A CALCIUM DEPENDENT PROTEIN KINASE INVOLVES H₂O₂ MEDIATED GUARD CELL SIGNALING IN ARABIDOPSIS

Disna Ratnasekera
Department of Agricultural Biology, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka

Accepted: 29th May, 2013

ABSTRACT

Drought is a major threat for plant growth and productivity. Plants lose over 90% of water by transpiration through stomatal pores. The cytosolic free Ca²⁺ elevated in guard cells in response to stress stimuli triggers stomatal closure. The plant-specific calcium-dependent protein kinases (CDPKs) play important roles in regulating downstream components of calcium signaling. In this study the biological function of Arabidopsis calcium-dependent protein kinase, CPK8, in response to ABA signaling in guard cells was characterized. The plants of T-DNA insertion mutant of cpk8 were more sensitive to drought stress than wild-type plants. The GUS staining studies confirmed that CPK8 expressed in leaves, specifically in guard cells. RT-PCR analysis showed that CPK8 expression was induced in response to drought stress. Further, pre-opened cpk8 stomata failed to close in response to H₂O₂ and Ca²⁺, which is consistent with the inability of cpk8 plants to reduce water loss upon drought. The drought susceptibility and stomatal impairment in response to H₂O₂ and Ca²⁺ of the cpk8 implicated that CPK8 plays a role in cellular environment in the control of H₂O₂ homeostasis and also as a compulsory molecule in the transduction of H₂O₂ signal in guard cells in response to drought stress.

Key words: Arabidopsis thaliana, Calcium-dependent protein kinases (CDPKs)

INTRODUCTION

Water is the most limiting resource for terrestrial plant growth and development and yield formation in many part of the world. Desiccation of crops during various growth stages causes severe and often irreversible damage and hence yields losses (Boyer 1982; Ainsworth and Long 2005). It would be beneficial for crop plants to show wide stomatal opening for CO₂ intake when water is available, but to close stomata during drought periods, thereby slowing desiccation and damage. Stomatal opening is driven by plasma membrane hyperpolarization proposed to drive K⁺ uptake into guard cells passively via inward-rectifying K⁺ (Kᵢᵣ) channels (Schroeder et al. 1987). Stomatal closing involves the influx of free cytosolic Ca²⁺ that down regulate the inward K⁺ channels and activate outward channels (Li et al. 2000). Ca²⁺ oscillations are a fundamental requirement for stomatal closure.

Several classes of Ca²⁺ binding sensory proteins have been identified in plants. CDPKs are the largest subfamilies of plant protein kinases among them. The completed Arabidopsis genome sequence has revealed 34 genes encoding CDPKs and they are highly homologous to each other (Cheng et al. 2002; Hrabak et al. 2003). Recently genome wide analysis of rice found that there are 29 genes encoding CDPKs and eight closely related kinase genes (Takayuki et al. 2005). Some other plants including soybean, tomato and maize also indicate the presence of multi-gene families (Harmon et al. 2001), but the reason for such a large number of CDPK genes is not yet known. Recent experiments indicate that functional specialization of individual CDPKs can occur through different types of regulation. For example, plants may use a combination of various strategies to functionally specialize individual CDPKs, as evidenced by two sandalwood CDPK isoforms that differ in tissue specific distribution, sub-cellular localization, and enzyme kinetics and properties (Anil et al. 2001).

CDPKs play diverse roles in various biological responses by interacting with other factors and act as key regulators of many signaling path-
ways. But very little is known about the particular CDPK that acts as the calcium sensor in each case. In this study we characterize the biological function of Arabidopsis calcium-dependent protein kinase CPK8 in response to ABA signaling in guard cells.

MATERIALS AND METHODS

Plant Materials and Growth Conditions: Arabidopsis thaliana ecotype Columbia was used in this study. The T-DNA insertion mutants of CPK8 (cpk8, SALK_036581) was obtained from Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). The homozygous cpk8 mutant was identified using gene-specific primers (forward primer 5′-CATGTTTGGGTGATGAT-3′ reverse primer 5′-GGCTTTAAGGGCTGAT GTC-3′).

Seeds were sterilized using NaOCl solution (0.5% NaOCl and 0.01% Triton x-100) for 10-15 min. and washed 5 times with sterilized distilled water under aseptic conditions in laminar flow cabinet and kept in dark at 4°C for 72 hours to break the dormancy.

Sterilized seeds were sown on MS plates and incubated in 20-22°C with 120 μmol/m²/s light intensity for seedling development. Seven day old seedlings were then transferred to 1:1 soil: peat medium. After transplanting, plants were covered with polythene cover to maintain high humidity and kept in growth chambers at 22°C with illumination at 120 μmol/m²/s for 16-h light/8-h dark cycle. The relative humidity was approximately 70% (±5%). One week later plants were discarded.

Vector Constructions and Generation of Transgenic Plants: The CPK8<sub>pro:</sub>GUS construct was generated by fusing the CPK8 promoter fragment (1.96 kb) in front of the β-glucuronidase (GUS) coding sequence in pCAMBIA1381 vector. The special primers for CPK8<sub>pro:</sub>GUS construct were 5′-CAGCTCTCCTAGGACGATAC-3′ and 5′-TTCGAATCTGAGAAGTCCG-3′. The GUS staining assays were carried out as described by (Xu et al. 2006).

Drought tolerance measurements: For drought experiments, Arabidopsis thaliana [Columbia ecotype, cpk8 knockout mutant] plants were grown on MS medium under continuous light for 7 days and transferred to peat soil in a controlled environment growth chamber with a 16:8 hour light: dark cycle and irrigated for 2 weeks. Then plants were subjected to drought by complete termination of irrigation. The plants (n=12 each) at the similar developmental stages were selected for the analysis. Watered plants were analyzed as control treatment. Pots were weighed after 3, 6, 9, 12, 15 and 18 days at the same time for relative water content measurements.

Water loss Measurements: Water loss experiments were conducted on weight basis and at the same time phenotype comparison at different time intervals were carried out. For measurement of water loss, plants were transferred from high (90%) to low humidity (50%) and then leaves were detached and incubated abaxial face up in 25°C with 50% RH. Their fresh weight was measured at different time intervals. Water loss was expressed as the percentage of initial fresh weight. To compare the phenotype, photographs were taken just after detaching and 5 hours after desiccation. Each experiment repeated 6 times with 4 replicates.

Stomatal aperture measurements: Plants were grown on MS medium under continuous light for 7 days and transferred to peat soil in a controlled environment growth chamber with a 12:12 hour light: dark cycle for 3 weeks and then placed in overnight dark before every treatment. To measure stomatal opening, detached leaves were floated in incubation buffer containing 50mM KCl with10mM MES/KOH and 0.1mM CaCl<sub>2</sub> for 3 hours under light to induce stomatal opening and measured the aperture width.

To measure the stomatal closing, detached leaves were floated in incubation buffer under
light for 2 hours and then kept in dark for another 2 hours to induce stomatal closing. After 2 hours, stomatal apertures were measured. For ABA-inhibition of stomatal opening, leaves were incubated with or without ABA (10 μM) under light for 2.5 hours. To study the effect of H$_2$O$_2$ and Ca$^{2+}$, stomata were opened by exposing plants for light and high humidity and incubating the leaves for 2 h in stomata-opening solution containing 50 mM KCl, 0.1 mM CaCl$_2$, and 10 mM MES/KOH, pH 6.15, in a growth chamber at 22 to 25°C under a photon flux density of 0.20 to 0.30 mmolm$^{-2}$s$^{-1}$. Stomatal apertures were measured 2 h after adding 100 μM H$_2$O$_2$ or 5mM Ca$^{2+}$.

**RESULTS**

**Phenotype Characterization of CPK8 T-DNA Insertion Mutant and Expression Patterns of CPK8:** No obvious morphological difference was observed between the cpk8 and wild-type, columbia plants under normal growth conditions (Fig. 1A). Fourteen days after withholding water, cpk8 showed severe wilting symptoms compared to wild type and re-watering did not allow the complete recovery of cpk8 plants (Fig. 1A). The site of T-DNA insertion in cpk8 was verified by conducting reverse transcription (RT)-PCR experiments (Fig.1B). RT-PCR analysis showed that there is no transcript of CPK8 in cpk8 homozygous plants (Fig. 1C), suggesting that CPK8 expression is completely eliminated in cpk8 mutant. To investigate the expression patterns of CPK8, transgenic plants harboring a GUS reporter gene fusing with CPK8 promoter was generated. High GUS activities were detected in the leaves and abundantly in stomatal guard cells, suggesting the potential role of CPK8 in regulation of stomatal movement (Fig. 1D).

**Drought Sensitivity:** When grown under well-watered conditions, the CPK8 T-DNA mu-

---

**Figure 1:** Characterization of cpk8 T-DNA insertion mutant in response to drought.
A-Phenotypic comparison between wild-type (WT) *Arabidopsis* (ecotype Columbia) and cpk8 during drought treatment
B-T-DNA insertion site in the cpk8 mutant. The T-DNA was inserted in the first exon of the CPK8 genomic DNA. Black boxes, solid line and diagonal boxes denote exons, introns and untranslated regions, respectively. Solid arrows indicate the primer locations for CPK8 kinase domain transcript.
C-RT-PCR verification of CPK8 expression in cpk8. Elongation factor 1a (EF) was used as loading control.
D-Expression patterns of CPK8 as determined by CPK8:GUS transgenic plants. Transgenic plants were stained with 5- bromo-4-chloro-3-indolyl-β-D-glucuronic acid solution for 12 hours Gus staining is shown in rosette leaves and guard cells.
tant plants did not display any visible phenotypic alteration. First, plants were grown with optimum irrigation up to two weeks and subjected to water stress by complete termination of watering. Fourteen days after withholding water, *CPK8* T-DNA mutant showed severe wilting symptoms compared to wild type. Re-watering did not allow the complete recovery of *CPK8* T-DNA mutant compared to wild type (Fig. 1A). RT-PCR verification of *CPK8* expression in *cpk8* is shown in figure 1C.

Relative water content (RWC) is a good indicator of a plant water status at any given time because it closely reflects the balance between water supply and transpiration rate. RWC were determined by weighing pots and expressing the weight loss as a percentage of initial fresh weight during desiccation. Transpirational water loss, as determined by RWC measurements after 3 days from the start of the treatment, was greatly decreased in the mutant compared to the wild type lines upon drought treatment (Fig 2).

**Water Loss Measurements of Detached Leaves:** Water loss from detached wild-type, and *cpk8* mutant rosette leaves were measured during incubation at 25°C and 50% relative humidity. This experiment repeats 5 times each with 4 replicates. After 3 hours of desiccation *cpk8* leaves displayed wilting symptoms and wild type leaves still remain turgid. By 5 hours after treatment mutant leaves completely wilted due to dehydration and wild type line still remained turgid (Fig. 3A). The fresh weight of detached leaves was measured at hourly time intervals. Throughout the duration of the desiccation treatment, mutant leaves consistently lost higher amount water than wild-type leaves (Fig.3B).

![Figure 2: Comparison of relative water content (RWC) between *cpk8* and wild type (ecotype Columbia) plants](image)

![Figure 3: Comparison of water loss measurements between *cpk8* and wild type (ecotype Columbia) plants](image)
Stomatal Aperture Measurements: To explore whether drought sensitivity observed for the cpk8 plants correlates with stomata performance, stomatal apertures were measured with different treatments. The cpk8 stomata closed to the same extent as the wild type in response to darkness (Fig. 4A). Similarly, the cpk8 mutations had no effect on the ability of pre-closed stomata to open in response to light (Fig. 4A).

It is well characterized that plants typically synthesize ABA in response to drought, which triggers the closing of stomata, thus reducing water loss and enhancing drought stress resistance (Schroeder et al., 2001; Luan, 2002). We tested whether the gene disruption affects the stomatal movements in the mutant treated with ABA. Leaf materials were incubated in 10μM ABA under light. ABA induced stomatal closure partially impaired in comparison to wild type (Fig. 4B).

The exogenous H2O2 induced elevations of cytosolic calcium and stomatal closure (Pei et al. 2000). However, reopened cpk8 stomata failed to close in response to H2O2 (Figure 5A), which is consistent with the inability of cpk8 plants to reduce water loss upon drought. Further, CDPKs have been predicted to function in response to cytoplasmic Ca2+ elevations in many physiological processes in plants. Extracellular Ca2+ causes stomatal closing, by initiating repetitive cytoplasmic Ca2+ elevations in guard cells (Pei et al. 2000). To investigate the role of CPK8 in guard cell signaling, detached leaves were incubated in 5mM CaCl2. Addition of [Ca2+]ext to pre-opened wild-type stomata caused closure, whereas in cpk8 stomatal closure was significantly attenuated suggesting that CDPKs function in [Ca2+]cyt perception and ion channel activation (Fig. 5B). These data indicate that the cpk8 mutations do not cause a general defect in stomatal functioning but specifically disrupt H2O2 and Ca2+ signaling in guard cells.

Excess H2O2 Accumulation in the cpk8 Mutant under Drought Stress: As an important signaling molecule, H2O2 had been identified to mediate ABA signal transduction in stomatal guard cells (Pei et al., 2000; Murata et al. 2001; Zhang et al. 2001b; Kwak et al. 2003; Bright et al. 2006; Miao et al. 2006; Yan et al. 2007). It is known that excess ROS (Reactive Oxygen Species) accumulation in living plant cells is toxic to cellular activities,

Figure 4: Light and Dark induced Stomata regulation and ABA-inhibition of stomatal opening
A. Average data for 3 representative experiments from each treatment are shown, n=150 total stomata).
B. Stomatal apertures were determined for wild-type and cpk8 mutant plants treated with 10μM ABA. Data were averaged across 4 separate experiments; n = 50 aperture measurements per experiment. Error bars represent standard errors.
so the cytosolic concentration of ROS must be stringently controlled (Mittler 2002; Apel and Hirt 2004). To test if H$_2$O$_2$ accumulation would be changed in cpk8 mutants, 3, 3’-diaminobenzidine (DAB) uptake method (Thordal-Christensen et al. 1997; Guan and Scandalios 2000) was applied to examine the production of H$_2$O$_2$ in leaves of cpk8, and wild-type plants. As shown in Fig. 6, ABA-induced H$_2$O$_2$ accumulation was significantly increased in the leaves of cpk8 mutants compared to wild-type plants. This demonstrated that CPK8 plays an important role in H$_2$O$_2$ homeostasis.

**DISCUSSION**

CDPKs have been found to function in response to cytoplasmic Ca$^{2+}$ elevations in many physiological processes in plants (Harmon et al. 2000). Evidences for a role of CDPKs in biotic stress signaling and environmental stress signaling were previously reported (Romeis 2001, Sheen 1996, Saij, 2000). The potential of CDPKs for engineering useful traits has also been suggested by alterations in the expression of rice OsCDPK7 that influenced cold and salt/drought tolerance in transgenic rice plants (Saijo 2000). Results of this study provided that AtCDPK8 plays a role in the transduction of an H$_2$O$_2$ signal in guard cells that mediates stomatal regulation in response to drought stress.

In addition, very strong expression of ATCDPK8 promoter GUS in transgenic plants was observed in guard cells of the leaf epidermis (Figure 1D), implying that ATCDPK8 specifically functions in guard cells. Stomatal

![Figure 5: H$_2$O$_2$ and Ca$^{2+}$-induced stomatal closure is impaired in cpk8 mutant. (A) H$_2$O$_2$-induced stomatal closing. (Average data from 3 representative experiments, n=150 total stomata). (B) External Ca$^{2+}$-induced stomatal closing. (Average data from 3 representative experiments, n=150 total stomata).](image)

![Figure 6: Comparison of ABA-induced H$_2$O$_2$ production in leaves of cpk8 and wild type ABA-induced H$_2$O$_2$ production in leaves of wild-type plants and cpk8 mutant assayed with 3,3-diaminobenzidine (DAB). The experiments were repeated for five times with similar results.](image)
aperture measurements showed that stomata from wild-type and AtCDPK8 plants closed to
the same extent in the dark and after exposure to light, both materials displayed same extent
in the opening of stomatal pores (Fig.4A). Pei et al (2000) reports that H2O2 induced eleva-
tions of cytosolic calcium and stomatal closure. However, pre-opened cpk8 stomata
failed to close in response to H2O2 and Ca2+ (Figure 5A, 5B), which is consistent with the
inability of cpk8 plants to reduce water loss upon drought suggesting that loss-of-function
mutation in CPK8 impairs H2O2 homeostasis and signal transduction in guard cells. Hence,
iti is possible that CPK8 acts as a key regulator that specifically modulates H2O2 homeostasis
and as an essential signal transduction mole-
cule in guard cell signal transduction pathway.
These data indicate that the c pk8 mutations do
not cause a general defect in stomatal func-
tioning but specifically disrupt H2O2 signaling
in guard cells. Recent reports revealed that
ABA- and Ca2+-induced stomatal closing were partially impaired in c pk3 c pk6 mutant
alleles (Mori et al. 2006). Similarly, stomatal
 closure in AtCDPK8 recessive plants was im-
paired in response to Ca2+ and H2O2 but not to
ABA whereas closure elicited by darkness and
opening elicited by light were unaffected sug-
gestig an alternate signaling pathway. On the
basis of the results presented here as well as
those reported previously (Mori et al. 2006;
Pei et al. 2000), allows to describe a divergent
oxidative signal transduction pathway from
sensing to the drought response in plants.

CONCLUSION

Results of this study confirmed the potential
location of the AtCDPK8 by GUS staining
and RT-PCR verified the presence and ab-
sence of AtCDPK8 in transgenic knockout
mutants and wild type plants respectively.
Phenotypic expression along with stomatal
d and DAB staining studies revealed that
AtCDPK8 plays a role in the transduction of
an H2O2 signal in guard cells mediating
stomatal regulation in response to drought stress via divergent oxidative signal transduc-
tion pathway.

ACKNOWLEDGMENTS

The laboratory facilities provided by State
Key Laboratory of Plant Physiology, Molecular
Biology and Biochemistry, College of Bio-
logical Sciences, China Agricultural Univer-
sity, Beijing, China are acknowledged.

REFERENCES

Ainsworth EA, Long SP 2005 What have we
learned from 15 years of free-air CO2 en-
richment (FACE)? A meta-analytic re-
view of the responses of photosynthesis,
canopy properties and plant production to
rising CO2. New Phytologist 165: 351-372
Anil VS, Harmon AC and Rao KS 2001 Calcium
dependent protein kinase in oil bodies: its
biochemical characterization and biologi-
cal significance. (Abstract no. 23) In
American Society of Plant Biologists
Meeting, MD. 30.
Apel K and Hirt H 2004 Reactive oxygen species:
metabolism, oxidative stress, and signal
transduction. Annu Rev Plant Biol; 55:373
-399.
Boyer JS 1982 Plant productivity and environ-
ment. Science 218: 443-448
Bright J, Desikan R, Hancock JT, Weir IS and
Neill SJ 2006 BA-induced NO generation
and stomatal closure in Arabidopsis are
dependent on H2O2 synthesis. Plant J. 45:
113-122.
Cheng SH, Willmann MR, Chen HC, Sheen J
2002 Calcium signaling through protein
kinases. The Arabidopsis calcium depend-
ent protein kinase gene family Plant
Physiol. 129,469-485.
Guan LM, Scandalios JG 2000 Hydrogen perox-
ide-mediated catalase gene expression in
response to wounding. Free Radical Biol
Med 28:1182-1190.
Harmon AC, Gribskov M , Gubrium E, Harper JF
2001 The CDPK super fam il y of protei n
kinases. New Phy tol 151: 175–183
Harmon AC, Gribskov M and Harper JF 2000
CDPKs - a kinase for every Ca2+ signal?
Trends Plant Sci. 5: 154-159.
Hrabak EM, Chan CW, Gribskov M, Harper JF,
Choi JH et al 2003 The Arabidopsis
CDPK-SnRK super family of protein
kinase. Plant Physiol. 132: 666-680
Kwak JM, Mori IC, Pei ZM et al. 2003 NADPH
oxidase AtrobohD and AtrobohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J* 22:2623-2633.

Li J, Wang SQ, Watson MB and Assmann SM 2000 Regulation of abscisic acid–induced stomatal closure and anion channels by guard cell AAPK kinase. *Science* 287,300-303.

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W, 2002 Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. *The Plant Cell* 14:S389-S400.

Miao Y, Lv D, Wang P et al. 2006 An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell*; 18:2749-2766.

Mittler R, 2002 Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci*; 7:405-410.

Mori IC, Murata Y, Yang Y, Munemasa S, Wang YF, Andreoli S, Tiriac H, Alonso JM, Harper JF, Ecker JR, Kwak JM and Schroeder JI 2006 CDPKs CPK6 and CPK3 function in ABA regulation of guard cell s-type anion- and Ca\(^{2+}\)- permeable channels and stomatal closure. *PLoS Biol* 4; 1749-1762.

Murata Y, Pei ZM, Mori IC and Schroeder JI 2001 Abscisic acid activation of plasma membrane Ca\(^{2+}\) channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in *abi1-1* and *abi2-1* protein phosphatase 2C mutants. *Plant Cell* 13; 2513-2523.

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI, 2000 Calcium channels activated by hydrogen peroxide mediate abscisic signalling in guard cells. *Nature* 406, 731-734.

Romeis T, Ludwig AA, Martin R, Jones JDG 2001 Calcium dependent protein kinases play an essential role in a plant defence response. *EMBO J.* 20, 5556-5567.

Saijo Y, Hata S, Kyozuka J, Shimamoto K and Izui K 2000 Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J.* 3; 19–27.

Schroeder JI, Raschke K, Neher E, 1987. Voltage dependence of K+ channels in guard cell protoplasts. *Proc. Natl. Acad. Sci. USA* 84:4108-12

Schroeder JI, Allen GJ, Hugouvieux V, Kwark JM, and Waner D, 2001 Guard cell signal transduction. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 627-658.

Sheen J 1996 Ca\(^{2+}\)-dependent protein kinases and stress signal transduction in plants. *Science* 274, 1900–1902.

Takayuki A, Tanaka N, Yang G, Hayashi N, Komatsu S. 2005 Genome wide identification of the rice calcium- dependent protein kinase and its closely related kinase gene families: Comprehensive analysis of the CDPKs gene family in rice. *Plant Cell Physiol.* 46(2): 356–366.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB, 1997 Subcellular localization of H2O2 in plants: H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* 11:1187-1194.

Tiriac H, Jose M, Alonso JM, Harper JF, Ecker JR, Kwak JM, Schroeder JI 2006 CDPKs CPK6 and CPK3 function in ABA regulation of guard cell S-type anion- and Ca\(^{2+}\) permeable channels and stomatal closure. *PLoS Biol* 4, 1749-1762.

Xu J, Li HD, Chen LH, Wang Y, Liu LL, He L and Wu WH 2006 A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in *Arabidopsis*. *Cell* 125; 1347-1360.

Yan J, Tsuichihara N, Etoh T and Iwai S 2007 Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening. *Plant Cell Environ.* 30: 1320-1325

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, and Song CP 2001b Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol.* 126: 1438-1448.