Pulsing of Membrane Potential in Individual Mitochondria: A Stress-Induced Mechanism to Regulate Respiratory Bioenergetics in Arabidopsis

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INTRODUCTION

Mitochondria are an essential feature of nearly all eukaryotic cells, providing the energy transformation capacity that is necessary to maintain and express a large genome (Lane and Martin, 2010). In addition, mitochondria perform many other essential roles, including provision of carbon skeletons for biosynthesis (Fernie et al., 2004), synthesis of a number of important cofactors (e.g., cytochromes, heme, iron sulfur clusters, tetrahydrofolate; Rebeillé et al., 1997; Meyer et al., 2005; Balk and Pilon, 2011), as well as occupying a central position in programmed cell death signaling (Kim et al., 2006; Scott and Logan, 2008). In green plant tissues, mitochondria are also critically important for efficient photosynthesis, catalyzing an essential step of the photosynthetic electron transport chain. Here, we report a mechanism that regulates bioenergetic balance in individual mitochondria: a transient partial depolarization of the inner membrane. Single mitochondria in living Arabidopsis thaliana root cells undergo sporadic rapid cycles of partial dissipation and restoration of membrane potential, as observed by real-time monitoring of the fluorescence of the lipophilic cationic dye tetramethyl rhodamine methyl ester. Pulsing is induced in tissues challenged by high temperature, H2O2, or cadmium. Pulses were coincident with a pronounced transient alkalinization of the matrix and are therefore not caused by uncoupling protein or by opening of a nonspecific channel, which would lead to matrix acidification. Instead, a pulse is the result of Ca2+ influx, which was observed coincident with pulsing; moreover, inhibitors of calcium transport reduced pulsing. We propose a role for pulsing as a transient uncoupling mechanism to counteract mitochondrial dysfunction and reactive oxygen species production.

The advent of live-cell fluorescent imaging has shown the mitochondrial population in a single plant cell to be highly heterogeneous in terms of size, shape, and motility of each mitochondrion (Logan, 2010). The morphological diversity within the mitochondrial population is also mirrored in the organization of the mitochondrial genome, which is heteroplasmic and unevenly distributed among the physically discrete mitochondria (Lonsdale et al., 1988; Arrieta-Montiel et al., 2009; Woloszynska, 2010). It is unclear, however, whether this structural heterogeneity reflects heterogeneity of function, since most studies of mitochondrial bioenergetics and metabolism are based on population measurements. One fundamental functional characteristic of mitochondria, the electrical potential across the inner mitochondrial membrane, can be assessed in vivo at the level of a single mitochondrion by quantifying the accumulation of fluorescent lipophilic cations using fluorescence microscopy. Using such an approach with living neurites, it was first noticed that a small proportion of mitochondria in a cell undergo spontaneous fluctuations of membrane potential during which membrane potential drops by ~20 mV and then returns to the starting value within 30 s (Loew et al., 1993). This bioenergetic phenomenon can also be seen in isolated animal mitochondria (Hüser et al., 1998) and is sometimes referred to as mitochondrial flickering (Duchen et al., 1998). The underlying cause of mitochondrial flickering remains a matter of debate and has been suggested to...
involves changes in ATP-synthase activity (Buckman and Reynolds, 2001), mitochondrial anion transport (O’Rourke, 2000), calcium flux (Duchen et al., 1998; Vergun and Reynolds, 2004, 2005), or the permeability transition pore (Hüser et al., 1998). Furthermore, the observation that an initially localized induction of flickering in a single mitochondrion can propagate in a coordinated fashion throughout the mitochondrial population (Aon et al., 2003; Kurz et al., 2010) is suggestive of a mobile cytoplasmic signaling component that can diffuse between mitochondria. Recently, a similar dynamic phenomenon, termed superoxide “flashes,” has been reported at the level of a single mitochondrion using circularly permutated yellow fluorescent protein (cpYFP; Wang et al., 2008), which appeared to coincide with membrane potential flickers (Wang et al., 2008; Fang et al., 2011). However, the specificity of the cpYFP probe for superoxide under these circumstances has been challenged (Muller, 2009; Meyer and Dick, 2010; Schwarzländer et al., 2011), raising questions as to the underlying cause of these flashes.

To date, there is no information on the bioenergetic dynamics of individual mitochondria in plant cells. Given the extensive heterogeneity of plant mitochondria in terms of genome content, morphology, and movement and the highly variable environment experienced by plant cells, we propose that heterogeneity of mitochondrial function is not only likely, but that it constitutes a fundamental and unexplored aspect of mitochondrial physiology and signaling in plants. To address this gap in our knowledge, we determined the in vivo dynamics of individual mitochondrial membrane potentials in Arabidopsis thaliana roots. Here, we report the occurrence of spontaneous transient fluctuations of mitochondrial membrane potential, termed “pulses,” in living plant cells. In addition, we demonstrate that these pulses are induced by environmental stress. Using isolated mitochondria, we investigate the mechanism for the measured decrease in membrane potential during a pulse. Based on this information, we put forward a model that suggests there is a similar underlying cause for experimental observations of pulsing phenomena in animal and plant cells and discuss how fluctuations in membrane potential might play a vital role in mitochondrial bioenergetics and signaling.

RESULTS

Transient Decrease in Membrane Potential of Single Mitochondria in Living Root Cells

The membrane potential of mitochondria was assessed in living plant cells by confocal laser scanning microscopy (CLSM) using the cationic lipophilic fluorescent dye tetramethyl rhodamine methyl ester (TMRM). TMRM accumulates reversibly in the mitochondrial matrix in a Nernstian manner in response to the inner mitochondrial membrane potential and therefore acts as a dynamic reporter of mitochondrial membrane potential (Brand and Nicholls, 2011). Arabidopsis roots expressing a mitochondrially targeted green fluorescent protein (mito-GFP; Logan and Leaver, 2000) were used to provide a reference image of each mitochondrion and equilibrated with 20 nM TMRM (Brand and Nicholls, 2011). TMRM accumulated in mitochondria as indicated by colocalization of TMRM fluorescence with the mito-GFP signal (Figure 1A). The overall TMRM signal varied slightly between cells, but within a cell the fluorescence intensities from individual mitochondria were very similar. Thus, the coefficient of variation for the ratio of TMRM to GFP signal from individual mitochondria within a cell ranged from 7.6 to 11.7% (491 mitochondria from n = 9 cells). All organelles that showed a GFP signal also showed a TMRM signal and vice versa, suggesting that all mitochondria had established a membrane potential. Analysis of the TMRM fluorescence intensity of individual

![Figure 1. Membrane Potential of Individual Mitochondria in Arabidopsis Root Epidermal Cells.](image)

(A) Colocalization of TMRM (20 nM; red) with mito-GFP (green) and merged overlay of both channels in two neighboring root cells (cell wall shows some unspecific signal in the TMRM channel). (B) Images at three time points of several mitochondria (same color coding as in [A]; one of which underwent a pulse (indicated by arrow). Bars = 2 μm. (C) Corresponding fluorescence intensity traces of TMRM (red) and mito-GFP (green) for the mitochondrion highlighted in (B). More than 300 cells were assessed and similar events were found consistently. A typical event is shown.
mitochondria over time showed that single mitochondria occasionally underwent a spontaneous transient decrease in membrane potential (Figures 1B and 1C; see Supplemental Video 1 online). Occurrences of decrease and recovery of membrane potential, which we refer to as pulses, took place within a 5- to 50-s timeframe (typically ~20 s) and involved a variable degree of TMRM loss ranging from ~20% down to background levels of TMRM fluorescence. In an unstimulated cell, there was an average rate of 0.15 pulses per 100 mitochondria per minute (1980 mitochondria from $n = 21$ cells/roots), although rates were quite variable between cells, leading to a spread in values (Figure 2, control). During a pulse the GFP fluorescence of the mitochondrion remained constant (Figures 1B and 1C), showing that the mitochondrion had not simply moved out of the focal plane of the microscope. Pulses were not an artifact caused by the presence of TMRM because the same phenomenon was also observed with the chemically distinct mitochondrial membrane potential dye 3,3'-dihexyloxacarbocyanine [DiOC$_6$(3)] (Matzke and Matzke, 1986; Liu et al., 1987) (see Supplemental Figure 1 online). Although pulses were rare events under control conditions, a first pulse for any given mitochondrion was often followed by another pulse within seconds or minutes. In vivo pulses frequently coincided with a spontaneous and transient change in shape of the individual mitochondrion involving a slight shortening of the longitudinal axis and concomitant rounding-up of the organelle (see Supplemental Video 5 online).

**Mitochondrial Pulsing Is Increased by Environmental Stress**

To establish whether the extent of mitochondrial pulsing depended on the conditions experienced by the cell, *Arabidopsis* roots were exposed to a variety of short- and medium-term abiotic stresses (Figure 2). The pulsing rate was strongly induced by heat treatments: nearly 30-fold in response to 42°C for 5 min and >10-fold in response to 40°C for 30 min. Repetitive pulses in individual mitochondria were common as a result of either regime. Treatments with H$_2$O$_2$ (10 mM) for 5 min also increased pulsing by 18-fold compared with the control. Although 10 mM H$_2$O$_2$ is a significant oxidative challenge, plant cells can detoxify such concentrations within minutes (Marty et al., 2009; Schwarzlnder et al., 2009). By contrast, the pulsing rate was not significantly changed in response to short- and medium-term salt treatments (500 mM NaCl for 10 min or 150 mM NaCl for 60 min) nor to osmotic treatments (1 M manitol for 10 min or 300 mM manitol for 60 min). Heavy metal exposure caused a 10-fold increase in pulsing when 3 mM CdSO$_4$ was applied for 60 min, but not in response to exposure to 5 mM CdSO$_4$ for 10 min. Although mitochondrial movement was partly inhibited in response to these stress treatments (data not shown), this was observed to be reversible; in addition, seedlings grew normally following transfer to standard culture conditions (data not shown). Together, these data show that pulsing can be strongly stimulated by certain stresses, while others had no obvious impact.

**Increased Pulsing Correlates with Mitochondrial Oxidation**

To investigate the relationship between stress treatments and mitochondrial pulsing further, we assessed the change in mitochondrial redox status in response to the different treatments. Increased mitochondrial reactive oxygen species (ROS) production, leading to oxidative stress, including oxidation of cellular redox buffers, such as the GSH pool, is a common outcome of a wide range of unfavorable conditions. Redox-sensitive GFP

![Figure 2. Effect of Abiotic Stress on the Occurrence of Pulsing and Mitochondrial Redox Status in *Arabidopsis* Root Epidermal Cells.](image)
biosensors provide a route to measure such changes with subcellular resolution (Meyer et al., 2007; Schwarzländer et al., 2008). Hence, mitochondrially localized roGFP1 (mt-roGFP1) was used to assess the impact of the stressors above on mitochondrial GSH redox status in roots (Morgan et al., 2008; Schwarzländer et al., 2009). Strikingly, significant changes in the oxidation of mt-roGFP1 were observed only under those conditions that also caused induction of pulsing (Figure 2B). Both heat treatments and H$_2$O$_2$ exposure led to strong oxidation of mt-roGFP1 in root epidermal cells, while salt and osmotic stress caused no significant change. This was despite obvious plasmolysis confirming the effectiveness of the treatments, causing slight detachment of the protoplast from the cell wall for 150 mM NaCl and 300 mM mannitol, and strong protoplast shrinkage for 500 mM NaCl and 1 M mannitol. Exposure of roots to 5 mM CdSO$_4$ for 10 min did not cause any oxidation of mitochondrial roGFP1, but 3 mM CdSO$_4$ for 60 min resulted in significant oxidation, which suggests that longer incubation times are required to allow effective Cd$^{2+}$ uptake even at relatively high external concentrations. The observed differential oxidation of mt-roGFP1 in response to different stressors demonstrates that change in mitochondrial redox status is not a universal feature of environmental stress. Moreover, the qualitative correlation between induction of pulsing and mitochondrial oxidative stress under different abiotic stress conditions suggests a close mechanistic link between mitochondrial ROS levels, or redox status, and pulsing.

**Isolated Mitochondria Show High Rates of Membrane Potential Pulsing**

The fact that mitochondrial pulsing in vivo is sensitive to environmental stress suggests that pulsing has physiological significance. To understand the role of pulsing and its physiological impact, it is necessary to understand what causes pulsing at the biochemical level. For this purpose, we chose to study isolated mitochondria, which offer practical advantages over in vivo experiments, including the ability to control the bioenergetic state through the provision of different substrates, straightforward application of inhibitors, particularly those that cannot be reliably used in vivo due to insufficient uptake by the plant cell, and improved quantitative imaging. This is particularly important in plants where mitochondria in vivo are highly motile, hindering quantitative imaging of single organelles over a time course, whereas isolated mitochondria can be immobilized by gentle centrifugation onto a cover slip. We therefore tested whether membrane potential pulsing could be observed in isolated mitochondria. Freshly isolated, well-coupled Arabidopsis mitochondria were provided with succinate as respiratory substrate and 100 nM TMRM and imaged by CLSM. Under these conditions, respiration operates in state 2, as ATP-synthase cannot operate in the absence of ADP and phosphate. In state 2, membrane potential is high (allowing TMRM imaging) but the electron transport chain (ETC) is also reduced, leading to a high rate of ROS production (Murphy, 2009). All mitochondria showed high rates of pulsing (Figure 3; see Supplemental Video 2 online). Pulses in isolated mitochondria displayed similar characteristics to those observed in vivo, both in terms of pulse interval (typically 5 to 50 s) and in terms of extent of TMRM loss (Figure 3B). These results suggest that the pulsing phenomena observed in vivo and in isolated mitochondria share a common underlying mechanism. It further demonstrates that mitochondria per se can sustain pulsing in the absence of the cellular context. The high rate of pulsing in isolated mitochondria could be attributed to either a loss of cellular factors that might control pulsing or to the high membrane potential and ROS generation associated with state 2 respiration.

**The Use of Inhibitors to Explore the Mechanism of Pulsing in Isolated Mitochondria**

To explore the mechanism responsible for pulsing, we exploited the isolated mitochondrial system to test a range of mitochondrial effectors. Mitochondrial membrane potential is determined
by the balance of proton pumping by the ETC and the influx of protons (and other cations) back into the matrix due to transport mechanisms, such as the ATP-synthase, uncoupling protein, and a variety of ion channels. In principle, therefore, pulsing could either be caused by a rapid decrease in ETC activity or by a rapid electrogenic flux of protons or other ions. Distinguishing between these mechanisms provided the focus for an inhibitor screen. To get a rough quantitative measure of pulsing activity, the TMRM signal for individual mitochondria over a time course was extracted from the CLSM imaging data. The mean of the coefficient of variation (mean CV) of the TMRM signals over time for individual mitochondria were used to determine the effect of treatments on pulsing (Figure 4). Mitochondria that are inhibited in pulsing will exhibit lower CV values than those that pulse dramatically and frequently. A large number of single mitochondria were analyzed for each sample (typically >150 mitochondria from ≥2 experimental repeats).

Pulsing is dependent on ETC activity to establish a membrane potential in the first place. However, respiratory components can be specifically inhibited or bypassed while maintaining active proton pumping and a membrane potential. This allows testing whether the activity of a specific ETC complex is responsible for pulsing. We compared pulsing rates under five different modes of electron transport (Figure 4A; see Supplemental Table 1 online) that engage different combinations of the ETC complexes and are known to drive differential ROS production. The highest pulsing activity (mean CV) was observed during succinate-driven respiration (complex II substrate). Pulsing was significantly less pronounced when ferrocyanide (complex IV substrate) was used as substrate, while a combination of the tricarboxylic acid cycle intermediates, pyruvate and malate, which provide reductant to complexes I and II, led to intermediate pulsing activity. Bypassing complex III and diverting electron flow to the alternative oxidase by myxothiazol treatment resulted in reduced pulsing under pyruvate/malate respiration to the level measured during ferrocyanide respiration (succinate cannot be used with myxothiazol as the activity of at least one proton pumping complex is required to maintain a membrane potential). Inhibition of complex IV with potassium cyanide further reduced pulsing to a small but significant degree. While the mechanistic link between pulsing and mode of electron transport is not clear, pulsing activity correlates with the ROS production that has been associated with the different ETC modes (Murphy, 2009). While pulsing activity was influenced by electron transport mode, pulsing continued under all combinations of substrates and inhibitors tested, suggesting that the pulsing mechanism does not involve changes in activity of any single ETC complex.

There are several mechanisms downstream of electron transport and proton pumping that could lead to pulsing, including (1) increased proton influx via ATP-synthase or uncoupling protein (Smith et al., 2004); (2) specific influx of other cations, such as potassium (Pastore et al., 1999) or calcium (Duchen et al., 1998); (3) efflux of anions via the inner mitochondrial membrane anion channel (O’Rourke, 2000); or (4) ionic equilibration due to the opening of a nonspecific pore, such as the permeability transition pore (mtPTP) (Hüser et al., 1998; Hüser and Blatter, 1999; Zorov et al., 2000; Jacobson and Duchen, 2002; for a review of the mtPTP in plants, see Logan, 2008). Proton translocation by ATP-

Figure 4. A Screen for Inhibition of Pulsing in Isolated Arabidopsis Mitochondria.

Pharmacological manipulation of mitochondrial electron transport and inner membrane channels led to inhibited ROS production. The mean CV of the TMRM signal of single mitochondria is used as a measure of pulsing activity. Mitochondria in minimal medium were supplemented with a respiratory substrate, oligomycin (25 μM), to inhibit ATP-synthase and BSA (0.1% [w/v]) to sequester free fatty acids that can cause uncoupling. (A) Pulsing activities during different electron transport modes driven by either succinate (succ), ferrocyanide (fer), or pyruvate/malate (pyr/mal) as respiratory substrates and inhibition of complex III or IV by myxothiazol (myx) or potassium cyanide (KCN), respectively (see Supplemental Table 1 online), n > 450; error bars, ± SE. (B) Change of pulsing activity, as measured by fold change in mean CV, in response to different inhibitors of mitochondrial inner membrane channels and redox-active compounds (see Supplemental Table 2 online). Atr, Atractosylide; CsA, cyclosporin A; DIDS, dithiocyanostilbene disulfonate; DTE, dithioerythritol; EGTA 150, 150 μM EGTA; EGTA 300, 300 μM EGTA; Glyb, glyburide; LaCl₃, lanthanum chloride; Nem, N-ethylmaleimide; RuRed, ruthenium red; Ryan, ryanodine; Succ, succinate; Temp, Tempol. Respiration was driven by 10 mM succinate. n > 150. Error bars = 95% confidence interval (logarithmic transformation) on the ratio of mean CV (with effector treatment) to mean CV (in corresponding control experiment). **P < 0.01 (Bonferroni corrected).
synthase is unlikely as a pulsing mechanism because ATP-synthase is inactive under the control conditions due to the absence of ADP and the inclusion of the inhibitor oligomycin. To test some of the other possible mechanisms, pulsing activity was measured during treatment with 19 pharmacological effectors, targeting different mitochondrial channels (uncoupling protein, K⁺ channel, Ca²⁺ channels, inner mitochondrial membrane anion channel, and mtPTP), redox active compounds, and ROS (see Supplemental Table 2 online), the rationale being that treatments that lead to a strong decrease, or arrest, of pulsing are likely to affect mitochondrial components that mediate pulsing. This screen was limited to treatments that did not strongly affect the mitochondrial membrane potential and, thereby, TMRM uptake. Although pulsing activity was changed by a number of treatments, there was no consistent behavior of all the agonists and antagonists that were expected to influence a specific target (Figure 4B). Interestingly, nine effectors caused an increase compared with the control, but only three caused a significant decrease in mean CV. Lanthanum chloride and mitoQ caused a close to complete arrest of observable pulsing (see Supplemental Table 2 online), the rationale being that treatments that lead to a strong decrease, or arrest, of pulsing are likely to affect mitochondrial components that mediate pulsing. This screen was limited to treatments that did not strongly affect the mitochondrial membrane potential and, thereby, TMRM uptake. Although pulsing activity was changed by a number of treatments, there was no consistent behavior of all the agonists and antagonists that were expected to influence a specific target (Figure 4B). Interestingly, nine effectors caused an increase compared with the control, but only three caused a significant decrease in mean CV. Lanthanum chloride and mitoQ caused a close to complete arrest of observable pulsing (see Supplemental Table 2 online), the rationale being that treatments that lead to a strong decrease, or arrest, of pulsing are likely to affect mitochondrial components that mediate pulsing. This screen was limited to treatments that did not strongly affect the mitochondrial membrane potential and, thereby, TMRM uptake. Although pulsing activity was changed by a number of treatments, there was no consistent behavior of all the agonists and antagonists that were expected to influence a specific target (Figure 4B). Interestingly, nine effectors caused an increase compared with the control, but only three caused a significant decrease in mean CV. Lanthanum chloride and mitoQ caused a close to complete arrest of observable pulsing (see Supplemental Table 2 online), the rationale being that treatments that lead to a strong decrease, or arrest, of pulsing are likely to affect mitochondrial components that mediate pulsing.

The relationship between membrane potential pulses and matrix alkalization was dissected to gain further insight into the pulsing mechanism. First, matrix pH was clamped by nigericin in the presence of K⁺. Clamping of pH had no affect on membrane potential pulses, but coincident changes in cpYFP signal were fully abolished (see Supplemental Figures 2B and 2C online). Second, the pH buffering capacity of the matrix was increased by loading mitochondria with 5 mM phosphate, which strongly attenuated the increase in cpYFP fluorescence during TMRM pulses, which themselves were not changed (see Supplemental Figures 2D and 2E online). These experiments clearly show that the pH change is a consequence, rather than cause, of the decrease in membrane potential. It was then assessed if increased proton pumping by the ETC is responsible for the increase in matrix pH. We used malonate to partially inhibit the ETC at complex II and thereby limit ETC capacity in isolated mitochondria respiring succinate. By careful titration, it was possible to identify the highest malonate concentration (1 mM) that did not affect the establishment of state 2 membrane potential (data not shown). Under these conditions, membrane potential pulsing still occurred, but the associated increase in pH was substantially attenuated (Figure 5C). This experiment demonstrates that matrix alkalization is caused by increased proton pumping by the ETC, as a result of a decrease in membrane potential. Pulses are therefore caused by an ion flux

Pulses Are Linked to Transient Matrix Alkalization

We infer from the effector screening that pulsing may be due to a specific ion flux that can be inhibited by lanthanum, rather than the opening of a nonspecific pore, such as the mtPTP. A powerful way of discriminating between the opening of a nonspecific pore and a specific channel is to measure and compare the effect on matrix pH. Opening of a nonspecific pore would allow the equilibration of ions across the inner mitochondrial membrane. Because of the pH gradient across this membrane, pore opening would cause a decrease in matrix pH. By contrast, specific electrogenic influx of cations other than protons (or specific efflux of anions) would cause an increase in matrix pH. This relationship was first formalized by Peter Mitchell (Mitchell, 1966): Electrogenic ion flux decreases the membrane potential and thereby allows increased ETC activity and increased proton pumping, decreasing proton concentration in the matrix.

Recently, we reported transient events of pronounced matrix alkalization (up to >1 pH unit) in single plant mitochondria (Schwarzländer et al., 2011). This finding was the result of a careful reassessment of the properties of a cpYFP that, although originally proposed to be a specific reporter of superoxide (Wang et al., 2008), is in fact extremely sensitive to pH changes in the mitochondrial matrix (Schwarzländer et al., 2011). Since the observed transient increases in cpYFP intensity were of a similar frequency and duration as membrane potential pulses both in vivo and in isolated mitochondria, we decided to investigate possible links between the two phenomena. In root cells of transgenic Arabidopsis expressing mitochondrially targeted cpYFP, transient increases in cpYFP fluorescence exactly coincided with a pulse as measured by TMRM fluorescence (Figure 5A; see Supplemental Video 5 online). Temporal coincidence between pulse and matrix alkalization was confirmed in isolated mitochondria (Figure 5B; see Supplemental Video 6 online). Indeed, there was a strict inverse correlation between TMRM signal intensity and cpYFP signal intensity for each mitochondrion (within the technical limitations set by the maximal scan speed of 0.5 s per frame and probe response times) (see Supplemental Figure 2A online). The correlative changes in fluorescence were not due to direct interaction between the two fluorescent probes because the cpYFP transients were also observed in the absence of TMRM (Schwarzländer et al., 2011). The strict temporal coincidence of events suggests that membrane potential pulses and transient mitochondrial alkalization events are manifestations of the same mitochondrial phenomenon.

The Mechanistic Link between Membrane Potential Pulses and Matrix Alkalization

The relationship between membrane potential pulses and matrix alkalization was dissected to gain further insight into the pulsing mechanism. First, matrix pH was clamped by nigericin in the presence of K⁺. Clamping of pH had no affect on membrane potential pulses, but coincident changes in cpYFP signal were fully abolished (see Supplemental Figures 2B and 2C online). Second, the pH buffering capacity of the matrix was increased by loading mitochondria with 5 mM phosphate, which strongly attenuated the increase in cpYFP fluorescence during TMRM pulses, which themselves were not changed (see Supplemental Figures 2D and 2E online). These experiments clearly show that the pH change is a consequence, rather than cause, of the decrease in membrane potential. It was then assessed if increased proton pumping by the ETC is responsible for the increase in matrix pH. We used malonate to partially inhibit the ETC at complex II and thereby limit ETC capacity in isolated mitochondria respiring succinate. By careful titration, it was possible to identify the highest malonate concentration (1 mM) that did not affect the establishment of state 2 membrane potential (data not shown). Under these conditions, membrane potential pulsing still occurred, but the associated increase in pH was substantially attenuated (Figure 5C). This experiment demonstrates that matrix alkalization is caused by increased proton pumping by the ETC, as a result of a decrease in membrane potential. Pulses are therefore caused by an ion flux
without equilibration of protons. This means that membrane potential pulsing cannot be mediated by mechanisms such as mtPTP, uncoupling protein, or ATP-synthase and suggests that pulsing is caused by transient opening of a channel specific for ions other than protons.

Pulses Coincide with an Increase in Matrix Calcium

We next sought to identify the ion responsible for pulsing. To determine which cations are able to stimulate an increase in matrix pH, the effect of different physiologically relevant cations on cpYFP fluorescence was assessed in populations of energized mitochondria using fluorimetry under the same conditions under which pulsing was analyzed (Figure 6). The divalent cations Mg\(^{2+}\) and Ca\(^{2+}\) both caused an increase in cpYFP fluorescence, whereas equal concentrations of the monovalent cations K\(^+\) and Na\(^+\) did not (Figure 6A). This suggests that the level of electrogenic influx of monovalent cations is insufficient to alter matrix pH, making influx of monovalent cations an unlikely cause of the pulsing phenomenon. By contrast, electrogenic influx of either Mg\(^{2+}\) or Ca\(^{2+}\) can occur at a rate sufficient to increase matrix pH. Furthermore, the increase in cpYFP signal was due to alkalinization and not caused by direct interaction between cpYFP and Mg\(^{2+}\) and Ca\(^{2+}\) since the response was suppressed after increasing matrix pH buffer capacity by pre-loading mitochondria with phosphate (Figure 6B). To further explore a link between Ca\(^{2+}\) and pulsing, we coloaded isolated mitochondria with TMRM and the calcium sensor Fluo-4 (Gee et al., 2000). The decrease in TMRM signal during a pulse coincided with a small (up to approximately one-third) increase in Fluo-4 fluorescence (Figure 7). The increase in Fluo-4 fluorescence was not due to pH changes of the magnitude estimated to occur in the matrix during a pulse (see Supplemental Figures 3A and 3B online) nor to changes in TMRM content of the matrix (see Supplemental Figure 3C online), ruling out a direct effect of pH on Fluo-4 or an artifact caused by interaction between Fluo-4 and TMRM. The fact that Fluo-4 was retained in isolated mitochondria during pulses also provides additional evidence against the involvement of a nonspecific pore, such as the putative plant mtPTP. In animals, the pore allows nonspecific passage of molecules smaller than \(\sim 1500\) D molecular mass (Bernardi, 1999), and Fluo-4 (molecular mass 737 D) would therefore be able to pass through. The retention of Fluo-4 during pulses was independently confirmed using calcein (623 D; data not shown). The transient increase in Fluo-4 fluorescence suggests that pulses in the mitochondrial membrane potential are linked to an increase in the free Ca\(^{2+}\) concentration in the mitochondrial matrix. Based on these results, influx of small amounts of Ca\(^{2+}\) provides a robust and consistent mechanistic explanation for pulsing.

DISCUSSION

Pulsing: A Novel Mode of Uncoupling

We observed an uncoupling phenomenon in single mitochondria in vivo, which is manifested by a sporadic transient decrease in the membrane potential of individual plant mitochondria. The extent of the depolarization (measured as a reduction of TMRM fluorescence in the mitochondria), as well as the duration, were variable and in this respect are similar to the transient membrane potential events reported previously (e.g., in toad myocytes) (O’Reilly et al., 2003). Moreover, the membrane potential pulses are coincident with a substantial increase in matrix pH, suggesting that pulsing is caused by transient opening of a channel specific for ions other than protons.
pH (up to >1 unit based on the calibration by Schwarzländer et al., 2011) and, therefore, a concomitant increase in the pH gradient across the inner mitochondrial membrane, measured both in vivo and in isolated mitochondria, which suggests specific electrogenic movement of ions other than protons are involved in pulsing.

**Is Pulsing Caused by Calcium Flux?**

An electrogenic cation influx while the inner membrane remains impermeable to passive proton movement could cause the observed decrease in membrane potential and the simultaneous alkalization of the matrix resulting from a stimulation of proton pumping by the ETC. In support of this view, we provide several lines of evidence that pulsing is linked to cation influx and show that influx of Ca$^{2+}$ ions can cause pulsing in isolated mitochondria. Nevertheless, pulsing was observed to continue in isolated mitochondria in the complete absence of added exogenous ions.

**Figure 6.** The Effect of Different Cations on cpYFP Fluorescence in Isolated Mitochondria.

Isolated mitochondria from mt-cpYFP Arabidopsis seedlings in minimal medium free of added metal ions (0.3 M Suc and 10 mM TES, pH 7.2 [Tris]) undergoing state 2 respiration (by provision with 10 mM Tris-succinate, pH 7.2) were supplemented with 100 μM or 1 mM NaCl, KCl, MgCl$_2$, or CaCl$_2$. The response of mt-cpYFP fluorescence intensity (excitation 485 nm/emission 535 nm) was determined in the absence (A) and presence (B) of 5 mM Tris-phosphate, pH 7.2, which increases matrix pH buffering capacity. $n = 3$, error bars = SE, *P < 0.05, and **P < 0.01 (t test). The experiment was repeated three times with similar results.

**Figure 7.** Membrane Potential Pulses Coincide with a Transient Increase in Free Calcium Levels in Isolated Mitochondria.

Isolated Flu-4 loaded wild-type Arabidopsis mitochondria were monitored by confocal microscopy upon energization in the presence of 100 nM TMRM. Intensities of Flu-4 (green) and TMRM (red) signals from three single mitochondria are shown. More than 40 mitochondria were analyzed in three independent experiments, and consistent behavior was observed. Representative events are shown.
metal ions (data not shown). This is most likely due to the presence of trace contaminants in the media constituents and internal stores of cations that are retained in the mitochondria during isolation and exported into the intermembrane space upon energization of the mitochondria to then reenter the matrix in an electrogenic fashion. Experimental estimations of free matrix calcium by Fluo-4 were consistent with the resting concentrations in rosette leaf mitochondria at ~200 nM measured in vivo using aequorin (Logan and Knight, 2003). This concentration range is sufficient to explain changes in membrane potential of the magnitude observed. In a simple model, a reduction of TMRM signal to 10% of its baseline intensity can be caused by an electrogenic influx of $1.5 \times 10^{-20}$ moles of Ca$^{2+}$, corresponding to ~9000 Ca$^{2+}$ ions (assuming a capacitance of the inner membrane of 1 $\mu$F per cm$^2$ [Cramer and Knaff, 1990], a spherical mitochondrion of 1 $\mu$m diameter, and ideal Nernst behavior of TMRM). The most parsimonious explanation for our current observations is that pulsing of isolated mitochondria involves calcium cycling between the matrix and the intermembrane space via a very low number of channels allowing electrogenic ion flux. Cycling of divalent cations has long been known, in principle, to cause mitochondrial uncoupling (Mitchell, 1966). The calcium channel blocker lanthanum increases coupling in our system (see Supplemental Figure 4 online), providing independent evidence for a calcium cycling mechanism being active in isolated mitochondria. Chelating calcium in the buffer with 300 $\mu$M EGTA caused pulsing inhibition, but we were unable to test the effect of even more rigorous chelation of mitochondrial calcium because very high concentrations of chelator are required to completely sequester cations in the mitochondrial matrix (Kushnareva et al., 1999), which caused membrane instability and prevented the establishment of a measurable membrane potential (data not shown). Our data demonstrate that influx of Ca$^{2+}$ is linked to membrane potential pulsing in isolated mitochondria, but we cannot rule out the involvement of other ions in vivo. Nevertheless, we emphasize that the coincidence of the decrease in membrane potential and increase in mitochondrial matrix pH in vivo provides direct evidence for selective ion transport as the mechanism for membrane potential pulsing. The recent identification of a calcium uniporter of animal mitochondria (Baughman et al., 2011; De Stefani et al., 2011) may provide a route to directly test the in vivo involvement of electrogenic calcium transport in plant mitochondrial membrane potential pulsing. However, the lack of effect on pulsing of Ruthenium Red and Ru360, inhibitors of the calcium uniporter, may suggest the involvement of a different class of mitochondrial transporter (Sparagna et al., 1995; Ryu et al., 2010) that is still to be characterized in plants.

**Pulsing May Be Triggered by ROS**

Pulsing was induced under mitochondrial oxidative stress in vivo and during electron transport modes associated with high ROS release in isolated mitochondria. Furthermore, pulsing was significantly inhibited by preincubation with mitoQ, a mitochondrial antioxidant (Kelso et al., 2001, 2002), raising the possibility that ROS may induce pulsing, possibly by activating the ion channel responsible. While other antioxidants increased pulsing rates, the specificity and distribution of these compounds is likely to be different, with mitoQ partitioning into the membrane, and this might provide an explanation for the differential effects. It is important to note that there is a possibility that photooxidative ROS may be generated during excitation of the fluorescent probes. Four recent studies have found that illumination itself can influence pulsing-like phenomena in mammalian mitochondria and bacterial cells (Hüser et al., 1998; Jacobson and Duchen, 2002; Falchi et al., 2005; Kralj et al., 2011), while others observed no such effect (Wang et al., 2008). To minimize the impact of photooxidation, we kept illumination to a minimum and used TMRM concentrations lower than in most previous studies (see Methods; Hüser et al., 1998; Falchi et al., 2005). Moreover, membrane potential transients were consistently observed with independent probes [TMRM, DiOC$_6$(3), and mt-cpYFP] and different excitation wavelengths, arguing against probe-specific photooxidative effects. The rarity of pulsing events in vivo and its induction under stress conditions argues that the stress treatment is the dominating stimulus under those conditions, rather than artifactual photooxidative effects. Additionally, the pulsing inhibitor lanthanum increased coupling as measured using an oxygen electrode, in which no photooxidation occurs. Together, this suggests that technical photooxidation plays a minor role in pulsing compared with biological effects.

**A Role of Pulsing in Maintenance of Mitochondrial Function and Quality Control: A Model**

Based on our analysis of the pulsing mechanism, and our knowledge of what triggers pulsing, we propose that one role of pulsing is to provide a means to avoid excessive mitochondrial ROS production. We developed a simple model that integrates abiotic stress signaling with pulsing to avoid excessive generation of mitochondrial ROS (Figure 8). Damage to, or imbalance of, the ETC leads to increased reduction of ETC complexes and ROS production. In turn, this triggers the opening of a channel or transporter capable of electrogenic cation transport (either a uniporter or an electrogenic exchanger). Electrogenic influx of cations into the matrix decreases the membrane potential and simultaneously leads to matrix alkalinization due to increased ETC proton pumping. In this state, the mitochondrial ETC will become more oxidized, reducing the rate of ROS production, and the channel will close allowing the reestablishment of a high membrane potential. If the fault in the ETC is not repaired, the pulsing cycle will repeat. This process could counteract excessive ROS production and subsequent oxidative damage to the organelle. In principle, the influx of calcium into the matrix during a pulse could also act as a signal. An alternative framework for signaling is provided by the conditional nature of matrix alkalinization in response to a pulse that could act as a quality control mechanism for individual mitochondria during stress. The low matrix phosphate concentration and high ETC capacity of a healthy, fully functional mitochondrion will lead to pronounced matrix alkalinization as a result of a pulse. However, in an unhealthy mitochondrion with a low rate of ATP synthesis due to ETC or ATP-synthase dysfunction, alkalinization would be less pronounced due to phosphate buffering of pH (phosphate will accumulate if the rate of its incorporation into ATP is low). pH
transients could be sensed by a pH-sensitive signaling protein (e.g., a kinase) mounting a constitutive signal in response to a short lived pH transient. Under stress, this signal could provide a mechanism for functional mitochondria to adjust to their changed environment by synthesis of mitochondrially encoded ETC subunits that are required for acclimation. This would represent an alternative mechanism to the redox control of mitochondrial genome expression that is predicted by the colocation for redox regulation hypothesis (Allen, 2003). Abiotic stress would increase the likelihood of faults developing in the mitochondrial ETC and thereby increase the frequency of pulsing. Abiotic stress is also known to trigger release of Ca\textsuperscript{2+} from intracellular stores, such as the endoplasmic reticulum (Knight et al., 1996), which may facilitate Ca\textsuperscript{2+} uptake into individual mitochondria.

A Unified Framework for Pulsing in Plants and Animals

Mitochondrial membrane potential pulses have also been observed in animal mitochondria. However, several contradictory explanations have been reported to account for the phenomenon. Pulsing has been linked to ATP-synthase activity (Buckman and Reynolds, 2001), mitochondrial anion transport (O’Rourke, 2000), and calcium and sodium flux (Duchen et al., 1998; Vergun and Reynolds, 2004, 2005; Poburko et al., 2011). However, most frequently, pulsing in animal mitochondria is attributed to opening of the mtPTP (Hüser et al., 1998; Hüser and Blatter, 1999; Zorov et al., 2000; Collins et al., 2002). Although it is entirely conceivable that pulses could be caused by different and mechanistically independent underlying events, we believe that the data presented here resolve a number of the current uncertainties. We simultaneously monitored the dynamics of both components of the proton motive force, membrane potential, and pH gradient (measured as matrix pH) in single mitochondria. This allowed the observation of a strong dynamic interdependency between the two parameters (see Supplemental Figure 2A online), which becomes particularly apparent during pulsing. As a result, greater bioenergetic detail is now available to reconstruct the mechanism as an important step toward a unifying concept of pulsing.

The observation that matrix alkalinization is attenuated by the buffering effect of free phosphate may also resolve the contradictory explanations that have been put forward for mt-cpYFP flashes in animal mitochondria (Fang et al., 2011; Ma et al., 2011; Wei et al., 2011). When characterizing mt-cpYFP flashes in living cardiomyocytes, Wang et al. (2008) and Fang et al. (2011) observed that each flash coincided with a membrane potential pulse. This was interpreted as a transient opening of the mtPTP, causing a burst of superoxide and resulting in a cpYFP flash. However, our recent finding that cpYFP is highly pH sensitive in the mitochondrial matrix (Schwarzländer et al., 2011) cast into doubt the ability of cpYFP to report mitochondrial superoxide flashes (although dynamic mitochondrial ROS and redox changes during pulses remain possible and even likely). Instead, matrix alkalinization is the predominant feature reported by mt-cpYFP. The amplitude (and therefore the detected frequency) of mt-cpYFP flashes is strongly dependent on the pH buffering capacity of the matrix and the proton-pumping capacity of the ETC. We have shown that free phosphate in the matrix strongly buffers against the pH change during a pulse. It is therefore relevant that several effectors of the mtPTP (such as bonkrekic acid or atractyloside) are actually inhibitors of the adenine nucleotide carrier, postulated to be one of the mtPTP components. The effect of these inhibitors on mt-cpYFP pulsing observed by Wang et al. (2008) could be explained by pH buffering, as a disruption of ATP/ADP homeostasis in the matrix will necessarily impact on matrix phosphate levels. Variability in the coincidence of pulses and flashes in vivo therefore probably reflects variation in matrix buffering and ETC capacity between

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**Figure 8. A Model of Mitochondrial Pulsing.**

(A) A high membrane potential and a low pH gradient contribute to the motive force. The rate of electron transport is thermodynamically limited by proton motive force, leading to ROS release.

(B) A channel opens in an ETC-dependent fashion (possibly due to ROS), allowing influx of divalent cations, such as calcium, into the matrix. The charge influx uncouples the mitochondrion, lowers the membrane potential, and removes the constraint on the rate of electron transport, thereby decreasing ROS release. The rate of electron transport increases, as does the rate of proton pumping, leading to increased matrix pH.

(C) The channel closes and cations are electroneutrally exported from the matrix in exchange for protons (directly or indirectly), reestablishing matrix pH and membrane potential. Note that charge stoichiometry is not considered in the schematic representation.
mitochondria. A mitochondrion with highly active electron transport and ATP-synthesis will show significant matrix alkalinization upon a pulse, while mitochondria with dysfunctional ETC and ATP-synthesis will show little pH response due to a relatively high matrix phosphate concentration and a relatively slow rate of proton pumping. Our discovery that single plant mitochondria can undergo transient and pronounced matrix alkalinization events (Schwarzländer et al., 2011) has now been matched by experiments using astrocytes (Azarias and Chatton, 2011) and a different pH sensing protein, Sypher, which is also based on a cpYFP (Poburko et al., 2011). The dynamic pH events in astrocytes also coincided with a transient decrease in membrane potential. This suggests that specific electrogenic ion flux across the mitochondrial inner membrane as a causative principle for membrane potential pulsing is conserved between plants and animals.

Conclusions and Perspectives

Pulsing of the membrane potential in individual mitochondria provides an autonomous uncoupling mode. We present a mechanistic framework to explain how those dramatic bioenergetic events occur and suggest a role for pulsing in maintaining mitochondrial functionality and quality control. Beyond its occurrence in plants, electrochemical pulsing also occurs in animal mitochondria and has recently been demonstrated in bacteria (Král et al., 2011). This latter observation raises the possibility that pulsing may have already been a characteristic of the prokaryotic endosymbiont and has remained conserved in modern mitochondria across the eukaryotic domain.

METHODS

Chemicals and Plant Material

Chemicals and inhibitors were purchased from Sigma-Aldrich. TMRM, DiOC6(3), Fluo-4 acetoxymethyl ester (AM), and calcine AM were bought from Invitrogen. Arabidopsis thaliana Columbia-0 was the background for all transgenic lines: mito-GFP (Logan and Leaver, 2000), mt-roGFP1 (Schwarzländer et al., 2008), and mt-cpYFP (Schwarzländer et al., 2011). For in vivo microscopy of roots, seedlings were cultivated for 7 to 10 d on vertical half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) agar plates under long-day conditions. Epidermal cells from the upper elongation zone and lower differentiation zone were imaged. For mitochondrial isolations, seedlings were cultured in hydroponic pots and the upper elongation zone and lower differentiation zone were imaged.

Isolation of Mitochondria

Mitochondria were freshly isolated from 14-d-old Arabidopsis seedling cultures as described before (Sweetlove et al., 2007). Coupling was measured in a Clark-type oxygen electrode (Oxygraph; Hansatech Instruments) at 20°C as described before (Sweetlove et al., 2002) either in basic incubation medium (0.3 M Suc, 5 mM KH2PO4, 10 mM TES, 10 mM NaCl, 2 mM MgSO4, 0.1% [w/v] fatty acid–free BSA, pH 7.2 [KOH]) for quality control or in minimal medium (0.3 M Suc, 10 mM TES, pH 7.2, adjusted with KOH or Tris) for pulsing assays.

Dye Loading and CLSM

Whole seedlings were equilibrated in 20 nM TMRM (in 0.5× MS medium) for ≥15 min before confocal imaging of roots bathed in fresh probe solution under the same conditions. All treatments were performed in the presence of TMRM. For Fluo-4 loading, isolated mitochondria were incubated in 20 μM Fluo-4 AM in minimal medium for 60 min at room temperature (to allow sufficient accumulation and internal hydrolytic cleave of the AM-ester groups to release free dye in the matrix) and washed twice. Mitochondria were immobilized onto round glass cover slides by centrifugation (2000g, 5 min, 4°C) in minimal medium. Immediately before imaging, mitochondria were energized by addition of medium containing succinate as respiratory substrate (if not stated otherwise) and labeled with 100 nM TMRM. Effectors were added as indicated and samples were imaged using conditions described before (Schwarzländer et al., 2011). Microscopy was performed using a Zeiss LSM510 META confocal microscope (Carl Zeiss MicroImaging) equipped with laser lines for 405-, 458-, 488-, and 543-nm excitation. Excitation and emission wavelengths were selected for the different probes as follows: TMRM, 543/565 to 615 nm; mito-GFP, 458/505 to 530 nm; roGFP1, 405,488/505 to 530 nm; cpYFP, 488 and 405/505 to 530 nm; DiOC6(3) and Flu-4, 488/505 to 530 nm. Imaging was performed with a ×63 lens (Zeiss Plan-Apochromat ×63, numerical aperture = 1.4, oil) and a ×40 lens for roGFP1 (Zeiss C-Apochromat ×40, numerical aperture = 1.2, water) in multitrack mode with line switching between channels. The pinhole diameter was 2 airy units. Laser output was kept under 2% of maximal power equaling <2.5 μW for any wavelength as measured in the defocused beam at the backplane of the objective. Pixel spacing was typically 50 to 100 nm, pixel dwell time was 1 to 2 μs, and image size was 512 × 512 pixels. Time series were collected with time intervals between 2 and 5 s if not indicated otherwise.

Data Analysis

CLSM time series imaging data of mitochondria were analyzed using a custom Matlab program (available from M.D.F. on request). For in vivo imaging, data spatial regions corresponding to single mitochondria were manually selected, and average values from a small (typically 5-pixel radius) circular region extracted for each time point following background subtraction. Analysis of mt-roGFP1 images was performed as described before (Morgan et al., 2008; Schwarzländer et al., 2008, 2009). The percentage of oxidized roGFP was calculated using in vivo calibration. For time series of isolated mitochondria, the positions of individual mitochondria were automatically identified as local maxima following convolution with a 2-D Mexican Hat kernel, scaled to match the typical mitochondrial size. Average fluorescence intensities for each channel were sampled from a circular region corresponding to half the radius of the mitochondrion from this point to generate time course traces for a large number of mitochondria (>30) per time series. CVs (the ratio of signal standard deviation to signal mean) were recorded for each mitochondrial time series. The mean CV of a large number of individual mitochondria was recorded as a summary statistic for its degree of pulsing. Signal traces from frequently and strongly pulsing mitochondria had high CVs (as a result of repeated large fluctuations). Significance was tested using a two-sample unpaired t test for the ETC screen and a one-sample location test (z-test), against unity, of the ratio of treated mean CV to control mean CV for the pharmacological screen. Significance values were Bonferroni corrected for the multiple comparisons performed. Meaningful error bars were derived for highly skewed pulsing frequency data by appropriate transformations (Figure 2A, square-root transformation; Figure 4B, logarithmic transformation), and the back-transformed mean and 95% confidence bands are shown.

Fluorimetry

Mitochondria were suspended in minimal medium in 96-well plates. The total assay volume was 200 μL. A Beckman Coulter DTX880 multimode
detector was used with filters for 485-nm (cpYFP, Fluo-4) or 535-nm (TMRM) excitation and 535-nm (cpYFP, Fluo-4) or 595-nm (TMRM) emission, respectively. Data were background-subtracted and processed in Microsoft Excel.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** DICO(3) Is a Dynamic Membrane Potential Probe Enabling the Visualization of Pulses in Single Mitochondria.

**Supplemental Figure 2.** Mt-cpYFP Dynamics during Pulses, at Clamped Matrix pH and Increased Matrix pH Buffering Capacity.

**Supplemental Figure 3.** Effect of pH and TMRM on Fluo-4 Fluorescence.

**Supplemental Figure 4.** The Effect of Lanthanum on Oxygen Uptake of Isolated Arabidopsis Mitochondria.

**Supplemental Table 1.** The Effect of Inner Membrane Channel Inhibitors and Redox-Active Compounds to Determine Their Effect on Pulsing.

**Supplemental Video 1.** Pulses of Single Mitochondria in an Arabidopsis Root Epidermal Cell.

**Supplemental Video 2.** Pulsing in Isolated Arabidopsis Mitochondria.

**Supplemental Video 3.** Pulsing in Isolated Arabidopsis Mitochondria in the Presence of 1 mM LaCl$_3$.

**Supplemental Video 4.** Pulsing in Isolated Arabidopsis Mitochondria in the Presence of 1 μM mitoQ.

**Supplemental Video 5.** Coincidence of TMRM Pulse, Change in Shape, and Increase in mt-cpYFP Fluorescence in a Single Mitochondrion in an Arabidopsis Root Epidermal Cell.

**Supplemental Video 6.** Coincidence of TMRM Pulse and Increase in mt-cpYFP Fluorescence in Isolated Arabidopsis Mitochondria.

**REFERENCES**


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