Mitochondrial and Cytosolic Calcium Dynamics Are Differentially Regulated in Plants[1]

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The role of mitochondrial calcium in plant cell signaling has received little attention due to the technical difficulties in measuring changes in the mitochondrial free calcium concentration ($[Ca^{2+}]_m$) in vivo. Here, we describe the unprecedented use of targeted aequorin to produce stably transformed Arabidopsis plants that both enable the analysis of mitochondrial calcium dynamics in planta and reveal independent regulation of $[Ca^{2+}]_m$.

The ability to monitor changes in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) in planta using recombinant aequorin has provided new insights into our understanding of plant Ca^{2+} dynamics (Knight et al., 1991, 1992, 1995; Knight and Knight, 1995). This technique has been refined to offer increased resolution of cellular Ca^{2+} dynamics by targeting aequorin to specific subcellular locations within the cell. Aequorin has been successfully targeted in this way to the nucleus (Brini et al., 1993), endoplasmic reticulum (Kendall et al., 1992), and mitochondria (Rizzuto et al., 1992) of mammalian cells and to the nucleus (Van der Luit et al., 1999), chloroplasts (Johnson et al., 1995), and the tonoplast (Knight et al., 1996) of plants. This paper describes the first successful targeting of aequorin to plant mitochondria, to our knowledge, and the data presented provide the first direct measurements of the elevations in $[Ca^{2+}]_m$ resulting from physiological or environmental stimuli.

The mitochondrial F_1-ATPase β-subunit of tobacco (*Nicotiana plumbaginifolia*) is nuclear encoded and contains an N-terminal extension that is cleaved upon import. Boutry et al. (1987) demonstrated that a 90-amino acid N-terminal segment of the β-ATPase could efficiently target the chloramphenicol acetyl transferase protein into mitochondria of transgenic plants. Subsequent work by Boutry and colleagues demonstrated that although the first 23 amino acids of the β-ATPase subunit was sufficient for targeting reporter proteins into tobacco mitochondria, the import efficiency increased significantly when 60 or 90 amino acid segments were used (Chaumont et al., 1994). In a previous study, efficient targeting of green fluorescent protein (GFP) to Arabidopsis mitochondria was achieved by engineering a chimeric construct in which the sequence encoding the first 87 amino acids of the tobacco β-ATPase was ligated upstream of the mGFP5 cDNA (Logan and Leaver, 2000). To both target aequorin to mitochondria and to allow confirmation of correct targeting by fluorescence microscopy, we engineered a chimeric construct comprising the aequorin cDNA downstream of the β-ATPase-mGFP5 cDNA to express a GFP-AEQ fusion protein in the mitochondria. The aequorin and mGFP5 cDNA chimera was PCR amplified from pGF-Aeq (C.A. Moore and M.R. Knight, unpublished data) with primers to add *Spe*I and *Sac*I restriction sites at the 5′ and 3′ ends, respectively (using the primers 5′-GCCGAAACTAGTAAAGGAGAA-3′ and 5′-GCGCGAGCTCGCAGAGTTTCTTAGGGGACA-3′; restriction sites underlined). This allowed the directional cloning of the AEQ-GFP sequence, downstream of the cDNA sequence encoding the ATPase N-terminal segment, into pBIN121 (Jefferson et al., 1987) in place of the GUS cDNA. Independent plasmid clones containing the β-ATPase-GFP-aequorin chimera (pBINAGA) were sequenced to confirm that the full chimera was in-frame. The construct was introduced into Arabidopsis Columbia-0 ecotype by *Agrobacterium tumefaciens*-mediated floral dip transformation (Clough and Bent, 1998), and Arabidopsis transformants were selected by growth on kanamycin. Analysis of positive transformants by epifluorescence microscopy demonstrated that this construct was stably inherited and correctly targeted to the mitochondria (Fig. 1).

The β-ATPase-GFP-aequorin fusion enabled specific measurement of the $[Ca^{2+}]_m$ in living plants and comparison with cytosolic free calcium concentration ($[Ca^{2+}]_c$; Fig. 2). In vivo reconstitution of aequorin and Ca^{2+} measurements were performed as described previously (Campbell, 1988; Knight et al., 1991, 1996). Arabidopsis plants (Columbia-0 background) expressing recombinant aequorin in the cytosol were engineered as described previously (Knight et al., 1991). The GFP-aequorin fusion protein behaves identically to unfused aequorin because

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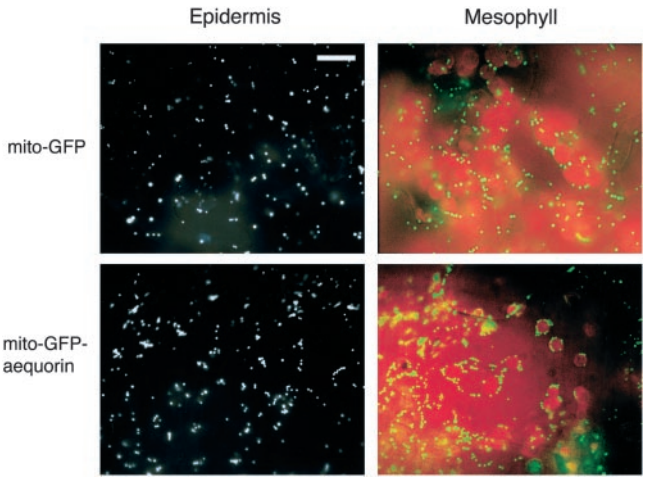

Figure 1. Epifluorescent micrographs of leaf epidermis or mesophyll tissue from plants expressing mitochondrial-targeted GFP (mito-GFP; Logan and Leaver, 2000) or the mitochondrial-targeted GFP-aequorin fusion protein (mito-GFP-aequorin). Color images were generated by false coloring GFP (green) and chlorophyll autofluorescence (red). Scale bar = 10 μM. Images were captured using a digital camera (F-View, Soft Imaging System GmbH, Munster, Germany) attached to an Olympus BX-40 epifluorescent microscope (Olympus Optical Co. [UK] Ltd., Southall, UK) fitted with cubes for GFP (Olympus U-M41001; excitation, 455–495 nm; dichroic mirror, 505 nm; barrier filter, 510–555 nm) and for chlorophyll autofluorescence (Olympus U-M41004; excitation, 534–588 nm; dichroic mirror, 595 nm; barrier filter, 609–683 nm). Visualization of mitochondria and chloroplasts was performed at 1,000× using an oil immersion objective (100× Universal Plan Fluorite numerical aperture =1.3, Olympus). False colored merged images were created using Confocal Assistant (Bio-Rad, Hemel Hempsted, UK), and all images were prepared for reproduction using Adobe Photoshop Elements (Adobe, San Jose, CA).

plants expressing either of these two proteins in the cytosol display the same calibrated $[Ca^{2+}]_c$ responses (C.A. Moore and M.R. Knight, unpublished data). For this reason, and because it has been shown that N-terminal fusions of aequorin do not affect aequorin activity (Rizzuto et al., 1992; Knight and Knight, 1995), calibration of $[Ca^{2+}]_m$ was performed using the same method and calibration curve as we had previously empirically determined for the unfused version of this aequorin isoform (Knight et al., 1996). The average resting $[Ca^{2+}]_m$ measured in vivo was 208 ± 6 nM, which is almost 2-fold higher than the resting concentration of Ca^{2+} in the cytoplasm (Fig. 2). The $[Ca^{2+}]_c$ value measured here concurs well with our previous studies (Knight et al., 1996, 1997). To our knowledge, this is the first published record of $[Ca^{2+}]_m$ obtained in whole plants. A similar value for $[Ca^{2+}]_m$ was obtained by Subbaiah et al. (1998) using the fluorescent indicator rhod2 in individual maize (*Zea mays*) suspension-cultured cells.

To compare the calcium dynamics of the cytosol and the mitochondria, we performed stress experiments. All stress treatment were performed as described previously (Price et al., 1994; Knight et al., 1995, 1996, 1997), involving seedlings floating in water and stresses subsequently added as liquids. Thus, this technique involves no transition from air to liquid, or vice versa; as a consequence, no anoxia/hypoxia is likely to be generated. Thus, all four stresses would be equal in this respect. In addition, measurements were made over less than 1 min in this present study, and the effects of anoxia/hypoxia upon calcium signaling require a longer time to manifest themselves (Sedbrook et al., 1996, C. Plieth and M.R. Knight, unpublished data). As shown by the data in Figure 3, challenging Arabidopsis seedlings with a cold or osmotic shock stimulated rapid increases in $[Ca^{2+}]_m$, peaking at 526 ± 60 and 504 ± 90 nM, respectively. In both cases, peak response of $[Ca^{2+}]_m$ was approximately one-half the $[Ca^{2+}]_c$ peak, although the "signatures"/temporal kinetics (McAinsh and Hetherington, 1998) from the two compartments were otherwise very similar. Although the rapid changes in $[Ca^{2+}]_m$ in response to cold or osmotic (drought) stress might simply reflect a role for mitochondria in buffering (through uptake) the $[Ca^{2+}]_c$ changes, it is possible that uptake of Ca^{2+} into individual mitochondria is individually controlled. With mitochondria dispersed throughout the cytoplasm, this latter scenario would provide a mechanism whereby the spatiotemporal features of a $[Ca^{2+}]_c$ signal could be modulated (Duchen, 2000). Changes in $[Ca^{2+}]_m$ in mammalian cells have been shown to have an impact on mitochondrial function. The main targets of $[Ca^{2+}]_m$ identified in this case are the dehydrogenases of the tricarboxylic acid cycle (McCormack et al., 1990; Rutter et al., 1996). Activa-

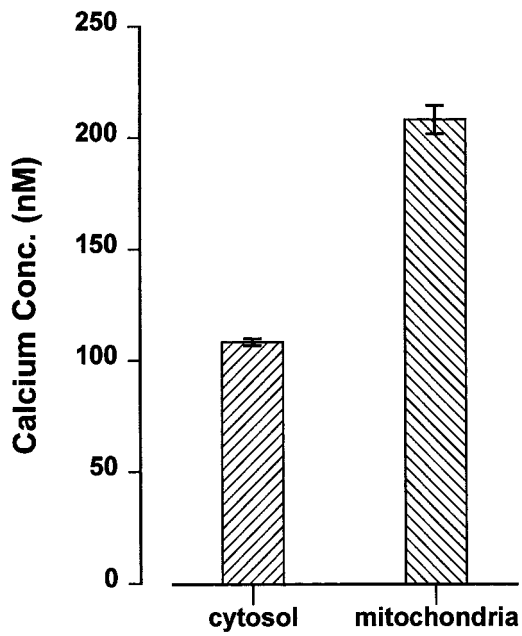

Figure 2. Resting free calcium concentrations in the cytosol or mitochondria. Average values measured in cytosolic-aequorin (n = 43; Knight et al., 1999) or mitochondrial-aequorin plants (n = 29). Error bars = SE.

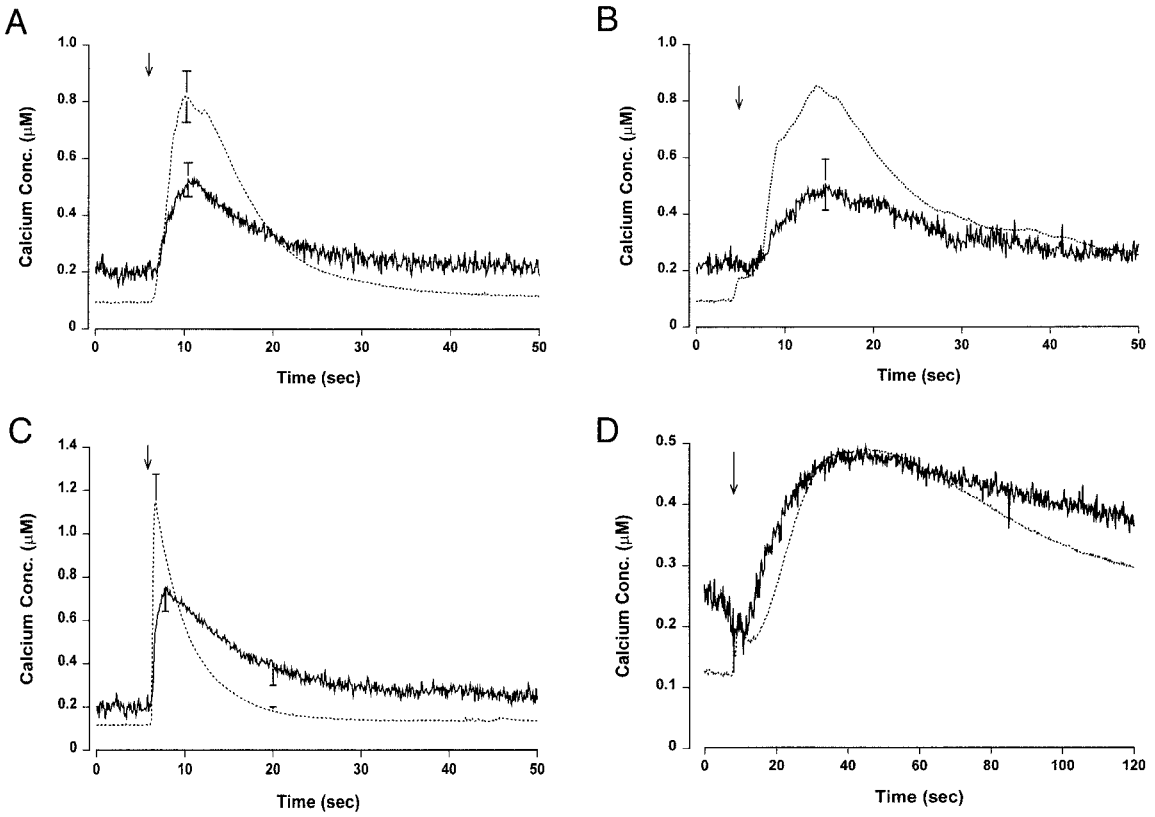

Figure 3. Effect of cold (A), mannitol (B), touch (C), or hydrogen peroxide (D) on $[Ca^{2+}]_m$. Seedlings from mitochondrial-aequorin plants (solid line) or control cytosolic-aequorin plants (Knight et al., 1999; dashed line) were treated as previously described (Knight et al., 1991, 1992, 1995, 1996, 1997; Price et al., 1994). In brief, cold, mannitol, or hydrogen peroxide treatments were effected by slowly (to prevent a touch response) injecting 0.5 mL of ice-cold water, 0.666 M mannitol, or 20 mM hydrogen peroxide, respectively, into a cuvette containing an Arabidopsis seedling floating in 0.5 mL of water at room temperature. Touch treatment was effected by the rapid injection of 0.5 mL of room temperature water into the cuvette. Time of injection is indicated with an arrow. Experiments using mitochondrial-aequorin plants were performed on two or three independent lines, and each gave the same pattern of results. The data presented are averages obtained from at least six replicate experiments using line pBINAGA211. Error bars = SE.

tion of these enzymes by $[Ca^{2+}]_m$ in the micromolar range stimulates respiration, which in turn increases the inner mitochondrial membrane potential, leading to an increase in ATP production. The extent and significance of $[Ca^{2+}]_m$-dependent regulation of tricarboxylic acid cycle dehydrogenases in plants is as yet unknown.

In contrast to the situation with cold and osmotic stress, experiments with mechanical and oxidative stresses (Knight et al., 1991, 1992, 1995; Price et al., 1994) revealed there to be independent regulation of $[Ca^{2+}]_m$ relative to $[Ca^{2+}]_c$ (Fig. 3). Touch stimulation induces a $[Ca^{2+}]_m$ response that is quite distinct from the $[Ca^{2+}]_c$ signature (Fig. 3C). As reported previously (Knight et al., 1991, 1992, 1995), touch induces an instantaneous elevation of $[Ca^{2+}]_c$ followed by a return to near-resting concentrations within 20 s. In contrast, although the elevation in $[Ca^{2+}]_m$ was equally rapid, the return to near-resting concentrations was much slower; the $[Ca^{2+}]_m$ was 37% of its measured maximum value after 20 s (Fig. 3C). This phenomenon may be due to the relatively lower buffering capacity of the mitochondria relative to the cytosol. That this effect manifests itself only in response to touch, as compared with cold and osmotic stress, may be a function of the higher absolute concentration of calcium achieved with mechanical stimulation (Fig. 3). These data demonstrate that $[Ca^{2+}]_m$ is differentially regulated in response to touch in Arabidopsis seedlings in terms of calcium signature. Exposure of Arabidopsis seedlings to 10 mM hydrogen peroxide resulted in an rapid increase in $[Ca^{2+}]_m$ to a peak of 495 ± 23 nM, which was equal in magnitude to the peak $[Ca^{2+}]_c$ response (490 ± 18 nM). The rapid increase in $[Ca^{2+}]_c$ in response to hydrogen peroxide has been described previously in Arabidopsis (Knight et al., 1998) and tobacco (Price et al., 1994). However, the relative (to $[Ca^{2+}]_c$) magnitude of the $[Ca^{2+}]_m$ response was surprising. Of the four stimuli described in this paper that induce rapid increases in $[Ca^{2+}]_m$, three of these induce transient elevations that peak at approximately 60% of the change in $[Ca^{2+}]_c$; therefore, the response to hydrogen peroxide is unique in this regard. These data indicate that

$[Ca^{2+}]_m$ is differentially sensitive to the hydrogen peroxide treatment relative to cold, touch, and osmotic stress (Fig. 3) and provide more evidence of independent regulation of $[Ca^{2+}]_m$ versus $[Ca^{2+}]_c$. Taken together, the mechanical (touch) and oxidative (hydrogen peroxide) perturbations indicate that mitochondria are not simply passively "sampling" the local $[Ca^{2+}]_c$ conditions but are capable of generating unique (relative to $[Ca^{2+}]_c$) calcium signatures. This implies that there is the potential for a discriminating and autonomous mitochondrial Ca^{2+} signaling pathway in plant cells, as there is in animal cells (for review, see Duchen, 2000; Rutter and Rizzuto, 2000; Rizzuto et al., 2000). The future challenge will be to determine which plant mitochondrial processes are coupled to $[Ca^{2+}]_m$ and under what circumstances independent regulation of $[Ca^{2+}]_m$ is needed.

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