A Cyclic Nucleotide-Gated Ion Channel, *CNGC2*, Is Crucial for Plant Development and Adaptation to Calcium Stress[1]

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To better understand the relationship between functions of cyclic nucleotide-gated channels (CNGCs) and plant physiology, we studied the effect of different ions on the growth of Arabidopsis *cngc2* mutants. Here, we report the novel finding that *cngc2* plants are specifically hypersensitive to calcium in their growth environments, leading to severe reductions in size and reproductive ability.

CNGCs are important for sensory transduction in animals. The best characterized CNGCs are from the visual and olfactory systems. This class of channels does not discriminate well between alkaline metals and allow a substantial Ca^{2+} flow. Physiologically relevant permeant ions are Na^+, K^+, and Ca^{2+}, and in both visual and olfactory transduction, this Ca^{2+} influx is important for desensitization and adaptation (Zagotta and Siegelbaum, 1996; Broillet and Firestein, 1999; Kaupp and Seifert, 2002).

Little is known about the physiological functions of CNGCs in plants. However, recent works have suggested that at least some members are involved in uptake and homeostasis of heavy metals such as Ni^{2+} and Pb^{2+} (Arazi et al., 1999; Sunkar et al., 2000). On the other hand, genetic studies in Arabidopsis have shown that a mutation in *CNGC2* (also called *DND1*; Yu et al., 1998) results in a near-complete loss of the hypersensitive response (HR), a form of programmed cell death closely associated with disease resistances (Heath, 2000). Moreover, *dnd1-1* (henceforth renamed as *cngc2-1*) mutants are dwarfed plants that exhibit pleiotropic phenotypes, including constitutively heightened defense against various pathogens, elevated levels of salicylate compounds, and increased expression of defense-related genes (Yu et al., 1998; Clough et al., 2000).

In heterologous expression systems, CNGC2 can form an ion channel that mediates Ca^{2+} and K^+ influxes but interestingly does not allow substantial Na^+ influx (Leng et al., 1999; Leng et al., 2002). Although the mechanism by which this ion channel mediates HR and defense responses is not well understood, fluxes of Ca^{2+} and K^+ are among the earliest detectable events during defense signaling. Importantly, Ca^{2+} influx and the subsequent rise in cytoplasmic Ca^{2+} have been correlated with the activation of many defense responses and the onset of HR (Jabs et al., 1997; Xu and Heath, 1998; Blume et al., 2000; Grant et al., 2000). These data suggest that the control of ion permeation through CNGC2 may be a critical component of HR and defense responses. Therefore, to better understand the role of CNGC2 in planta, we examined the effect of different ions on the growth of Arabidopsis *cngc2* mutants.

RESULTS

CNGC2 Mutant Alleles

Experiments reported herein utilized two homozygous mutant lines of Arabidopsis. The first allele is *cngc2-1* and contains a premature stop codon at W290 (Clough et al., 2000). The second allele, *cngc2-2*, contains a T-DNA inserted 42 bases into the fourth intron (after L411) and was isolated from the Arabidopsis Knockout Facility. Both mutations are expected to completely or severely disrupt gene functions, and we showed that *cngc2-2* is an RNA-null mutant (data not shown). Both alleles also behaved as recessive mutants in all of the phenotypic tests discussed below.

Ca^{2+} Sensitivity of *cngc2-1* and *cngc2-2* on Defined Media

Both *cngc2-1* and *cngc2-2* plants showed Ca^{2+}-hypersensitive growth phenotypes. Growth of mutant plants on Murashige and Skoog control media (with or without 1% [w/v] supplemented Suc) was not significantly different from their respective wild types for about the first 3 weeks under our normal growth condition (for details, see "Materials and

[1] This work was supported by the National Science Foundation (grant no. IBN–9728563 to M.R.S.) and by the U.S. Department of Agriculture (grant no. 95–37304–2364 to M.R.S.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.102.019216.

Methods"). However, when the media contained increased amounts of $CaCl_2$, both mutants showed drastically stunted growth. A representative example is shown in Figure 1. This phenotype was clearly visible after about 12 d of growth, and mutants remained smaller than their wild-type counterparts by all measurable criteria (rosette size, root mass, and overall stature) throughout the rest of their life spans. Whereas wild-type plants were not affected by moderate increases in $[Ca^{2+}]$ (up to 30 mM extra $CaCl_2$ and growth for up to 6 weeks on petri plates), both mutants showed significant size reduction in all $[Ca^{2+}]$ tested (Fig. 2A; Student's t test, $P \ll 0.001$). This growth defect was specific to Ca^{2+}, because mutants and wild types were indistinguishable in their responses to changes in various other ions. Conditions tested include: increasing $[Mg^{2+}]$ (up to 20 mM additional $MgCl_2$), $[K^+]$ (up to 100 mM additional KCl), and $[Na^+]$ (up to 75 mM additional NaCl), and a 10-fold increase or decrease in $[H^+]$ (Student's t test showed no significant difference, $P < 0.05$; also, see below). As an example, Figures 1 and 2B show that the growth of mutants and wild types on increasing [NaCl] was very comparable. Taken together, these results suggest that *cngc2* mutants are not hypersensitive to ionic or osmotic stresses in general but rather are specifically impaired in coping with excess external Ca^{2+}.

cngc2 mutants maintained their Ca^{2+} sensitivity in later stages of development. We can grow plants in magenta cubes on Murashige and Skoog media to maturity. After about 2 months, wild-type plants had

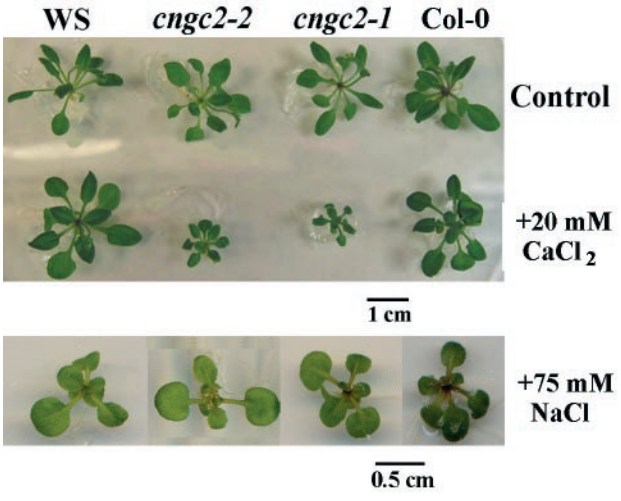

Figure 1. *cngc2-1* and *cngc2-2* are both hypersensitive to Ca^{2+} but not other ions. Mutant plants and their corresponding wild types were grown on control media or media with additional 20 mM $CaCl_2$ or 75 mM NaCl. The top panel shows that at 3 weeks old, mutant and wild-type plants were practically indistinguishable under the control condition. However, mutants were drastically smaller when $[Ca^{2+}]$ was elevated. Typically, rosette diameter of mutants is about a third or less of their corresponding wild types. The bottom panel shows that both mutants and wild types were equally sensitive to Na^+ (16-d-old plants).

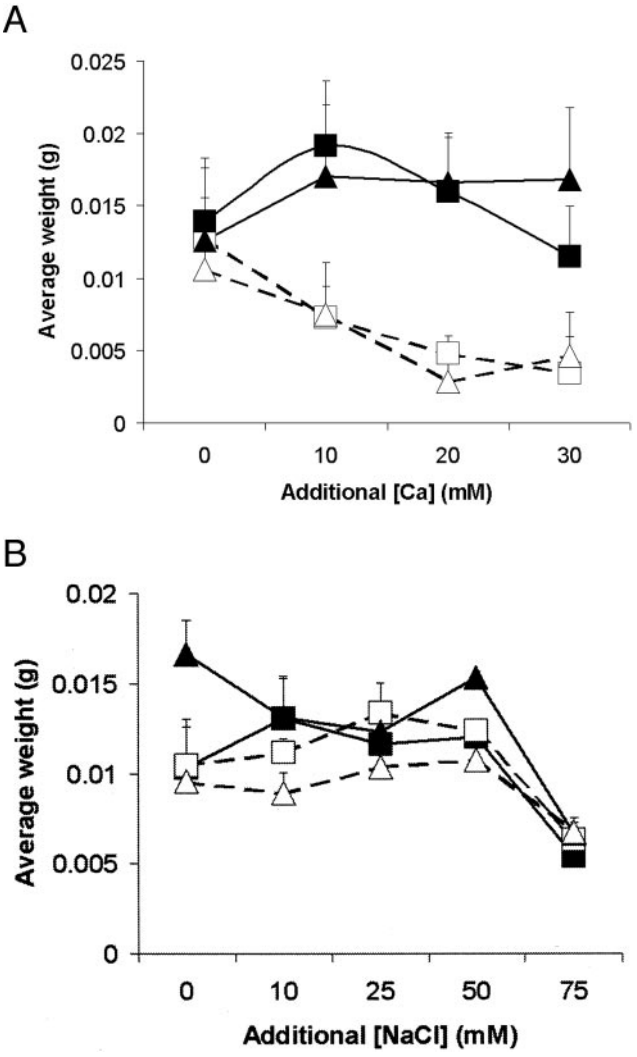

Figure 2. A, Ca^{2+} response of *cngc2* mutants. Fresh weights of plants grown on control media or media containing increasing amounts of $CaCl_2$ were quantified, and the result of a typical experiment is shown. Each data point (with corresponding SD) represents the average fresh weight of 25-d-old plants grown under the specified condition (n = 12–23 for each point). The result shows that mutants are hypersensitive to Ca^{2+} in the growth medium. Qualitatively similar results have been obtained at least four more times. ■, Wassilewskija (WS); □, *cngc2-2*; ▲, Columbia (Col-0); △, *cngc2-1*. B, Na^+ response of *cngc2* mutants. Fresh weights of plants grown on control media or media containing increasing amounts of NaCl were measured. Plants were grown on indicated media for 2 weeks, and the weight of aerial portions was measured from three groups of plants, each of 15 individuals. The average weight of an individual in each group was obtained, and then the average weights and corresponding SDs of individual plants in each growth condition were calculated and plotted. The result shows that mutant and wild-type plants are similarly sensitive to external NaCl. ■, WS; □, *cngc2-2*; ▲, Col-0; △, *cngc2-1*.

healthy seed sets despite the addition of Ca^{2+} to the medium (10–30 mM). Conversely, *cngc2* mutants in such conditions were severely dwarfed and produced no or very few seeds (data not shown). This shows that defects in *cngc2* that are exaggerated in

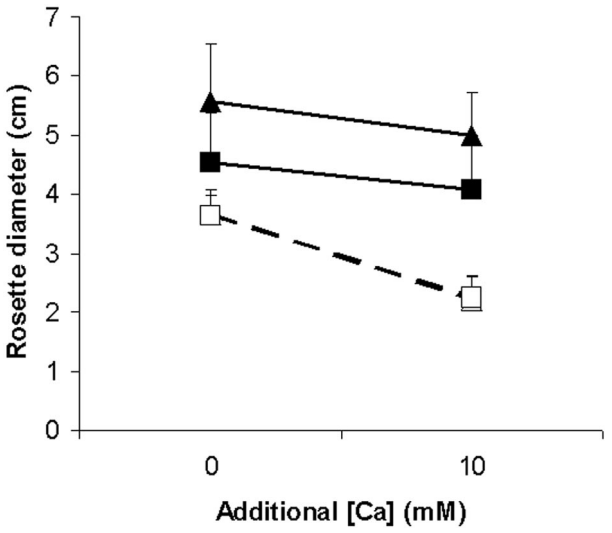

Figure 3. *cngc2* mutants are hypersensitive to increased [Ca^{2+}] in soil. Plants were directly grown in a soil mix and watered with distilled water or distilled water with 10 mM $CaCl_2$ as indicated. Each data point represents the average diameter of rosette leaves (and corresponding SD) after 4 weeks of growth (n = 9–11 for each point). The result shows that mutants are hypersensitive to Ca^{2+} in soil. ■, WS; □, *cngc2-2*; ▲, Col-0; △, *cngc2-1*.

elevated external [Ca^{2+}] affect both vegetative and reproductive development.

Ca^{2+} Sensitivity of *cngc2-1* and *cngc2-2* in Soil

When grown directly in a soil mixture, mutant plants similarly showed hypersensitivity to Ca^{2+}. Analogous to the scenario described above, the size difference between soil-grown mutant and wild-type plants was exacerbated when they were watered with 10 mM $CaCl_2$ instead of distilled water (Fig. 3). Whereas wild-type plants were minimally affected by watering with 10 mM $CaCl_2$ in both size and seed sets, mutants were significantly stunted in vegetative and reproductive growth and led to a complete absence of seed production (Fig. 3; data not shown).

DISCUSSION

We show that null mutations in *CNGC2* lead to a specific and dramatic Ca^{2+} hypersensitivity that results in severe reductions in plant size and seed yield. To our knowledge, this is the first report of a significant and stable phenotype caused by a single gene mutation when plants are challenged with a nominal increase in extracellular [Ca^{2+}]. The only other mutant we are aware of with Ca^{2+}-sensitive growth is *smt1*. Such plants are defective in a sterol methyltransferase, leading to Ca^{2+} sensitivity in roots on agar media during the first 2 weeks of growth (Diener et al., 2000). This is in stark contrast to *cngc2* mutants, which display diminutive growth through different developmental stages and growth media. Our finding is especially intriguing because the Arabidopsis genome potentially contains 19 other CNGCs (see http://www.cbs.wmn.edu/Arabidopsis/), and yet disrupting the function of CNGC2 alone has such a large impact. Ca^{2+} levels used in this study are commonly found in natural growth environments (for example, see Kelling and Schulte, 1998). Therefore, our data support the hypothesis that CNGC2 is particularly important for plant growth in physiological calcified conditions.

We emphasize that *cngc2* mutants are not hypersensitive to stress in general. The responses of *cngc2* and wild-type plants to various other challenges are indistinguishable. These include increasing [Na^+] and [Mg^{2+}], increasing and decreasing pH and [K^+], decreasing Ca^{2+} (by omitting Ca^{2+} from the medium or by chelating Ca^{2+} with 1.5 mM BAPTA), and cold and freezing conditions (see "Results"; C.W.M. Chan and M.R. Sussman, unpublished data). The phenotypic difference between mutant and wild-type plants upon increasing external [Ca^{2+}] is not affected by the inclusion of 1% (w/v) Suc and is independent of the light regimen (we obtained qualitatively similar results with 8-h-light/16-h-dark cycles; data not shown). We also noticed that *cngc2-2*, just like *cngc2-1*, did not display the HR upon pathogen infection (G.I. Jurkowski and A.F. Bent, unpublished data; Yu et al., 1998). Because the two mutant alleles are from different ecotypes, our data strongly provide a causal link between CNGC2 function and the adaptive response of plants to both abiotic (for example, Ca^{2+} stress) and biotic challenges.

Mutant phenotypes cannot be attributed solely to the constitutive induction of salicylic acid and defense-related genes in *cngc2*. Depletion of salicylate compounds in *nahG*$^+$ *cngc2-1* transgenic plants removes the constitutively elevated pathogen resistance without completely reversing the dwarf and lack of HR phenotypes (Clough et al., 2000). Results from preliminary experiments also suggested that *nahG*$^+$ *cngc2-1* transgenic plants retained the Ca^{2+} hypersensitivity (C.W.M. Chan and M.R. Sussman, unpublished data). Therefore, mutant developmental defects are largely independent of salicylic acid levels.

One of the simplest explanations for the Ca^{2+}-hypersensitive phenotype in *cngc2* mutants is that mutant plants are dwarf due to Ca^{2+} toxicity. This Ca^{2+} toxicity can be the result of Ca^{2+} hyperaccumulation or of a defect in sensing and/or adapting to elevated levels of Ca^{2+} in the growth environment. Preliminary results suggested that mutant plants did not accumulate more Ca^{2+} than wild type either in control growth conditions or when placed under Ca^{2+} stress (C.W.M. Chan, J.F. Harper, and M.R. Sussman, unpublished data). Therefore, our current working model is that mutants are perhaps defective in signaling pathways that allow for normal growth in low tens of millimolar Ca^{2+}.

CONCLUSIONS

In summary, our data support the hypothesis that CNGC2 is critical for plant development. Mutants are affected in vegetative and reproductive growth, programmed cell death, and adaptive responses to biotic and abiotic stimuli. Our data represent a starting point for further analyses into relationships between CNGC2, calcium signaling, and the aforementioned physiological processes. Importantly, our result strongly suggests that CNGC2 is a key determinant for growth under physiologically relevant [Ca^{2+}].

MATERIALS AND METHODS

Mutant Isolation

Two mutant Arabidopsis lines were used. *cngc2-2* (WS ecotype) was isolated from the Wisconsin T-DNA α collection using standard methods (Krysan et al., 1999). The primers used were against the T-DNA left border (JL202) and CNGC2 (5′-tctcccatggtggttcctctatttcaatc-3′). The isolation of *cngc2-1* (*dnd1-1*; Col-0 ecotype) has been published (Yu et al., 1998).

Growth Conditions

Plants were routinely grown on 0.5× Murashige and Skoog salt (Invitrogen, Carlsbad, CA), 2.5 mM MES, and 0.8% (w/v) washed agar (Sigma-Aldrich, St. Louis), with pH adjusted to 5.7 with KOH as necessary. Murashige and Skoog (0.5×) media contains 1.5 mM $CaCl_2$. Media were supplemented with 1% (w/v) Suc and additional ions as indicated in the text. Alternatively, plants were grown in a soil mixture (Jiffy mix:medium grade vermiculite, 2:1 [v/v]) and watered with distilled water (or distilled water supplemented with 10 mM $CaCl_2$ where indicated) about once a week. All plants were grown in growth chambers (AR-75L, Percival, Boone, IA) that were set to 21°C and constant light (about 40 μE).

Received December 16, 2002; returned for revision December 21, 2002; accepted January 20, 2003.

LITERATURE CITED

Arazi T, Sunkar R, Kaplan B, Fromm H (1999) Plant J **20:** 171–182
Blume B, Nurnberger T, Nass N, Scheel D (2000) Plant Cell **12:** 1425–1440
Broillet MC, Firestein S (1999) Ann NY Acad Sci **868:** 730–740
Clough SJ, Fengler KA, Yu IC, Lippok B, Smith RK Jr, Bent AF (2000) Proc Natl Acad Sci USA **97:** 9323–9328
Diener AC, Li H, Zhou W, Whoriskey WJ, Nes WD, Fink GR (2000) Plant Cell **12:** 853–870
Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) Plant J **23:** 441–450
Heath MC (2000) Plant Mol Biol **44:** 321–334
Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Proc Natl Acad Sci USA **94:** 4800–4805
Kaupp UB, Seifert R (2002) Physiol Rev **82:** 769–824
Kelling KA, Schulte EE (1998) A2523 Soil and Applied Calcium. University of Wisconsin-Extension, Cooperative Extension Publications, Madison, Wisconsin, pp 1–2
Krysan PJ, Young JC, Sussman MR (1999) Plant Cell **11:** 2283–2290
Leng Q, Mercier RW, Hua BG, Fromm H, Berkowitz GA (2002) Plant Physiol **128:** 400–410
Leng Q, Mercier RW, Yao W, Berkowitz GA (1999) Plant Physiol **121:** 753–761
Sunkar R, Kaplan B, Bouche N, Arazi T, Dolev D, Talke IN, Maathuis FJ, Sanders D, Bouchez D, Fromm H (2000) Plant J **24:** 533–542
Xu H, Heath MC (1998) Plant Cell **10:** 585–597
Yu IC, Parker J, Bent AF (1998) Proc Natl Acad Sci USA **95:** 7819–7824
Zagotta WN, Siegelbaum SA (1996) Annu Rev Neurosci **19:** 235–263