Spectral and Dose Dependence of Light-Induced Ion Flux Responses from Maize Leaves and their Involvement in Leaf Expansion Growth

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Introduction

Leaf expansion growth is a complex process strongly influenced by light quantity and quality. The mechanisms underlying light-driven leaf growth involve the orchestrated action of several photoreceptor systems such as phytochrome, blue light (BL) receptors and chlorophyll (Eskins 1992, Neff and Van Volkenburgh 1994, Stahlberg and Van Volkenburgh 1999, Van Volkenburgh 1999, Stiles and Hanson 1992, Bellando et al. 1995, Stiles et al. 2003). The exact role of each photoreceptor in leaf growth, as well as the specific signal transduction pathways, remains obscure (Van Volkenburgh 1999). At the cellular level, light stimulates leaf growth through acidification of the apoplast, probably caused by pumping H⁺ ions out of the cell and acid-induced wall loosening, thus enabling cell expansion (Brock and Cleland 1989, Marré et al. 1989). Apoplastic acidification appears to be coupled with K⁺ uptake (Briskin and Hanson 1992, Bellando et al. 1995, Stiles et al. 2003).

It is generally accepted that in dicots, both BL and red light (RL) promote leaf expansion, but the mechanisms underlying this leaf growth promotion seem to be different. The leaf growth of Arabidopsis and bean plants is stimulated by RL (Eskins 1992, Lavee et al. 2002), most probably through activation of phytochrome (Kozuka et al. 2005), while BL induces less leaf elongation than RL at the same intensity (Jackson and Jenkins 1995). The effect of different light qualities on leaf growth, however, appears to be tissue specific. Kozuka et al. (2005) showed that leaf blade expansion is promoted and leaf petiole elongation inhibited by increasing RL and BL intensities. Continuous RL caused steady swelling of the parenchyma but not epidermal protoplasts from etiolated leaves of many grass species (Long and Iino 2001, and references within). The extension of bean leaves was stimulated by BL after a short lag period of 2 min, while it was slowed by RL for 12 min before stimulating it (Blum et al. 1992). Overall, the kinetics of light-induced changes in leaf expansion growth appear to be very complex. Frequently, leaf growth is temporarily inhibited upon illumination, before accelerating to a new growth rate (Stahlberg and Van Volkenburgh 1999). The underlying ionic mechanisms of this process remain obscure.

Due to the comparatively small volume of the leaf apoplast, addition or removal of relatively few ions will change the apoplastic solute concentration to a much larger extent than that in the cytoplasm. Thus, dumping of solutes into the apoplast, or extraction of solutes from the wall space, represents an efficient way for cells to control the osmotic gradient across the plasma membrane, and consequently turgor and growth rate (Van Volkenburgh 1999). The precise roles of light-stimulated ion fluxes, apart from H⁺ efflux, are far from being understood. It is generally believed that light-induced extrusion of H⁺ into the apoplast (required to activate cell wall loosening) and uptake of K⁺ (needed to adjust the osmotic potential of the cytoplasm) are pivotal processes in cell extension (Elzenga and Van Volkenburgh 1997, Van Volkenburgh 1999).

Abbreviations: BL, blue light; MIFE, microelectrode ion flux estimation; RL, red light; WL, white light.
However, to the best of our knowledge, no direct evidence for RL- or BL-stimulated K⁺ uptake into the growing leaf tissues has been reported. More often, the requirement of K⁺ for leaf growth and the physiological role of light-induced K⁺ fluxes is usually attributed to charge balancing for H⁺ extrusion (Staal et al. 1994, Stiles and Van Volkenburgh 2004). Furthermore, most electrophysiological studies related to the specificity of light effects on various leaf tissues have been carried out on dicotyledonous species (Spalding and Cosgrove 1992, Spalding and Goldsmith 1993, Elzenga and Van Volkenburgh 1997, Stahlberg et al. 2000, Stiles and Van Volkenburg 2002, Stiles and Van Volkenburg 2004). Thus, the extent to which the above conclusions can be extrapolated to monocots remains unclear.

The aim of this study was to assess the spectral and dose dependence of light-induced ion flux responses from maize leaves and relate them to leaf growth by measuring and comparing differences in ion flux kinetics from intact leaves (epidermis attached) and isolated mesophyll tissue. We present evidence that light-induced K⁺ flux kinetics are different between growing and non-growing tissues and attribute this difference to the direct involvement of BL- and RL-induced K⁺ flux in turgor-driven leaf expansion growth, controlled by the epidermis, as well as to the charge-balancing role of K⁺ in the leaf mesophyll. We also show a much stronger influence of RL on Ca^{2+} fluxes in the basal region compared with BL, which argues in favor of an important role for RL in Ca^{2+} signaling during leaf growth.

Results

Leaf segments were exposed to different light intensities (3–300 μmol m⁻² s⁻¹ range) in order to test the dose dependence of light-induced ion flux responses from maize leaves. In this study, we present the transient kinetics of BL- and RL-induced ion flux changes upon maximal intensity (300 μmol m⁻² s⁻¹) which is well in excess of the light compensation point for maize [~40–45 μmol m⁻² s⁻¹ for white light (WL)].

K⁺ flux profiles

Near zero steady-state net K⁺ fluxes were measured from all samples under dark conditions. The onset of RL illumination caused an almost immediate net K⁺ efflux from leaf mesophyll segments (Fig. 1B). This efflux peaked about 5 min after the treatment and then the K⁺ flux gradually increased, resulting in a steady K⁺ uptake 20 min after illumination. No significant (P < 0.05) difference was found between tip and base mesophyll tissues (Fig. 1B) in terms of RL-induced K⁺ flux kinetics. When fluxes were measured from the intact leaf segments, a substantial difference was found between tip and base responses (Fig. 1A).
uptake into intact leaf segments in the growing basal region, as is evident from the dose–response curves (Fig. 5).

**Ca²⁺ flux profiles**

The exposure of leaf segments to either RL or BL caused an increase in net Ca²⁺ influx (Figs. 2, 4). Near zero net Ca²⁺ fluxes were measured in the dark from all samples. RL-induced changes in net Ca²⁺ flux were also very similar between tip and base mesophyll segments (Fig. 2B), with transient Ca²⁺ uptake peaking between 5 and 10 min after the onset of RL. This uptake was short lived, and net Ca²⁺ fluxes returned to the baseline 20 min after the light treatment. In intact segments, however, qualitatively different Ca²⁺ flux kinetics were measured between basal and tip leaf regions (Fig. 2A). In the growing base, RL-induced Ca²⁺ flux kinetics from intact leaf segments (Fig. 2A, filled symbols) were generally similar to those measured from the mesophyll, while in the non-growing tip, RL-induced Ca²⁺ uptake was significantly (P < 0.05) smaller, but stayed elevated for the entire duration of the RL treatment (at least 40 min; Fig. 2A, open symbols). Opposite results were obtained using BL treatment at the same fluence rate (Fig. 4C). Here, the basal intact segments showed essentially no response to BL, while prolonged net Ca²⁺ uptake was measured in the non-growing tip (Fig. 4C). Overall, the effect of RL on Ca²⁺ uptake compared well with that of BL for all fluence rates, in the growing basal region of intact leaves (Fig. 5).

**H⁺ flux profiles**

Hydrogen flux responses were not significantly (P < 0.05) different between the base and tip regions when measured from intact leaf segments in response to either RL (Fig. 3A) or BL (Fig. 4B), with a rapid increase in apparent net H⁺ uptake observed within 1–2 min after light treatment. Mesophyll responses were more complex, with an RL-induced H⁺ efflux measured immediately after RL onset. This efflux peaked 2–3 min after the treatment, followed by a gradual H⁺ uptake over the next 30–40 min (Fig. 3B). The magnitude of this apparent uptake was much stronger in the photosynthetically more competent tip region (Fig. 3B, open symbols). Analysis of the dose dependency data also suggested that RL is more efficient in causing such an apparent uptake of H⁺ compared with BL (Fig. 5).

**Growth experiments**

The relative efficiency of BL and RL on leaf growth was studied in experiments using leaf disks. In the basal leaf segments, 30 μmol m⁻² s⁻¹ of BL was twice as efficient in enhancing leaf expansion growth (Table 1). Leaf expansion growth in the tip region was almost an order of magnitude lower, and not significantly different (at P < 0.05) between BL- and RL-exposed segments (Table 1).

**Discussion**

Leaf expansion growth is a result of the integrated responses of epidermal, mesophyll and vein cells (Staal et al. 1994, Elzenga and Van Volkenburgh 1997, Van Volkenburgh 1999). Understanding the specific details of such integrations is a challenging task. Light-stimulated transport of ions across cell membranes appears to be crucial for this process.
Potassium uptake and leaf growth

Light-stimulated leaf growth depends on K\(^+\) uptake. WL-stimulated growth of poplar leaves was promoted in 50–100 mM KCl (Stiles and Van Volkenburgh 2002), and application of the K\(^+\) channel blocker tetraethylammonium (TEA) inhibited growth of tobacco leaves (Stiles et al. 2003, Stiles and Van Volkenburgh 2004). It is often assumed that the primary role of K\(^+\) uptake is to provide an electrical counterbalance to light-induced H\(^+\) efflux as opposed to a role in solute accumulation and turgor maintenance (Stiles et al. 2003, Staal et al. 1994, Stiles and Van Volkenburgh 2004). This process is believed to be largely independent of photosynthesis (Van Volkenburg and Cleland 1990, Van Volkenburg et al. 1990, Van Volkenburg 1999, Stahlberg et al. 2000). However, photosynthetic activation of K\(^+\) efflux (K\(_{\text{out}}\)) channels by WL was reported for

**Fig. 3** Red light (RL, 300 μmol m\(^{-2}\) s\(^{-1}\))-induced H\(^+\) flux responses from intact (A) and mesophyll (B) leaf segments isolated from growing (leaf base; filled symbols) and non-growing (leaf tip; open symbols) leaf regions. Data are means ± SE (n = 5–8).

**Fig. 4** Blue light (BL, 300 μmol m\(^{-2}\) s\(^{-1}\))-induced K\(^+\) (A), H\(^+\) (B) and Ca\(^{2+}\) (C) flux responses from intact leaf segments isolated from growing (leaf base; open symbols) and non-growing (leaf tip; filled symbols) leaf regions. Data are means ± SE (n = 5–8).

Arabidopsis mesophyll cells (Spalding et al. 1992, Spalding and Goldsmith 1993), presumably via photosynthetically produced ATP (Harada et al. 2002, Goh et al. 2004). At the same time, using chlorophyll-deficient mutant cells from rice, it was also shown that the K\(_{\text{out}}\) channel is controlled by a cytosolic pool of ATP that is predominantly derived from oxidative phosphorylation (see Goh et al. 2004 for references). Thus, it appears that regulation of K\(^+\) fluxes across the plasma membrane is a very complex process controlled by both photosynthetic and non-photosynthetic (phytochrome) sensing systems.
The above reports deal with photosynthetically active WL. In our study on mesophyll cells, onset of RL caused a rapid transient $K^+$ efflux followed by a significant $K^+$ uptake in both growing and non-growing leaf regions (Fig. 1B). It is unlikely that such net uptake could be the result of activation of inward $K^+$ conductance. A more reasonable explanation would be a blockage of $K^+$ efflux by some cytosolic factor. Li and Assmann (1993) reported the inhibition of outward $K^+$ currents from mesophyll cells by GTP-binding proteins and $Ca^{2+}$. Interestingly, although there was no significant ($P < 0.05$) difference in RL-induced $K^+$ efflux between basal and tip regions, the magnitude of $K^+$ flux recovery was twice as large in tip segments (Fig. 1B). Thus, RL-induced increases in the cytosolic ATP pool (according to Spalding and Goldsmith 1993) are not likely to be involved, as leaf basal cells contain only 60% of chlorophyll compared with tip cells (Zivanovic et al. 2005) and thus are much less photosynthetically competent. It appears therefore that that some other factors are involved. As higher net $K^+$ accumulation is expected to lead to increased cell turgor pressure, one possibility is that $K^+$ is used as an osmoticum in the growing basal region.

The above suggestion is further supported by comparing $K^+$ flux responses from intact leaf segments. No RL-induced $K^+$ efflux was measured from the growing basal leaf segment. Instead, net $K^+$ uptake started 7 min after the onset of illumination, consistent with a role for $K^+$ as a major inorganic osmolyte required for leaf expansion growth (Fig. 1A). Importantly, the apparent $K^+$ efflux recorded from basal regions correlates nicely with reported transient inhibition of leaf growth observed during the first 2–6 min upon illumination in some species (Stahlberg and Van Volkenburgh 1999). This confirms a possible causal link between RL-induced $K^+$ flux and leaf growth. BL treatment of intact segments had a greater effect on $K^+$ fluxes compared with RL illumination, as shown by dose dependence studies (Fig. 5). This is in good agreement with the growth experiments on leaf disks undertaken under the same ionic conditions, showing that BL-exposed leaf segments grew twice as fast as those exposed to RL at the same intensity (Table 1). The initial $K^+$ efflux upon RL illumination may fulfill the charge-balancing role, as suggested by other authors (Stiles et al. 2003, Staal et al. 1994, Stiles and Van Volkenburgh 2004).

It should also be noted that qualitatively, the transient $K^+$ flux kinetics in response to 300 $\mu$mol m$^{-2}$ s$^{-1}$ RL reported in this work (Fig. 1) were very similar to leaf responses to 2,600 $\mu$mol m$^{-2}$ s$^{-1}$ WL reported earlier (Zivanovic et al. 2005), in both mesophyll and intact leaf segments. Also, a great deal of similarity was found between RL and WL changes in $Ca^{2+}$ and $H^+$ fluxes. It appears therefore that, electrophysiologically, RL and WL have an

<table>
<thead>
<tr>
<th>Leaf region</th>
<th>RL</th>
<th>BL</th>
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<tr>
<td>Base</td>
<td>5.31 ± 1.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.27 ± 1.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tip</td>
<td>1.25 ± 1.14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.54 ± 1.17&lt;sup&gt;c&lt;/sup&gt;</td>
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Values are given as the mean ± SE ($n = 5–6$).

Values with the same letters are significantly different from each other at $P < 0.05$. 

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almost identical ‘action spectrum’ at relatively high fluence rates.

**Calcium uptake and RL signaling**

It was previously reported that transient Ca\(^{2+}\) uptake is one of the earliest events associated with plant electrophysiological responses to light. RL-induced Ca\(^{2+}\) uptake was measured in moss caulonemal filaments (Ermolayeva et al. 1997, Johannes et al. 1997), in algal cells (Dreyer and Weisenseel 1979), in protoplasts of maize etiolated leaves (Das and Sopory 1985) and in oat leaves (Chae et al. 1990). The protoplast swelling in wheat leaves depended on the presence of Ca\(^{2+}\) in the bathing medium (Bossen et al. 1988) and was required for phytochrome signaling in RL responses (Long and Iino 2001). In contrast to RL, BL-induced protoplast shrinking without Ca\(^{2+}\) uptake implicates the involvement of cryptochrome in protoplast shrinking (Long and Iino 2001). No swelling was observed in protoplasts pre-treated with the Ca\(^{2+}\) channel blocker verapamil (Bossen et al. 1991). The physiological role of this Ca\(^{2+}\) influx is at least 3-fold. First, rapid Ca\(^{2+}\) influx may be responsible for the transient membrane depolarization observed in response to leaf illumination (Weisenseel and Ruppert 1977, Takagi and Nagai 1988, Spalding and Cosgrove 1992, Elzenga et al. 1995, Shabala and Newman 1999). Secondly, phytochrome-mediated Ca\(^{2+}\) influx may be important for exocytosis and vesicle fusion to the plasma membrane, both of which are under the control of cytosolic Ca\(^{2+}\) (Thiel and Battey 1998). This ultimately links light-induced Ca\(^{2+}\) uptake with leaf expansion growth. Thirdly, transient RL-induced rises in cytoplasmic Ca\(^{2+}\) (Shacklock et al. 1992) are likely to function in a signal transduction chain, most probably involving calcium–calmodulin signaling (Elzenga et al. 1997).

The results presented in this study are generally consistent with the above reports. Transient net Ca\(^{2+}\) influx was measured from both basal and tip mesophyll segments (Fig. 2B) in response to RL illumination. The eventual contribution of photosynthesis to Ca\(^{2+}\) influx is not likely to be significant as there was no difference in Ca\(^{2+}\) fluxes between the basal (lower chlorophyll content) and tip (higher chlorophyll content) leaf region from mesophyll tissue (Fig. 2B). These results are in line with previous findings (Živanović et al. 2005) that showed that the photosynthetic inhibitor DCMU did not affect net Ca\(^{2+}\) influx. Also enhanced was Ca\(^{2+}\) uptake measured near the epidermal surface of intact leaf segments (Fig. 2A). However, the ‘signatures’ of Ca\(^{2+}\) uptake were strikingly different between the growing (base) and the non-growing (tip) regions, suggesting non-photosynthetic photoreceptor-mediated Ca\(^{2+}\) influx in RL-driven leaf growth. The effect of BL on Ca\(^{2+}\) fluxes was completely different from that with RL (Fig. 4C). As the basal tissue was less responsive to BL than the tip, it is unlikely that BL-induced Ca\(^{2+}\) fluxes are directly involved in leaf growth. Also the stronger influence of RL on Ca\(^{2+}\) fluxes in the basal region compared with BL argues in favor of an important role for RL in Ca\(^{2+}\) signaling during leaf growth.

**Hydrogen fluxes: masking effect of photosynthesis**

A rapid shift towards net H\(^{+}\) influx was observed upon both RL and BL illumination near intact leaf segments (Figs. 3A, 4B). This is consistent with our previous reports for WL (Živanović et al. 2005) and is explained by the masking effects of apoplastic alkalization due to RL- or BL-induced CO\(_2\) uptake, required to drive photosynthesis. This alkalization masks any acidification of the apoplast resulting from light activation of H\(^{+}\)-ATPase pumps as reported elsewhere (Staal et al. 1994, Stahlberg and Van Volkenburgh 1999, Van Volkenburg 1999). Accordingly, interpreting the effects of RL and BL on H\(^{+}\) flux kinetics in photosynthesizing leaf tissues is highly speculative, and even misleading, unless the bath CO\(_2\) concentration can somehow be maintained at a constant level. For the same reasons, correlative analysis between the kinetics of H\(^{+}\) and other fluxes is also misleading. These issues remain a great challenge for future studies.

**Materials and Methods**

**Plant material**

Maize plants (Zea mays L.) were grown from seed under glasshouse conditions in a standard potting mixture, essentially as described by Živanović et al. (2005). The experiments were carried out on 14- to 18-d-old plants. Small leaf segments (~4 × 7 mm) were excised from two different regions of the third leaf: (i) the basal part (growing region; ~15 mm above the ligule) and (ii) the tip region (non-growing tissue; ~15 mm from the leaf tip) (see Živanović et al. 2005 for details). To avoid any blocking effects of the cuticle on ion fluxes thus enabling ion flux measurements, the leaf cuticle was gently removed by methanol as described in our previous publications (Shabala and Shabala 2002, Živanović et al. 2005). We refer to these segments as ‘intact’ (e.g. having the epidermis attached to the mesophyll tissue). Mesophyll segments were prepared by removing the abaxial epidermal tissue from leaf segments using fine forceps (Eye-Instruments, Albert Heiss H3376, Tutlingen, Germany) as described earlier by Živanović et al. (2005).

Isolated leaf segments were left floating on experimental solution for several hours in order to avoid wounding effects and to stabilize ion fluxes. The ionic composition of the experimental solution was: 0.1 mM KCl, 0.1 mM NaCl, 0.1 mM MgCl\(_2\), 0.05 mM CaCl\(_2\), 0.05 mM NH\(_4\)Cl, 5 mM sucrose (pH 5.1, non-buffered). Under these conditions, the overall electrochemical K\(^{+}\) gradient favors passive K\(^{+}\) efflux which gradually decreases over several hours. Accordingly, all flux measurements were taken about 4 h after segment isolation.

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vibrating microelectrodes (the MIFE technique) as previously reported (Shabala et al. 1997, Shabala and Newman 2000, Živanović et al. 2005). Briefly, electrode blanks were pulled from borosilicate glass capillaries (GC150-10, Clark Electrochemical Instruments, Pangbourne, Berks, UK), dried overnight in an oven at 220°C and silanized with tributylchlorosilane (Fluka 90796). Cooled microelectrodes were backfilled with solutions (15 mM NaCl + 40 mM KH2PO4, adjusted to pH 6 by NaOH for H+; 200 mM KCl for K+; and 500 mM CaCl2 for Ca2+). Commercially available ionophore cocktails (H 95297; K+ 60031; Ca2+ 21048; all from Fluka) were used for filling the electrode tips. Ion-selective microelectrodes were calibrated in a set of standard solutions before and after use. The average slope was 53–54 mV per unit of ion activity for monovalent and Ca2+ ions, respectively.

**Experimental protocol and treatments**

Excised leaf segments were mounted in a Perspex holder and placed in a measuring chamber filled with aerated measuring solution (0.1 mM KCl and 0.1 mM CaCl2; pH 5.1, non-buffered). Microelectrodes were mounted on a 3D-micromanipulator. Electrodes were positioned near the leaf surface under dim green microscope light. Leaf samples were kept in the dark for 0.5 h before the onset of illumination. Ion fluxes were recorded 40 μm above the leaf surface and calculated according to previously described procedures (Shabala and Newman 1999, Newman 2001). Leaf segments were illuminated with different light qualities using a cool light source (KL1500 LCD, Schott, Mainz, Germany) with a flexible light guide and blue or red light filters (BG37 for 481 nm, RG610 for 625 nm; Schott, Mainz, Germany). Light intensity was measured by a Li-Cor quantum photometer (LI-250 light meter; Li-Cor Inc., Lincoln, NE, USA). The effects of BL and RL (3–300 μmol m−2 s−1 range) on ion fluxes were tested by exposing the leaf segments to 40 min/40 min light/dark cycles. Transient K+, H+ and Ca2+ fluxes were measured from leaf segments isolated from either basal or tip regions (growing vs. non-growing), from both intact and mesophyll tissues (see Plant material section).

The dose dependency of RL- and BL-induced K+, H+ and Ca2+ fluxes was studied by measuring transient ion flux responses (as above) at three fluence rates: 3, 30 and 300 μmol m−2 s−1. These measurements were undertaken from intact leaf segments isolated from growing (basal) parts. For K+ and H+, the magnitude of the ion flux response was determined as the difference between the steady-state dark and light values. These values were then plotted against fluence rate in Fig. 5. For Ca2+ flux, the values shown in Fig. 5 are the average change in the leaf diameter per unit time over the entire growth period.

**Acknowledgments**

This work was supported by an ARC Grant (A00001144) to S.S.

**References**


(Received January 17, 2007; Accepted February 21, 2007)