FINAL TECHNICAL REPORT

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Title: Physiology and Regulation of Calcium Channels in Stomatal Guard Cells.
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Research supported by DOE Grant Number DE-FG03-94-ER20148 focused on characterization of a plasma membrane hyperpolarization-activated calcium-permeable channel current (I_{Ca}) and its roles in guard cell abscisic acid signal transduction. The goal of this research project was to gain an understanding and characterization of these plasma membrane ion channels in *Arabidopsis* guard cells and to determine their roles in controlling transpirational water loss of plants during drought stress. The proposed research investigated the hypothesis that I_{Ca} currents represent an important Ca^{2+} influx channel pathway during abscisic acid (ABA) signal transduction in guard cells. The goals of the present DOE research were: To identify Ca^{2+} channel blockers that inhibit I_{Ca} activity, to characterize the ionic selectivity and the voltage dependence of I_{Ca}. To identify second messengers that are required for I_{Ca} activation. To determine whether and, if so, how ABA and other stimuli, such as elicitors or CO_2, may mediate activation of I_{Ca}. To characterize whether and how the recessive ABA-insensitive mutant, *gca2*, affects I_{Ca} activation and Ca^{2+} signaling.

Important advances in understanding physiological functions, regulation and molecular genetic mutations that affect I_{Ca} channel activation and Ca^{2+} signaling in guard cells have been made. Results from DOE-supported research in the PI's laboratory are summarized below. Research from other laboratories is not reviewed here for clarity. A list of publications resulting from DOE grant DE-FG03-94-ER201 48 -supported research can be found at the end of this final report.

Publications From DOE DE-FG03-94-ER201 48-Supported Research: Several findings supported by the preceding period of this DOE grant which was focused on plant cation channels were published after submission of the previous DOE renewal proposal in June of 1999 as summarized briefly here. We found that a dominant negative mutation in the guard cell-expressed inward K^+ channel, KAT1, represses inward K^+ channel activity in guard cells, and reduces light-induced K^+ uptake into guard cells, light-induced stomatal opening and transpiration in *Arabidopsis* (Kwak et al., 2001). These findings provide molecular support of the model the K^+ channels contribute to K^+ uptake during light-induced stomatal opening (Schroeder et al., 1984 & 1987). This DOE-funded research on K^+ channels (Kwak et al., 2001) led to recent approval of a patent (Schroeder et al., 2003). In wheat roots we found that K^+ starvation enhances the activity of inward-rectifying K^+ currents (Buschmann et al., 2000). We further isolated a wheat root K^+ channel cDNA, *TaAKT1*, and showed that the *TaAKT1* transcript level is enhanced by K^+ starvation (Buschmann et al., 2000).

Methods for Monitoring Cytosolic Ca^{2+} Changes in Guard Cells. We adapted and developed three separate approaches for measuring cytosolic calcium changes in *Arabidopsis* guard cells. These include acid loading of Fura-2 and Indo-1 (Allen, 1999a) and Fura-2 AM loading into guard cells (Kuchitsu, 2002). Furthermore, we adapted transgenic expression of the Ca^{2+}-sensitive protein cameleon to guard cells (Allen et al., 1999b).

Reactive Oxygen Species Activate Ca^{2+}-permeable Cation Currents in the Plasma Membrane of *Arabidopsis* Guard Cells. In initial research we found that application of hydrogen peroxide to *Arabidopsis* guard cells produced activation of a hyperpolarization-induced current. We found activation of ionic currents in the plasma membrane of *Arabidopsis* guard cells by reactive oxygen species and showed that they carry Ca^{2+} influx (Pei et al., 2000). These I_{Ca} currents showed enhanced activation at hyperpolarized membrane potentials (Pei et al., 2000). Experiments showed that this current, activated by reactive oxygen species (ROS) can be carried by the cations Ca^{2+}, Mg^{2+} and Ba^{2+} but not by TEA^+ suggesting that they are non-specific

cation-permeable currents (Pei et al., 2000). The current was therefore named "I_{Ca}". These currents reversed at the Ba^{2+} equilibrium potential in $BaCl_2$ solutions (Pei et al., 2000) and discrete step changes in current activity were resolved suggesting that ion channels carry I_{Ca} (Z. Pei and Schroeder, unpublished data). ROS were further shown to induce cytosolic Ca^{2+} rises in patch clamped guard cells and intact guard cells (Pei et al., 2000). Putative Ca^{2+} channel blockers, that are known to inhibit ABA responses in guard cells, inhibited the I_{Ca} currents in guard cells (Pei et al., 2000), although such blockers are known to inhibit several channel types in plants.

A Role for I_{Ca} Channels in Guard Cell ABA Signaling. In further experiments, stomatal movement analyses using reactive oxygen species, extracellular Ca^{2+} buffering, ABA and the NADPH oxidase inhibitor DPI indicated a possible role for I_{Ca} channels in ABA-induced stomatal closing (Pei et al., 2000). In patch clamp experiments application of ABA 5 minutes after establishing whole cell recordings showed activation of hyperpolarization-induced cation influx currents similar to I_{Ca} (Pei et al., 2000). Further research showed that the cation selectivity, voltage-dependence and time-dependence of ABA- and ROS-activated currents are indistinguishable confirming that ABA activates I_{Ca} channels (Kwak et al., 2003). Furthermore ABA caused an increase in H_2DCF fluorescence (Pei et al., 2000), which reports changes in ROS levels in guard cells. Recent independent studies of others have also reported ABA-induced ROS production and hyperpolarization-induced Ca^{2+} currents in maize embryos and in *Vicia* guard cells. Molecular genetic evidence for a role of ROS and I_{Ca} in ABA signal transduction was revealed by analyzing the ABA-insensitive mutant *gca2* in collaboration with Dr. Erwin Grill's laboratory (TU Munich). The *gca2* mutant shows ABA-insensitivity in stomatal closing, a lack of ROS activation of I_{Ca} currents in guard cells and a lack of ROS-induced stomatal closure (Pei et al., 2000). Together these data suggest that ROS-activated Ca^{2+}-permeable channels are important mechanisms for ABA-induced stomatal closing and that ABA-induced ROS production contributes to ABA signal transduction (Pei et al., 2000).

ABA activation of I_{Ca} requires NADPH and is differentially disrupted in *abi1-1* and *abi2-1* PP2C Mutants. We found that the activation of I_{Ca} currents by ABA in cytoplasmic dialyzed (whole cell patch clamped) guard cells required addition of NADPH or NADH to the patch clamp electrode solution (Murata et al., 2001), that dialyzes the cytoplasm of guard cells. Furthermore interestingly the dominant ABA insensitive protein phosphatase 2C (PP2C) mutants, *abi1-1* and *abi2-1*, impaired ABA activation of I_{Ca} currents (Murata et al., 2001). This finding correlated with reduced ABA-induced cytosolic calcium elevations in guard cells in the *abi1-1* and *abi2-1* mutants (Allen et al., 1999a). Unexpectedly stomatal movement assays, patch clamp and DCF fluorescence assays consistently suggest that *abi1-1* and *abi2-1* differentially disrupt ABA signaling upstream and downstream of ROS production, respectively (Murata et al., 2001). We previously showed that the ABA hypersensitive mutant, *era1*, causes ABA hypersensitive activation of slow anion channels and stomatal closing (Pei et al., 1998). Ca^{2+} imaging, patch clamp and stomatal movement analyses have now shown that the *era1* mutant causes ABA hypersensitive cytosolic Ca^{2+} elevations and ABA hypersensitive I_{Ca} activation in guard cells (Allen et al., 2002). Together genetic analyses of I_{Ca} regulation by ROS and ABA in the *gca2*, *abi1-1*, *abi2-1* and *era1* mutants provide strong genetic support for this newly recognized branch of the ABA signal transduction network that includes ROS, I_{Ca} channels and these four mutants (Allen et al., 2002; Murata et al., 2001; Pei et al., 2000) (for rev. (Allen and Schroeder, 2001).

Genetic evidence for the function of reactive oxygen species in ABA activation of I_{Ca} channels was obtained by showing that double mutants in the two highly guard cell-expressed NADPH oxidases, AtrbohD and AtrbohF, impair ABA activation of I_{Ca} channels and partially impair ABA-induced stomatal closing (Kwak et al., 2003). I_{Ca} channels were further shown to be Na^+ permeable (Kwak et al., 2003).

ABA, Elicitor and Extracellular Ca^{2+} Signaling in Guard Cells. In further research we found that pathogenic elicitors cause Ca^{2+} elevations in guard cells and activation of I_{Ca} channels (Klüsener et al., 2002). Interestingly, elicitor activation of I_{Ca} currents in guard cells also required cytoplasmic NADPH in the patch pipette solution indicating that ROS activation of I_{Ca} represents a shared branch for several stomatal closing and stress signals in guard cells (Klüsener et al., 2002). The time-dependence of I_{Ca} currents activated by ABA, ROS and elicitors were similar showing rapid activation in response to hyperpolarizing voltage pulses and "spiky" behavior (Klüsener et al., 2002; Pei et al., 2000). In *Arabidopsis* guard cells rapid removal of extracellular Ca^{2+} is sufficient to disrupt continued elicitor- and ABA-induced Ca^{2+} elevations suggesting that Ca^{2+} influx and Ca^{2+} release mechanisms may be interdependent in *Arabidopsis* (Klüsener et al., 2002).

Ca^{2+} Oscillations and Signal Transduction in Guard Cells. The adaptation of the cameleon Ca^{2+} indicator to Ca^{2+} imaging in guard cells (Allen, 1999b), led to important new insights into understanding the importance of Ca^{2+} oscillations in guard cell signal transduction. In brief, research analyzed stomatal movements in response to four stomatal closing stimuli, ABA, H_2O_2, cold and external Ca^{2+}. The *det3* mutant was found to disrupt stomatal closing by certain stimuli but not by ABA. Interestingly those stimuli for which stomatal closing was impaired in *det3*, showed prolonged stimulus-induced cytosolic Ca^{2+} elevations rather than repetitive Ca^{2+} transients (Allen et al., 2000). In further experiments the *det3* mutant phenotype could be suppressed and stomatal closing recorded by experimentally imposing Ca^{2+} oscillations, whereas imposing Ca^{2+} plateaux in wildtype guard cells resulted in lack of long-term stomatal closing (Allen et al., 2000). In further research a defined "window" of Ca^{2+} oscillation parameters was shown to cause long-term (programmed) stomatal closure (Allen et al., 2001a). In addition it was found that long-term Ca^{2+} responses are programmed by Ca^{2+} oscillation parameters, whereas short-term Ca^{2+} reactive stomatal closure occurred whenever Ca^{2+} levels increased (Allen et al., 2001a). Interestingly, Ca^{2+} oscillations in the ABA-insensitive *gca2* mutant were shown to differ from WT (Allen et al., 2001a). The ABA-insensitivity of *gca2* together with the lack of ROS activation of I_{Ca} channels in *gca2* (Pei et al., 2000) and the rapid Ca^{2+} oscillations in *gca2* (Allen et al., 2001a) implicate a feedback regulatory role of *gca2* which may communicate cytosolic Ca^{2+} elevations back to ICa Ca^{2+} channel regulation, thus tuning oscillation periods.

Ca^{2+} Signaling in *abh1* and CO_2 Signaling. A new ABA hypersensitive mutant *abh1*, was isolated and shown to cause ABA hypersensitive Ca^{2+} elevations in *Arabidopsis* guard cells (Hugouvieux et al., 2001). ABH1 was shown to function as a dimeric mRNA cap-binding protein *in* vitro implicating a link between RNA processing and modulation of early ABA signal transduction (Hugouvieux et al., 2001).

CO_2 is an important environmental regulator of stomatal apertures. CO_2 was found to modulate cytoplasmic Ca^{2+} oscillations in guard cells (J. Young, S Mehta, E. Grill and J.I. Schroeder, submitted). This study reveals several new important findings into the mechanisms of CO2 signal transduction in guard cells (J. Young, S Mehta, E. Grill and J.I. Schroeder, submitted).

Published Reviews and Commentaries: Reviews and model/perspectives articles were prepared with DOE support that describe advances in understanding guard cell signal transduction, the newly recognized I_{Ca} signaling branch (Schroeder, 2001a; Schroeder, 2001b), and plant Ca^{2+} signal transduction (Allen and Schroeder, 2001). Commentaries on K^+ channel functions (Schroeder, 2003) and biotechnology issues (Beachy et al., 2002) were published. In collaboration with Dr. Jerome Giraudat (CNRS Gif-sur-Yvette) a journal issue of Current Opinions in Plant Biology was edited on the subject of plant signal transduction and gene regulation (Giraudat and Schroeder, 2001). We developed a clickable electronic guard cell signaling model as a learning tool for *The Arabidopsis Book* (Mäser et al., 2003).

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