micromanipulator (MM3A; Kleindiek Nanotechnik, Reutlingen, Germany). The microelectrodes were pulled from glass capillaries (Hilgenberg GmbH, Malsfeld, Germany) as described earlier (Roelfsema *et al.* 2001). The electrodes were filled with 300 mM KCl, and their resistances ranged from 80 to 150 $\text{M}\Omega$. The electrodes were connected via AgCl/CL half cells to headstages (HS-2A $\times 0.01$; Axon Instruments, Foster City, CA, USA). The membrane potential was clamped to test voltages using an integrated microelectrode and differential amplifier (Geneclamp 500, Axon Instruments). Voltage step protocols were applied via an ITC-16 interface (Instrutech Corp., Elmont, NY, USA) under control of Pulse software (HEKA, Lambrecht, Germany). The data obtained by short pulses were low pass filtered at 200 Hz with eight-pole Bessel (type 92, Frequency Devices, Haverhill, MA, USA) and were sampled at 1 kHz; during long-time sampling, the data were filtered at 10 Hz and sampled at 33.3 Hz.

Protoplast isolation and patch clamp

Guard cell protoplasts were isolated from leaves of 2–4-week-old *V. faba* plants as described by Raschke & Hedrich (1989).

Anion currents were studied in the whole-cell configuration, using patch pipettes prepared from Kimax-51 glass (Kimble Products, Vineland, NY, USA) and coated with silicone (Sylgard 184 silicone elastomer kit; Dow Corning, Auburn, MI, USA). Currents were recorded with an EPC-7 patch-clamp amplifier (HEKA) and low pass filtered with an eight-pole Bessel filter. Whole-cell data were filtered at a cutoff frequency of 2 kHz and sampled at 5 kHz. The data were digitized (ITC-16, Instrutech), stored on hard disk and analysed with Wavemetrics software (WaveMetrics, Inc., Lake Oswego, OR, USA). The pipette solution contained the following (in mM): tetraethylammonium chloride (TEACl), 150; MgCl_2 , 2; ethylene glycol bis(2 aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10; MgATP, 1; and 2-[4-(2-(Hydroxyethyl)-1-piperazinyl)]-ethanesulphonic acid (HEPES)-tris(hydroxymethyl) aminomethane (Tris) pH 7.2, 10. The bath solution was composed of the following (in mM): CaCl_2 , 40; LaCl_3 , 100; and methanesulphonic acid (MES)-Tris pH 5.6, 10. (\pm)-Cis, trans ABA (Lancaster, Newgate, UK) was used from ethanol stocks and added to the pipette solution at the desired concentration.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence images were obtained with a confocal laser scanning microscope (LSM 5 Pascal, Zeiss). Chlorophyll was excited at 488 nm with an argon laser (RMC 7812Z, Lasos, Ebersburg, Germany), and emitted

light was passed through a long-pass filter (560 nm). Images were analysed with Axio Vision software (Zeiss).

Quantitative chlorophyll fluorescence measurements were carried out with a microscopy pulse-amplitude-modulation (PAM) fluorometer (Heinz Walz GmbH, Effeltrich, Germany) adapted to an upright fluorescence microscope (Axioskop 2FS, Zeiss). Light emitted from a blue light-emitting diode (type NBSG 500; Nichia, Tokyo, Japan) served for fluorescence excitation. This was filtered with a blue filter (BG 39; Schott, Mainz, Germany) and directed via a dichroic beamsplitter mirror (DT Yellow; Balzers, Liechtenstein) towards the objective lens of the microscope. Fluorescence emitted by the chloroplasts was passed through the dichroic mirror, filtered with a red glass filter (RG 645, Schott) and detected with a photomultiplier (type PC/PM-MC, Walz). On its way to the detector, the fluorescence passed an iris diaphragm that could be used to limit the field of detection to a single stoma (Goh, Schreiber & Hedrich 1999). The measurements were carried out with measuring light pulses given at a frequency of 18 Hz in the dark, which corresponds to $0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$. The fluorescence signal was damped with a time constant of 1 s. Actinic light and saturating light pulses were given at relatively low photon flux densities of 13 and $325 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. These values were optimized for observing CO_2 -dependent changes in chlorophyll fluorescence with *C. comosum* (data not shown). During illumination with actinic light, the measuring frequency was increased to 688 Hz. For PAM recordings, the leaves were mounted on a microscope table for impalement studies as previously described, except that an upright microscope was used and the abaxial side of the leaf was thus facing upwards.

RESULTS

Nf bleaches mesophyll and guard cell chloroplasts

Treatment of *V. faba* seedlings with 100 μM Nf, twice a week, resulted in plants that lack chlorophyll and remain smaller than green control plants (Fig. 1a). Leaves of these albino plants often did not unfold completely and developed necrotic spots at high light intensities, indicating injury due to photooxidation (not shown). Nf-treated plants were essentially chlorophyll free which resulted in a lack of photosynthetic activity (Fig. 2b, lower trace).

Inspection under a confocal laser scanning microscope revealed the loss of chlorophylls in guard cells as well (compare Fig. 1b & c). Interestingly, the guard cells of Nf-treated plants were bigger than those of control plants (Fig. 1b & c), resulting in protoplasts with an average diameter of $16.8 \mu\text{m}$ ($\text{SD} = 2.1$, $n = 77$), compared with $14.5 \mu\text{m}$ ($\text{SD} = 1.6$, $n = 63$) in green control plants (Fig. 1d & e). Possibly, the increase in guard cell size is linked to low endogenous levels of ABA in Nf-treated plants, because ABA also decreased the guard cell size in *Tradescantia virginiana* (Franks & Farquhar 2001).

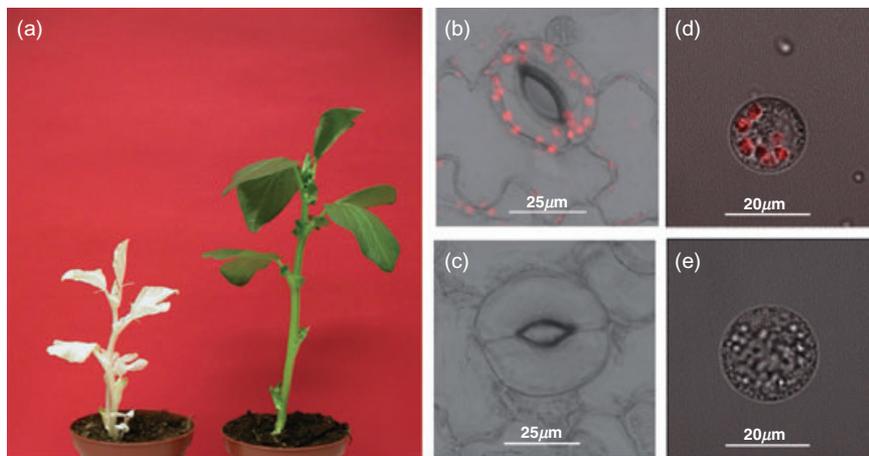


Figure 1. Norflurazon (Nf) bleaches *Vicia faba* plants and increases the size of guard cells. (a) The growth of Nf-treated plants is retarded compared with the green controls, and their leaves often do not unfold completely. Guard cells of Nf-treated plants were larger than those of control plants and completely lack chlorophyll fluorescence (compare b and c). These differences were maintained at the level of guard cell protoplasts (d and e). An average diameter of $16.8 \mu\text{m}$ for albino protoplasts and $14.5 \mu\text{m}$ for green controls was calculated on the basis of whole-cell capacitances of 8.9 pF ($\text{SE} = 0.14$, $n = 77$) and 6.6 pF ($\text{SE} = 0.08$, $n = 63$) and assuming spherical guard cell protoplasts.

Guard cells in albino leaf patches lack a red light response

Responses to light by Nf-treated *V. faba* plants were studied in gas exchange experiments with subsequent red- and blue-light illumination. A high quantum flux density of red light ($630 \mu\text{mol m}^{-2} \text{s}^{-1}$) triggered a time-dependent increase in transpiration in green control plants, but not in Nf-treated plants, indicating that the herbicide rendered the stomata insensitive to red light (Fig. 2a & b). In contrast, blue light at a low quantum flux density ($24 \mu\text{mol m}^{-2} \text{s}^{-1}$) stimulated transpiration in green as well as in Nf plants, although in Nf plants, this response was reduced in amplitude (Fig. 2a & b).

The lack of a response to PAR in albino *V. faba* plants may be due to the loss of photosynthetic capacity in mesophyll cells, but may also have been caused by the loss of chlorophyll in guard cells (Fig. 1b & c). Variegated leaves of *C. comosum* differ in this respect, because mesophyll cells in the white areas of these leaves (Fig. 3a) do not

contain chlorophyll, whereas guard cells contain normal chloroplasts (Fig. 3b). The photosynthetic performance of guard cell chloroplasts was studied with a microscopy PAM. Single stomata in the white area of *C. comosum* leaves were excited at a photon flux density of $0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$ to obtain a value for the dark-adapted chlorophyll fluorescence F_0 , and the first saturating light pulse was applied to determine maximum fluorescence (F_m) and variable fluorescence (F_v) ($F_v = F_m - F_0$, Fig. 3c & d). The ratio $F_v:F_m$, which is normally considered a measure of the maximal quantum yield of PSII, was 0.49 ($\text{SD} = 0.04$, $n = 10$). This value is considerably smaller than the normal value of 0.83 found for mesophyll cells of most plant species (Krause & Weis 1991). This difference may reflect a true deficiency in the efficiency of PSII, but it may also reflect a relatively large contribution of non-variable photosystem I (PSI) fluorescence to the overall signal (Schreiber 2004). After the first and simultaneously with the second saturation pulse, continuous actinic light was switched on at a photon flux density of $13 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 250 s. During the application

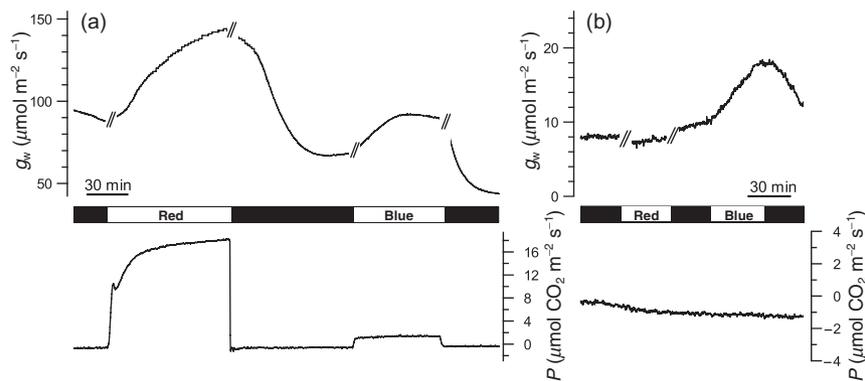


Figure 2. Light-induced stomatal opening in leaves of green (a) and Nf-treated (b) *Vicia faba* plants. Changes in stomatal conductance to H_2O (g_w) (upper traces) and photosynthetic activity (P) (lower traces) were recorded after application of $630 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light, or $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light (time points of irradiation are indicated by the bar between the traces). Note that red light induces a time-dependent increase in conductance in green plants but not in Nf plants, while blue light stimulates transpiration in both. The traces are disrupted during temperature transients. Typical responses of Nf-treated and control plants are shown; the responses were reproduced at least three times.

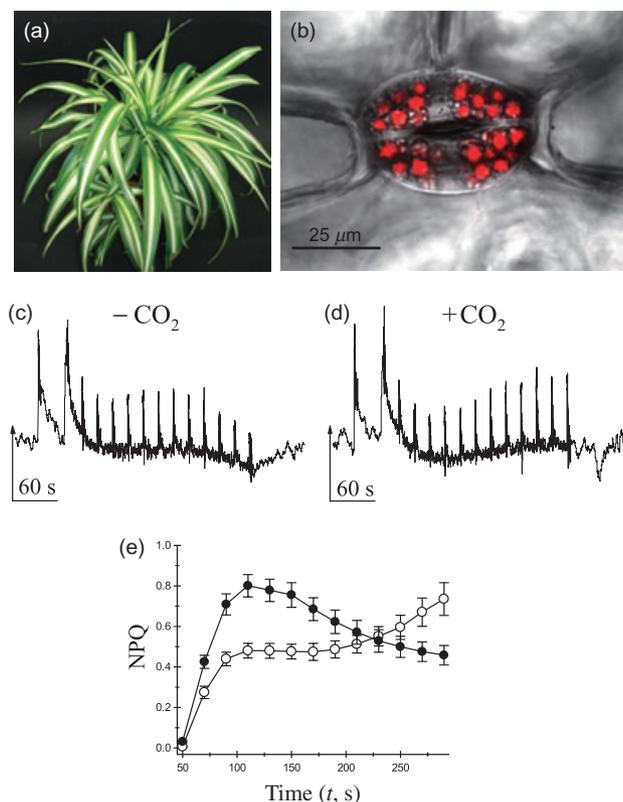


Figure 3. Chlorophyll fluorescence of guard cells located in the white area of variegated *Chlorophytum comosum* leaves. (a) Variegated leaves of *C. comosum* with large albino sections along the central veins. (b) Laser scanning microscope image presenting an overlay of transmitted light and chlorophyll fluorescence from guard cells in the albino leaf section. (c & d) Chlorophyll fluorescence induction curve measured for single stomata in albino leaf patches of *C. comosum*. Chloroplasts were studied with measuring light ($0.06 \mu\text{mol m}^{-2} \text{s}^{-1}$), while actinic light and saturating light pulses were given at photon flux densities of 13 and $325 \mu\text{mol m}^{-2} \text{s}^{-1}$, respectively. Single stomata were measured in CO_2 free air (c) or in air with $350 \mu\text{L l}^{-1} \text{CO}_2$ (d). (e) Changes in the non-photochemical quenching (NPQ) measured for single stomata during induction curves as shown in (c) and (d). Stomata were measured in CO_2 free air (○) and air with $350 \mu\text{L l}^{-1} \text{CO}_2$ (●). Data are the average from 10 stomata; error bars represent SE.

of actinic light, saturating light pulses were applied every 20 s for assessment of the time-dependent changes of F_m' and the derived fluorescence parameters of effective PSII quantum yield $[(F_m' - F)/F_m']$ and non-photochemical quenching (NPQ) $[(F_m - F_m')/F_m']$ (Schreiber 2004). The responses of single stomata were measured in CO_2 free air (Fig. 3c) and in air with $350 \mu\text{L l}^{-1} \text{CO}_2$ (Fig. 3d). While the application of CO_2 did not have much effect on the PSII quantum yield (data not shown), it caused significant differences in the NPQ parameter (Fig. 3e). After the initial increase of NPQ due to 'membrane energization', only in the presence of CO_2 was the typical relaxation of NPQ observed; this response is generally attributed to the onset of ATP consumption in the Calvin-Benson cycle (Schreiber 2004). This strongly suggests that guard cells in albino leaf

patches of *C. comosum* are capable of photosynthesis. A similar conclusion has been drawn on the basis of experiments with isolated guard cells of other species (Outlaw 1989).

The stomata of *C. comosum* were tested for their responsiveness to red light ($630 \mu\text{mol m}^{-2} \text{s}^{-1}$) and blue light ($24 \mu\text{mol m}^{-2} \text{s}^{-1}$). When both green and white areas of the leaf were illuminated, the stomata opened in response to both light qualities (Fig. 4a). Visual inspection with a microscope revealed that the stomata in both white and green areas opened (data not shown). In contrast, if red light was projected only on the white area of the leaf, no stomatal opening was observed (Fig. 4b, $n = 4$). Red light also did not cause a measurable uptake of CO_2 , even though the guard cells in these albino leaf patches contain chloroplasts (Fig. 4b). The response of the stomata to blue light illuminating white leaf sections was variable; in two experiments, the transpiration was not stimulated by blue light (not

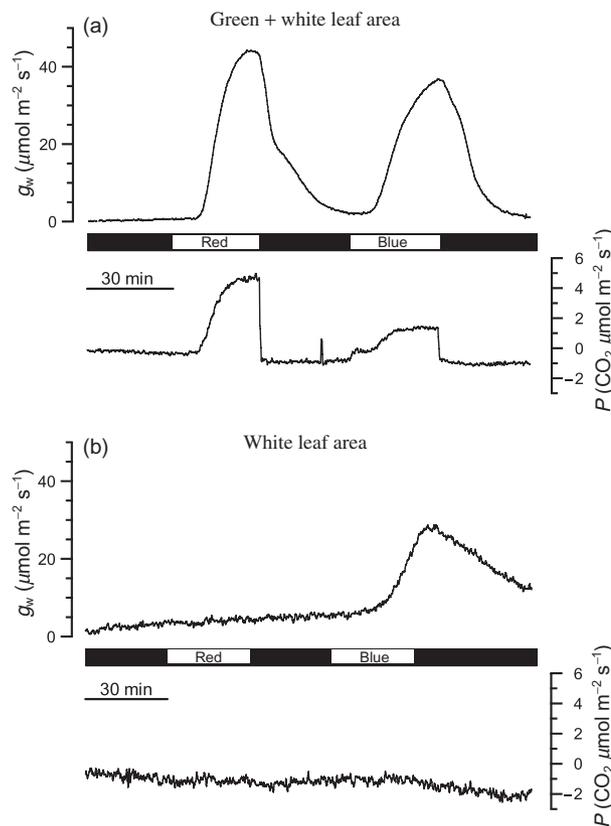


Figure 4. Light-induced stomatal opening in variegated leaves of *Chlorophytum comosum*, illuminated on the green and white areas (a) or white area only (b). Changes in stomatal conductance to H_2O (g_w) (upper traces) and photosynthetic activity (P) (lower traces) were recorded after application of $630 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light, or $25 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light (time points of irradiation are indicated by the bar between the traces). Note that red and blue light shined on both green and white areas induce photosynthesis and a time-dependent increase in conductance. When green areas are covered, photosynthesis cannot be measured, and red light does not lead to an increase in conductance, while blue light induced stomatal opening in two out of four leaves.

shown), while blue light triggered stomatal opening in two other experiments (Fig. 4b).

Stomata of Nf-treated leaves still respond to CO₂

Nf not only induces the loss of chlorophyll, but also alters the expression of a large number of genes including many nuclear-encoded genes of chloroplast proteins (McCormac & Terry 2004a). Albino leaves of *V. faba* therefore may also have lost guard cell sensitivity to intercellular CO₂ concentration. This possibility was tested by comparing the stomatal responses of Nf-treated and control plants to CO₂ (Fig. 5a & b). The stomata of Nf-treated plants opened in CO₂ free air and closed again in air containing 700 $\mu\text{L l}^{-1}$ CO₂, but the response to CO₂ was smaller in amplitude compared with green control plants (Fig. 5a & b). Stomatal closure was also triggered with 100 μM ABA fed through the petiole of leaves, in both Nf-treated and control plants.

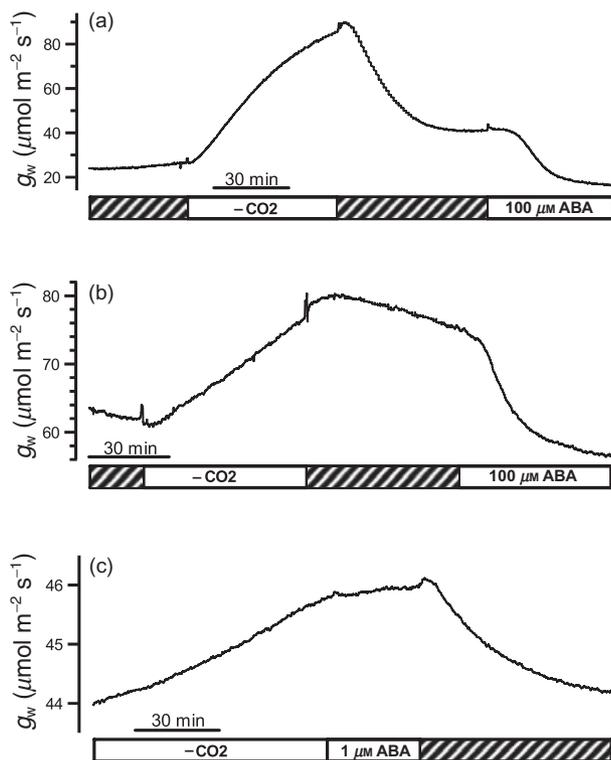


Figure 5. Stomatal responses to CO₂ and abscisic acid (ABA) in green (a) and Nf-treated (b & c) *Vicia faba* leaves. (a & b) Stomatal conductance to H₂O (g_w) recorded in darkness; the atmospheric CO₂ concentration was changed from 700 $\mu\text{L l}^{-1}$ (striped area in the bar below the traces) to CO₂ free air (as indicated in the bar) and afterwards, 100 μM ABA was fed via the petiole (concentration given in the bar). (c) An Nf-treated leaf was kept in CO₂ free air (as indicated in the bar), fed with 1 μM ABA and subsequently exposed to 700 $\mu\text{L l}^{-1}$ CO₂ (striped area in the bar below the traces). Note that CO₂ responsiveness increased, compared with (b), after feeding 1 μM ABA via the petiole. The traces shown represent typical responses that were reproduced at least three times.

In contrast with the smaller response to CO₂, no difference in the ABA-induced velocity of stomatal closure was apparent, between Nf and control leaves (Fig. 5a & b). CO₂-induced stomatal closure in Nf-treated leaves could be accelerated by application of low concentrations of ABA (1 μM) at the petiole. Although this concentration did not initiate stomatal closure (Fig. 5c), its presence accelerated the CO₂-induced decrease in transpiration rate from 13 nmol H₂O m⁻² s⁻² (SD = 9) in the absence of ABA, to 86 nmol H₂O m⁻² s⁻² (SD = 40) in the presence of 1 μM ABA. These results are in accordance with those obtained with *Xanthium strumarium* L., showing that the stomata of this species require low levels of ABA to respond to CO₂ (Raschke 1975a).

Plasma membrane ion transport

Blue light-induced guard cell swelling and stomatal opening most likely depend on the activation of the plasma membrane proton pumps (Assmann, Simoncini & Schroeder 1985; Kinoshita & Shimazaki 1999; Taylor & Assmann 2001). Because of the activation of H⁺ ATPases, blue light can hyperpolarize the plasma membrane of guard cells and drive the uptake of K⁺ via inward rectifying K⁺ channels (Roelfsema *et al.* 2001). The blue light response of guard cells of Nf plants was tested using an impalement technique with double-barrelled microelectrodes. Guard cells were impaled using red light at a photon flux density of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Stomata that were only slightly opened (aperture ~3 μm) were selected. To obtain this degree of stomatal opening, Nf-treated plants were recorded with CO₂-free air, while green controls were exposed to 700 $\mu\text{L l}^{-1}$ CO₂.

After impalement, all guard cells were in the depolarized state (Roelfsema *et al.* 2001), the average membrane potential of albino guard cells was -59 mV (SD = 10, $n = 10$) and -67 mV with green controls (SD = 14.5, $n = 8$). A beam of blue light (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) projected on guard cells hyperpolarized the plasma membrane of control (not shown) as well as Nf-treated plants (Fig. 6a). In many cases, the guard cells displayed irregular membrane potential changes from the hyperpolarized state to the depolarized state after prolonged illumination (not shown), but returned to a stable depolarized state only after the offset of blue light (Fig. 6a). The changes in plasma membrane current, which underlie the blue light-induced hyperpolarization, were recorded at a holding potential of -100 mV. At this potential, voltage-dependent K⁺ channels are inactive (Fig. 6b–d) and thus pump currents can be recorded at high resolution. The average peak current triggered by blue light was 37 pA (SD = 23, $n = 4$) in guard cells of Nf-treated plants and 51 pA (SD = 14, $n = 5$) in control cells. Blue light thus induced similar plasma membrane currents, despite the higher atmospheric CO₂ concentration used for guard cells in green plants. High concentrations of CO₂ lead to an enhanced blue light response at the guard cell plasma membrane (own unpublished results).

K⁺ uptake and release in guard cells is mediated via two voltage-dependent K⁺ channel types (Roelfsema &

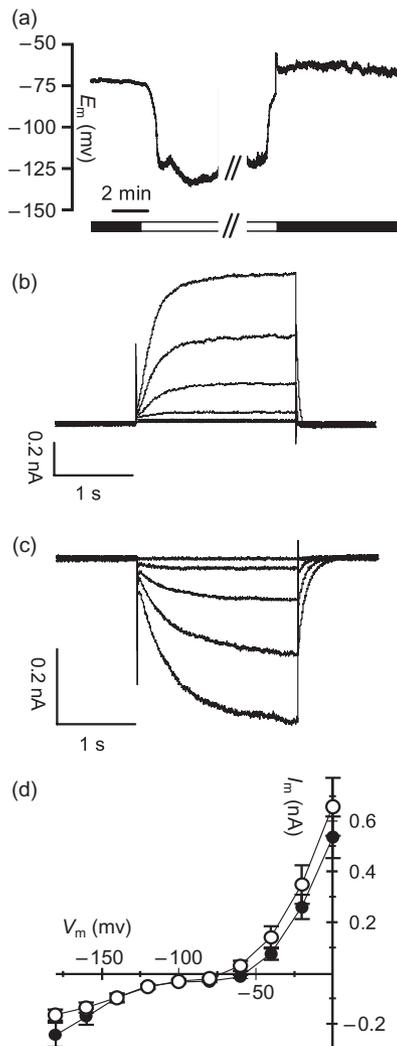


Figure 6. Electrical properties of guard cells in intact albino *Vicia faba* plants. (a) Membrane potential response of a guard cell exposed to $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ blue light (white area in the bar below the graph), given in addition to $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ red light background. Blue light induced a hyperpolarization of the plasma membrane, interrupted by fluctuations towards more depolarized values. After termination of blue light illumination, the membrane potential returned a stable depolarized state. (b & c) Voltage-dependent K^+ channels in Nf-treated albino guard cells. Time-dependent K^+ currents were elicited with voltage pulses (2 s) applied from a holding potential of -100 mV with 20 mV increments to 0 mV (b) or to -180 mV (c). (d) Steady-state current–voltage relation averaged for eight guard cells of Nf-treated plants (\circ) and seven cells from green plants (\bullet). The steady-state currents were sampled during the last 100 ms of test pulses. Error bars indicate SE. E_m , free running membrane potential; I_m , membrane current; V_m , clamped membrane potential.

Hedrich 2005). The properties of these channels in guard cells of intact plants were studied using a voltage-clamp approach with double-barrelled microelectrodes (Roelfsema *et al.* 2001). Starting from a holding potential of -100 mV , test voltages were applied stepwise to more

positive or negative values to resolve the time-dependent activation of outward and inward K^+ channels, respectively. The properties of both K^+ channel types (Fig. 6b & c) were similar to those of control plants (Roelfsema *et al.* 2001). On average, the conductance of K^+ channels in Nf-treated guard cells was not significantly altered (Fig. 6d). These results indicate that both blue light-stimulated H^+ ATPases and inward- and outward-rectifying K^+ channels function in a similar manner in guard cells of Nf-treated and green control leaves.

Nf does not cause a loss in ABA sensitivity

Because ABA is mainly derived from carotenoids, synthesized in plastids, we expected the Nf-generated albino plants to contain less ABA than the green controls. Indeed, albino leaves of *V. faba* contained only 3 pmol gFW^{-1} (SD = 0.85 , $n = 3$) which represents 4% of the ABA content measured in green *V. faba* leaves (79 pmol gFW^{-1} , SD = 34 , $n = 3$). In water-stressed leaves (50% rest water content) the ABA content of albino plants increased only 2.5 times (10 pmol gFW^{-1}), while that of green plants increased 67 times ($5300 \text{ pmol gFW}^{-1}$). This shows that Nf-treated plants are not able to synthesize ABA in response to dehydration.

The stomata of Nf-treated plants, grown at these low endogenous ABA levels, still close in response to ABA that was fed via the petiole, just as control leaves (Fig. 5a & b). Previous studies documented that the activation of anion channels in guard cells represents a key step in ABA-induced stomatal closure (Pei *et al.* 1997; Roelfsema, Levchenko & Hedrich 2004). ABA activates both rapid (R-type)- and slow (S-type) anion channels, two channel populations that are characterized by different voltage-dependence and activation kinetics (Keller, Hedrich & Raschke 1989; Hedrich, Busch & Raschke 1990; Schroeder & Keller 1992). To study whether the low ABA content of albino plants changes the ABA-dependence of anion channel activation in guard cells, the patch clamp technique was applied. After gaining whole-cell access, $10 \mu\text{M}$ ABA diffusing from the pipette into the cytoplasm activated anion channels in a transient manner (Fig. 7a) (Levchenko *et al.* 2005). A peak activity was found for guard cells of Nf-treated plants after 220 s (SE = 18 , $n = 14$), while the anion channel activity peaked 142 s after whole-cell access was achieved in control cells (SE = 32 , $n = 10$). In the majority of albino (Fig. 7b) and control guard cells, S-type anion channels were activated by ABA. The weak voltage dependence of the S-type channel is reflected by its almost linear current–voltage relationship (Schroeder & Hagiwara 1989; Schroeder & Keller 1992). Under the same experimental conditions, activation of R-type anion channels (Keller *et al.* 1989; Hedrich *et al.* 1990) was observed in Nf-treated (8 of 68 cells) and green guard cells (7 of 52 cells) as well. The depolarization-activated R-type anion channels were identified by their typical current–voltage curve with a peak current at approximately -40 mV (Fig. 5c) (Kolb, Marten & Hedrich 1995; Schulz-Lessdorf, Lohse & Hedrich 1996).

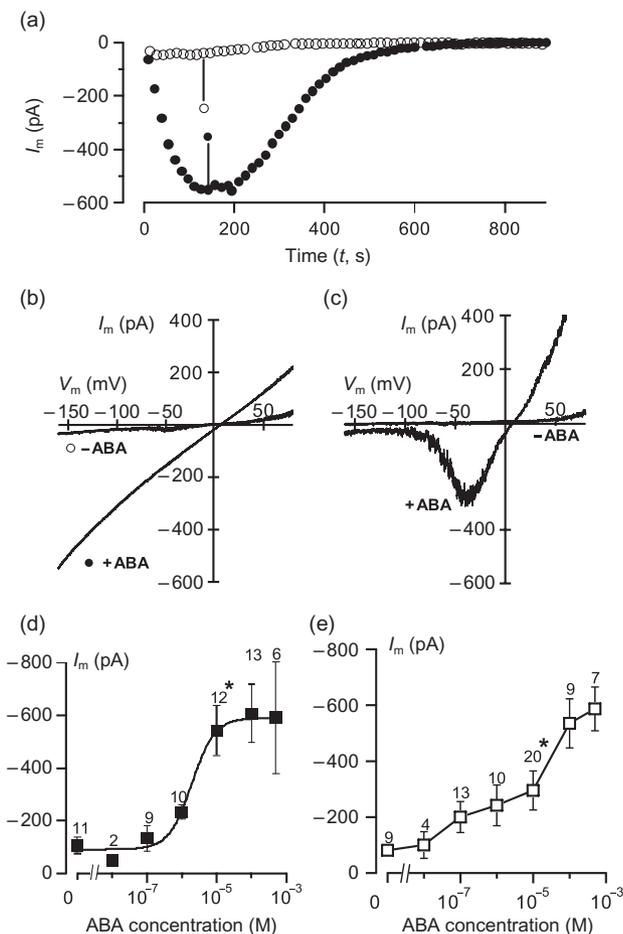


Figure 7. Abscisic acid (ABA) activation of anion currents in guard cell protoplasts. (a) Time courses of anion channel activation in guard cell protoplasts of Nf-treated plants, in the absence (○) or presence (●) of $10 \mu\text{M}$ ABA in the patch pipette. Measurements were started ($t = 0$) after obtaining the whole-cell configuration. The activity of slow (S-type) anion channels is given as the current measured at -160 mV . Data are from the same cell as in (b), with corresponding symbols. (b & c) Current–voltage relation of guard cell protoplasts of Nf plants in the whole-cell configuration. Cells were patched in the absence and presence of $10 \mu\text{M}$ ABA in the pipette. The voltage ramps (1500 ms) were applied from a holding potential of -160 to $+80 \text{ mV}$. In the cell presented in (b), ABA activated S-type anion channels (60 of 68 cells), while ABA activated rapid (R-type) anion channels (8 of 68 cells) in the cell shown in (c). (d & e) Dose–response curves of ABA-dependent anion channel activation in guard cells of control (d) and Nf-treated (e) plants. The maximum currents during ABA transients were plotted as a function of the ABA concentration (error bars represent n , the number of experiments). The asterisks indicate a significant difference in the degree of anion channel activation ($P < 0.05$). E_m , free running membrane potential; I_m , membrane current; V_m , clamped membrane potential.

With $0 \mu\text{M}$ ABA in the patch pipette, only a background anion channel activity was observed. With the increase in ABA concentration above $0.1 \mu\text{M}$, however, anion channel activity rose in a dose-dependent manner (Fig. 7d & e). Dependence of anion channel activation on ABA was fitted with a sigmoidal curve in control guard cells, revealing

half-saturation constants of $2 \mu\text{M} \pm \text{cis, trans ABA}$ and a Hill coefficient of 1.2 (Fig. 7d). For most concentrations of ABA, the difference in anion channel activation was not significant between Nf-treated and control cells. However, at a concentration of $10 \mu\text{M}$, the activation of anion channels in Nf-treated cells was significantly smaller ($P < 0.05$) than in the controls (Fig. 7e). Apparently, Nf treatment decreased the ABA sensitivity, while the same maximum values of anion channel activation were found at high ABA concentration. Note that the maximum amplitude of anion currents was similar, although the albino guard cell protoplasts had a $\sim 34\%$ larger membrane surface than green guard cells (Fig. 1d & e).

DISCUSSION

Light-induced stomatal opening

The stimulation of stomatal opening by light was recognized by botanists as early as 1812 (Moldenhauer 1812). A role for the intercellular CO_2 concentration in this response was postulated about 100 years later (Stahl 1920). CO_2 may represent a feedback signal that couples the degree of stomatal opening to the photosynthetic activity in the leaf (Raschke 1975b; Roelfsema & Hedrich 2005). In the light, photosynthesis will lower the intercellular CO_2 concentration (Hanstein *et al.* 2001), which will in turn cause the stomata to open and enhance the uptake of CO_2 (Roelfsema *et al.* 2002). With this theory, stomata in non-photosynthesizing leaves should not open in response to PAR. Indeed, we found no stimulation of stomatal opening by red light with Nf-treated leaves of *V. faba* nor with the white sectors of *C. comosum* leaves.

The albino leaf areas of the two plants differ because guard cells in *V. faba* displayed no chlorophyll fluorescence (Fig. 1), but guard cells in the white area of *C. comosum* had normal chloroplasts (Fig. 3). These chloroplasts most likely are capable of CO_2 fixation, because the NPQ was characteristically altered in response to changes in the atmospheric CO_2 concentration. Guard cells thus seem to have a functional Calvin cycle (Outlaw 1989), but can also provide ATP for stomatal movement (Mawson 1993; Tominaga, Kinoshita & Shimazaki 2001). This would be in agreement with the relatively low F_v/F_m and associated low F_m/F_0 values, which could be due to a large PSI/PSII ratio. The excess of PSI could provide ATP required for stomatal movement via cyclic electron transport. Despite the role of guard cell chloroplasts in providing energy for stomatal movement, the stomata of *C. comosum* do not open if red light is only given to the white areas of the variegated leaves. This result contradicts the observation that guard cells in isolated epidermal strips, bathed in experimental solutions, open in response to red light (Olsen *et al.* 2002). The difference can be explained by the low rate of diffusion for CO_2 in solution. While isolated guard cells bathed in experimental solutions may alter their own CO_2 concentration, the CO_2 concentration of guard cells in intact leaves probably equilibrates rapidly with the

intercellular gas space. In intact leaves, guard cells therefore likely monitor the intercellular CO₂ level (Mott 1988), which depends predominantly on the photosynthetic capacity of the mesophyll.

In addition to the response of stomata involving photosynthesis, double-beam (red plus blue) experiments revealed a specific response of stomata to blue light (Iino, Ogawa & Zeiger 1985). On the basis of data obtained with epidermal strips of *npq1*, a mutant with a defect in violaxanthin deoxidase, a role of carotenoids in this blue light response was suggested (Frechilla *et al.* 1999). However, Nf-treated leaves of *V. faba* can still respond to blue light, indicating that guard cells have at least an additional blue light receptor that does not depend on carotenoids. Two additional blue light receptors have been suggested to mediate the blue light-specific opening of stomata. Phototropins represent good candidates, because stomata of the *phot1/phot2* double mutant of *Arabidopsis thaliana* do not display a blue light-specific response, but still are responsive to red light (Kinoshita *et al.* 2001). Alternatively, cryptochromes may be involved, because blue light does not provoke an increase in stomatal opening in the *cry1/cry2* double mutant (Mao *et al.* 2005). However, it is not known if the stomata of the latter mutant are still responsive to other stimuli, such as PAR. The blue light responsiveness of stomata may be variable, because the stomata in the white area of *C. comosum* leaves opened in response to blue light in only half of the experiments.

The CO₂ sensor is active in albino guard cells

Although stomata of Nf-treated plants do not respond to red light, they still respond to CO₂ in darkness. CO₂-induced stomatal closure occurs, however, with a lower velocity in Nf plants compared with green controls (Fig. 5a & b). This difference is not due to a general failure of stomata closure in Nf-treated plants, because ABA still induces fast stomatal closure. Furthermore, low concentrations of ABA enhance the CO₂ response of Nf-treated stomata (Fig. 5c), which shows that both signals act synergistically on stomatal movement. The lowered CO₂ response may be linked to altered transcription rates of genes that function in this signalling pathway. Potential candidates for CO₂ sensors are phosphoenolpyruvate (PEP) carboxylases and carbonic anhydrases. The transcription levels of the four PEP carboxylase genes (*AtPPC1-4*) in *Arabidopsis* were not much altered by Nf, but genes encoding cytoplasmic (CA 2) and chloroplastic (CA 1) carbonic anhydrases were down-regulated by Nf (McCormac & Terry 2004b). This suggests a role for carbonic anhydrases in the guard cell response to CO₂.

ABA-responses of guard cells are Nf resistant

Albino leaves obtained with Nf contained only 4% of the ABA content found for green control leaves. Some of the residual ABA could originate from seed stores or it may be formed from a precursor pool existing of E,E- and E,Z-

farnesol (Cowan 2001). The *Arabidopsis aba1* mutant lacks zeaxanthin epoxidase activity and also is unable to synthesize ABA (Cutler & Krochko 1999). The ABA content of *aba1* rosette leaves is reduced from 6 ng gFW⁻¹ (~23 pmol gFW⁻¹) in wild type to 1 ng gFW⁻¹ (~4 pmol gFW⁻¹) in *aba1* leaves (Koornneef *et al.* 1982). Mature *aba1* plants display a retarded growth and tend to wilt, a defect that was restorable by exogenous ABA supply. Wilting was not obvious with Nf-treated *V. faba* plants, presumably because the stomata of these plants also lack the response to PAR, and their stomata therefore do not open as wide as those of the ABA-synthesis mutants.

Guard cells of ABA-depleted plants were expected to have an altered ABA sensitivity, because ABA affects the transcription rate of several genes that encode protein phosphatases 2C and are involved in ABA signalling (Rodriguez, Leube & Grill 1998; Leonhardt *et al.* 2004; Sanchez, Duque & Chua 2004). These protein phosphatases are probably negative regulators of ABA responses in plant cells, because overexpression of HAB1 resulted in reduced ABA sensitivity (Saez *et al.* 2004), while an *abi2* protein with low phosphatase activity (Merlot *et al.* 2001) or loss of function of *AtP2C-HA* (Leonhardt *et al.* 2004) increased ABA sensitivity. It is tempting to speculate that these protein phosphatases create a negative feedback loop, acting on ABA-activated protein kinases encoded by AAKP (Li *et al.* 2000) in *V. faba* and OST1 (Mustilli *et al.* 2002) in *Arabidopsis*. As expected from their ABA-dependent gene activity, *ABI1*, 2 and *AtP2C-HA* were down-regulated in Nf-treated *Arabidopsis* plants, while *HAB1* and 2 transcripts were virtually unchanged (McCormac & Terry 2004b). With these data, guard cells of Nf plants were thus expected to have a higher ABA sensitivity than the control cells. The situation appeared different because guard cells of Nf-treated plants were less responsive to 10 μM ABA in the patch pipette. Apparently, Nf induced a small shift of the dose-response curve, with anion channels requiring higher concentrations of ABA for activation (Fig. 7d & e). However, the maximum activity of anion channels was not affected by Nf. Future experiments should be directed to identify proteins that influence the ABA responsiveness of anion channels through direct manipulation of genes involved in ABA signalling.

In summary, the herbicide Nf has dramatic effects on the phenotype of *V. faba* plants, resulting in albino plants retarded in growth and with strongly reduced ABA content. The stomata of Nf-treated *V. faba* plants do not respond to red light, just as the stomata in the white leaf sectors of *C. comosum*. The absence of a response to PAR is in line with a role for the intercellular CO₂ concentration as an intermediate signal between red light and stomatal opening. Treatment with Nf did not disable stomatal responses to blue light, CO₂ and ABA. The stomata of Nf-treated plants still close in response to ABA, although the dose-response curve of anion channel activation in guard cells was significantly modified. The responsiveness of guard cells to ABA thus seems to be affected by the stress hormone itself. Guard cells thus seem to possess robust

signalling pathways coupling CO₂, blue light and ABA sensors to stomatal movement, which remain active even after depleting plants of carotenoids, chlorophyll and ABA.

ACKNOWLEDGMENTS

We thank K. Neuwinger, E. Reisberger and S. Neimanis (University of Würzburg) for technical assistance concerning the gas exchange recordings, B. Röger (University of Würzburg) for the ABA measurements, U. Schreiber (University of Würzburg) for assistance with the chlorophyll fluorescence measurements, and E. Van Volkenburgh (University of Washington) for English-language proofing. Nf (Zorial Rapid 80) was a gift from Syngenta. This work was supported by a grant of the A. von Humboldt foundation to G. Psaras and grants of the Deutsche Forschungsgemeinschaft to R. Hedrich.

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Received 8 February 2006; received in revised form 18 March 2006; accepted for publication 31 March 2006