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A cyclic nucleotide-gated channel is necessary for optimum fertility in high-calcium environments

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Summary

- *Arabidopsis cngc2* plants are hypersensitive to external calcium and exhibit reduced plant size and fertility, especially when they are treated with elevated but physiologically relevant levels of calcium. This report focuses on the role of cyclic nucleotide-gated channel 2 (CNGC2) in plant fertility.
- To determine the cause of the reduced fertility, we investigated the flower structure and growth potential of both male and female reproductive organs in *cngc2* plants grown in high-calcium conditions.
- *cngc2* mutants had short stamens that may limit pollen deposition and pistils that were not conducive to pollen tube growth.
- Our data indicate that sporophytic, but not gametophytic, defects are the main cause of the observed reduction in seed yield in *cngc2* plants, and suggest that correct cyclic nucleotide and calcium signaling are important for cell elongation and pollen tube guidance.

Introduction

Cyclic nucleotide-gated channels (CNGCs) are a family of cation channels that are activated by cyclic nucleotide monophosphates and typically permeable to Na^+, K^+ and Ca^{2+} to various extents (Kaupp & Seifert, 2002; Kaplan *et al.*, 2007). These channels have important functions in sensory signal transduction in animals (Kaupp & Seifert, 2002), but are less well understood in plants. Recent evidence suggests that they may be involved in a wide variety of plant physiological processes, including phototransduction, biotic and abiotic stress responses, growth and development, and ion homeostasis (Kaplan *et al.*, 2007). Additional data suggest that some CNGCs contribute to the uptake of toxic metals, such as cesium and lead (Sunkar *et al.*, 2000; Hampton *et al.*, 2005). They may also be important for processes in which cyclic nucleotide and Ca^{2+} signaling pathways converge (for example, Talke *et al.*, 2003; Frietsch *et al.*, 2007).

There are potentially 20 genes encoding CNGCs in *Arabidopsis thaliana* (Kaplan *et al.*, 2007). CNGC1 is thought to play a role in Ca^{2+} uptake and primary root growth in seedlings (Ma *et al.*, 2006), as well as the uptake of K^+ for nutritional needs (Hampton *et al.*, 2005). CNGC3 is likely to be involved in ion homeostasis, ion transport from the xylem and, possibly, salinity adaptation (Gobert *et al.*, 2006). CNGC10 aids in potassium uptake and is probably part of a light signal transduction pathway (Borsics *et al.*, 2007). Through transcriptome analysis, CNGC7, CNGC8, CNGC16 and CNGC18 are presumed to be expressed only or primarily during pollen development (Kaplan *et al.*, 2007), although Talke *et al.* (2003) presented massively parallel signature sequencing and expressed sequence tag data to suggest that CNGC8 and CNGC18 are also expressed in some vegetative tissues. CNGC18 is also known to be necessary for pollen tube growth (Frietsch *et al.*, 2007). CNGC2 (also known as DND1) and CNGC4 (also known as HLM1) are both involved in a form of developmentally regulated programmed cell death, called the hypersensitive response, and pathogen defense (Clough *et al.*, 2000; Balague *et al.*, 2003). Indeed, mutations in a *CNGC4* ortholog in barley also cause similar pathogen-related phenotypes (Rostoks *et al.*, 2006). Interestingly, among all the CNGCs studied to date, CNGC2 seems to uniquely play a role in adapting to growth in high-calcium environments (Chan *et al.*, 2003), although it is not known to cause any significant change in Ca^{2+} accumulation *in planta* (Chan *et al.*, 2003; Hampton *et al.*, 2005).

cngc2 mutant plants display broad-spectrum disease resistance that is associated with a constitutively high level of salicylic acid (SA; Clough *et al.*, 2000). The basis for this elevation is currently unclear. The lack of hypersensitive response, however, is most probably a result of a lack of CNGC2-dependent

Ca^{2+} influx in the mutant. CNGC2 has been shown to mediate cyclic nucleotide monophosphate-dependent Ca^{2+} influx in both a heterologous expression system (Leng *et al.*, 1999) and in *Arabidopsis* guard cell protoplasts (Ali *et al.*, 2007). Ca^{2+} influx, in turn, is necessary for the production of nitric oxide, which subsequently leads to the initiation of the hypersensitive response (Ali *et al.*, 2007). The additional growth and developmental phenotypes of *cngc2* are probably related to defects in Ca^{2+} signaling. Mutant plants exhibit a reduction in size and reproductive ability when grown in media with elevated but physiologically relevant Ca^{2+} concentrations (Chan *et al.*, 2003). Transcriptional data suggest that this may be attributed to improper regulation of Ca^{2+}-dependent signal transduction events (Chan *et al.*, 2008). Therefore, we decided to further explore the relationship between Ca^{2+} hypersensitivity and various growth defects in *cngc2* as a way to better understand the role(s) played by CNGC2 in cyclic nucleotide signaling, Ca^{2+} signaling and downstream developmental pathways.

This report specifically focuses on our investigation of the basis of low fertility in *cngc2* plants. During normal fertilization, after a pollen grain comes into contact with the stigma, a pollen tube is formed that passes through the style and transmitting tract. It eventually reaches an ovule where the male gametes are released. Cues from various parts of the pistil are thought to contribute significantly to pollen tube guidance (Cheung & Wu, 2001; Palanivelu & Preuss, 2006). Both Ca^{2+} and cyclic adenosine monophosphate are involved in pollen tube growth and orientation (Moutinho *et al.*, 2001; Rato *et al.*, 2004; Frietsch *et al.*, 2007). In a series of experiments, we analyzed the fertility, flower phenotypes and growth potential of male and female reproductive tissues of mature *cngc2* plants grown in elevated Ca^{2+}. Our results suggest that defects in both the male and female sporophytic tissues are largely responsible for the decrease in seed yield in the mutant.

Materials and Methods

Plant material, growth conditions and growth parameters

The majority of experiments reported here utilized the previously characterized *Arabidopsis thaliana* (L.) Heynh. *cngc2-2* mutant [*Wassilewskija* (*WS*) ecotype], which was isolated from the Wisconsin T-DNA collection using standard methods (Krysan *et al.*, 1999; Chan *et al.*, 2003). Some experiments also used *cngc2-1* [also known as *dnd1-1*; *Columbia* (*Col*) ecotype], and its isolation has also been published (Yu *et al.*, 1998). The double homozygous mutant *cngc2-1*/*sid2* was a gift from Professor Andrew Bent at the University of Wisconsin-Madison, Department of Plant Pathology (SID2 is necessary for the synthesis of SA; Wildermuth *et al.*, 2001).

Seeds were germinated on 0.5 × Murashige and Skoog salt, 2.5 mM 2-(*N*-morpholino)ethanesulfonic acid and 0.8% (w/v) washed agar, with the pH adjusted to 5.7 with KOH as necessary, to minimize the potential effect of external calcium on germination. After 10 d, healthy seedlings were transferred to a soil mixture [Jiffy mix : medium-grade vermiculite, 2 : 1 (v/v)]. The Jiffy mix used contains 48–52% sphagnum peat moss and 48–52% vermiculite and lime. The acid-extractable Ca^{2+} of this soil mix was estimated to be 0.5–1 mM via atomic absorption spectroscopy (B. M. Asby & C. W. M. Chan, unpublished). Plants were watered twice a week with 1 l of distilled water, or the same volume of distilled water supplemented with $CaCl_2$ or KCl in the concentrations specified to keep the soil surface moist. Under our growth conditions, we did not observe any measurable depletion in soil Ca^{2+} during the course of our experiments (*c.* 3 wk growth in soil): the Ca^{2+} concentration of the used soil remained at *c.* 0.5–1 mM when plants were watered with distilled water, and approximated the concentration of Ca^{2+} in the watering solution in high-Ca^{2+}-treated plants. All plants were grown in standard laboratory conditions (constant light at *c.* 40 μmol and 23°C), and were analyzed after 21 d of growth in soil.

The plant growth parameters measured were the height, rosette leaf size (length of the largest three leaves from each plant for a total of five plants per sample), dried shoot mass (aboveground plant material was isolated from 15 individuals per sample, and dried in a 70°C oven for 16 h before weighing) and seed yield (at least 15 siliques per sample were analyzed). Where indicated, siliques were dissected open with a sharp needle and fixed in ethanol : acetic acid (3 : 1). All chemicals were from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

Pollen tube growth assays

In vitro pollen germination was performed by isolating anthers from flowers of the specified genotype that had just opened, and placing them in an agar pollen growth medium containing 18% sucrose, 0.01% boric acid, 2 mM $CaCl_2$, 2 mM $Ca(NO_3)_2$, 1 mM $MgSO_4$ and 1% low-melting-point agar. To compare the effect of wild-type and mutant pistil extracts on pollen tube growth, 0.5 μl of specified pistil extract was also applied to each anther. Pistil extracts were prepared from young (closed) flower buds from *cngc2-2* and *WS*, respectively. All tissues, except the pistils, were removed, and 1 μl of a liquid pollen growth medium (which has the same composition as the agar medium described above, except for the exclusion of agar) was added for each pistil used in the sample (usually 20 pistils were used). Pistils were mechanically disrupted with a plastic pestle fitted to the bottom of a 1.5 ml Eppendorf tube, and the resulting extract was applied immediately to the anthers. Four anthers were isolated from each *WS* flower, with two anthers exposed to the *cngc2-2* pistil extract and the other two to the *WS* extract. A total of 24 flowers was tested for each kind of pistil extract. Images of pollen tubes were taken with an Olympus BX60 microscope (Center Valley, PA, USA) after 16 h of growth on the specified medium.

To check for semi *in vivo* and *in vivo* pollen tube growth, developing flower buds of the same developmental stage (flowers that had just opened unless otherwise specified) were dissected

open, emasculated and deposited with fresh pollen. Semi *in vivo* pollen tube growth was assayed essentially according to Palanivelu & Preuss (2006). Briefly, pollen grains were allowed to interact with stigmas for 3 h before unbound pollen was washed off with liquid pollen growth medium (washed three times). Stigmas with attached styles were then removed (i.e. ovules were not included) and placed on the agar pollen growth medium to allow pollen tubes to grow for 16 h. Images of pollen tubes were taken with an Olympus BX60 microscope as above, and the experiment was performed on at least 15 pistils for each sample. For *in vivo* growth assays, pollen tubes were allowed to grow on pistils for 24 h before the pollinated flower buds were fixed in ethanol : acetic acid (3 : 1) for 3 h and softened in 1 M NaOH for 10 min. They were washed with distilled water and stained with 0.1% aniline blue overnight. To observe pollen tubes, samples were washed with distilled water, compressed under glass slides and observed with an Olympus BX60 epifluorescence microscope. The epifluorescence images were captured digitally using an Olympus DP70 device camera. Each pollination attempt, using specified pollen and ovule donors, was performed at least three times.

Environmental scanning electron microscopy (ESEM)

For ESEM, fresh plant tissue was placed on adhesive PELCO Tabs (Ted Pella, Inc., Redding, CA, USA) and analyzed in a Quanta 200 ESEM (FEI Company, Hillsboro, OR, USA). Samples were scanned at 20 kV under 2–4 Torr pressure.

Results

CNGC2 is required for optimal growth in elevated concentrations of Ca^{2+}

We first compared the growth responses of the wild-type with *cngc2-1* (*Col* ecotype) and *cngc2-2* (*WS* ecotype), both RNA-null mutants (Clough *et al.*, 2000; Chan *et al.*, 2003, 2008), to varying concentrations of Ca^{2+} in the medium. Plants were grown in a soil mix, and watered with one of the following solutions: distilled water, distilled water supplemented with 20 mM $CaCl_2$, and distilled water supplemented with 40 mM $CaCl_2$. When watered with distilled water, *cngc2* plants of both ecotypes were slightly shorter than the wild-type (Fig. S1a,b, see Supporting Information). Distilled water supplemented with either 20 mM or 40 mM $CaCl_2$ had no effect on the height of wild-type plants (Fig. S1c–f), but substantially affected both *cngc2* mutants. As shown in Fig. S1c–f, *cngc2* mutants were much shorter than wild-type plants in distilled water supplemented with 20 mM $CaCl_2$, and were extremely short in distilled water supplemented with 40 mM $CaCl_2$. Data on rosette leaf size and shoot mass also mirrored the height data presented above. Just as *cngc2-2* were shorter than *WS* plants in progressively higher Ca^{2+} growth conditions, they also had smaller rosette leaves and lower shoot mass (Fig. S1g,h).

The effect of Ca^{2+} on the fertility of *cngc2* mirrored that observed for the overall size of the plants. In distilled water, *cngc2-2* produced slightly shorter siliques that contained a reduced number of seeds compared with the wild-type (Fig. 1a,g; see the next section for the quantification of seed number). Distilled water supplemented with 20 mM and 40 mM $CaCl_2$ did not affect the silique length (or seed number) of wild-type plants (Fig. 1c,e). However, in distilled water supplemented with 20 mM $CaCl_2$, *cngc2-2* plants produced very short siliques (Fig. 1i). In distilled water supplemented with 40 mM $CaCl_2$, *cngc2-2* plants did not flower at all, and hence produced no siliques or seeds. Qualitatively similar results were observed for *cngc2-1* (data not shown). All the above phenotypes can be complemented by introducing a *35S CaMV:CNGC2* construct into *cngc2-2* plants via *Agrobacterium*-mediated transformation (Clough & Bent, 1998). *cngc2-2* plants carrying an ectopic copy of *35S CaMV:CNGC2* had the same overall size and silique length (and seed content) as wild-type plants, regardless of Ca^{2+} supplementation (Fig. S1a,c,e,and Fig.1k,m,o). These results show that CNGC2 is required for optimal vegetative growth (mature plant height, leaf size and shoot mass) and reproductive potential (seed development) in elevated levels of external Ca^{2+}. These results confirm previous reports that mutations in *CNGC2* cause plants to be diminutive and to have low fertility (for example, Clough *et al.*, 2000).

The *cngc2-2* mutant is sensitive to Ca^{2+}, but not to various other ions in normal growth medium. For instance, growth of the mutant (in plant height, rosette leaf size and shoot mass) in response to treatment with distilled water, or distilled water supplemented with 40 mM or 80 mM KCl, is not significantly different from the wild-type (Fig. S1g,h and data not shown, $P > 0.01$), implying that *cngc2-2* is not hypersensitive to K^+ or Cl^-. This is consistent with previous observations that both *cngc2-1* and *cngc2-2* mutants respond similarly to the wild-type to a range of concentrations of $MgCl_2$, KCl and NaCl (Chan *et al.*, 2003).

cngc2-2 plants are impaired in seed development

We then focused on the reduced fertility of *cngc2-2* and examined its impairment in seed development in detail. In distilled water, *cngc2-2* had shorter siliques than the wild-type, as described in the previous section. Dissected siliques showed gaps between normally developing seeds, as indicated by the asterisks in Fig. 1h (siliques from the wild-type plants had no gaps; see Fig. 1b). The gaps represent ovules that failed to develop into seeds. However, healthy seeds were interspersed throughout the length of the siliques (Fig. 1q), suggesting that at least some pollen tubes can extend to the end of the pistil and fertilize ovules there. When *cngc2-2* was treated with distilled water supplemented with 20 mM $CaCl_2$, very few or no healthy seeds were observed (Fig. 1j). This impairment in seed development in *cngc2-2* was completely reversed in plants that carried an ectopic copy of *CNGC2* (note that the seed sets in the wild-type over a

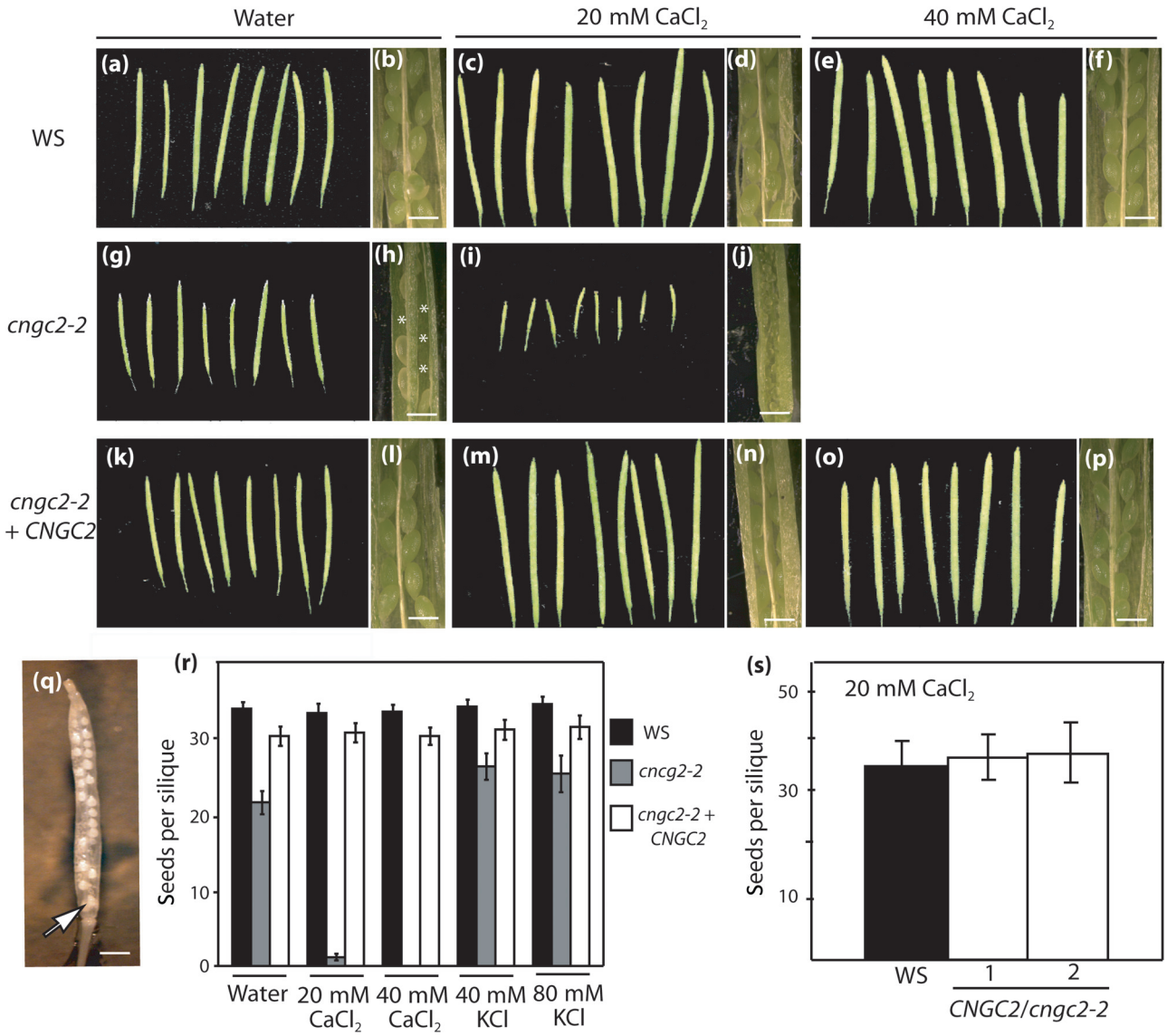


Fig. 1 *cngc2* plants have shorter siliques and lower seed yield. Arabidopsis plants were grown in a soil mix and watered with distilled water, or distilled water supplemented with $CaCl_2$ or KCl in the concentrations specified. Representative siliques from the primary stems of wild-type (a–f), *cngc2-2* (g–j) and *cngc2-2* plants carrying an ectopic copy of *CNGC2* (k–p) are presented. All images of whole siliques were taken under the same magnification, and some siliques were dissected open to show the seeds therein (bars, 0.25 mm). Asterisks in (h) indicate empty spaces inside mutant siliques. Under our experimental conditions, only *cngc2-2* showed a reduction in silique length and the number of seeds within when the external Ca^{2+} concentration was increased. In (q), a representative silique from *cngc2-2* plants watered with distilled water was dissected open and fixed. It showed that healthy seeds were distributed along the whole length of the silique (note healthy seeds, highlighted by the arrow, towards the bottom of the silique; bar, 2 mm). (r) Average number of seeds per silique from wild-type and *cngc2-2* plants grown under various conditions as indicated. As shown, mutant plants had significantly lower seed yield than wild-type plants under all growth conditions tested [$P < 0.005$, Student's *t*-test; error bars represent ± standard error (SE)], and this defect was completely reversed by ectopically expressing wild-type *CNGC2* in the *cngc2-2* mutant ($P > 0.01$). There was also no significant difference in seed number between *cngc2-2* plants grown under control (watered with distilled water) and high-KCl conditions ($P > 0.01$), showing that the mutant is specifically sensitive to Ca^{2+} and not to K^+ or Cl^-. (s) Average number of seeds per silique from wild-type and two *CNGC2/cngc2-2* plants watered with 20 mM $CaCl_2$, showing that the seed yield from these plants was indistinguishable (error bars are ± SE).

range of Ca^{2+} concentrations, illustrated by Fig. 1b,d,f, and the corresponding seed sets in the complemented *cngc2-2*, illustrated by Fig. 1l,n,p, are indistinguishable). Figure 1r shows the quantification of the significantly lower seed yield in *cngc2-2* compared with the wild-type, especially in high-calcium growth conditions. Whereas *WS* plants contained an average of *c.* 34 seeds per silique under all experimental conditions tested, siliques from *cngc2-2* contained *c.* 21–26 seeds on average when plants were watered with distilled water or distilled water supplemented with KCl (40 or 80 mM). The seed yield of

Table 1 Segregation of the *cngc2-2* mutant allele

(a) Genotypic analysis		
Wild-type	*cngc2-2* heterozygote	*cngc2-2* homozygote
40 (39.25)	85 (78.5)	32 (39.25)
(b) Phenotypic analysis		
Wild-type		Mutant
101 (100.5)		33 (33.5)

We analyzed the genotypes of progenies from self-pollinated *CNGC2/cngc2-2* plants that were watered with distilled water supplemented with 20 mM $CaCl_2$. Progenies were also treated with distilled water plus 20 mM $CaCl_2$. The genotypes of the progenies were determined by PCR. The number of plants of each of the three possible genotypes is given in (a), and the expected number according to normal Mendelian segregation is given in parentheses. Our results indicated normal segregation of the *cngc2* allele (chi-squared test, $P > 0.05$). We further confirmed this result through phenotypic analysis of progenies of such self-pollinated *CNGC2/cngc2-2* plants. The number of plants belonging to each of the two possible phenotypes (wild-type, tall plants with normal siliques; mutant, small plants with very short, empty siliques) is given in (b), and this result agrees with normal Mendelian segregation (chi-squared test, $P > 0.05$; the expected number of plants in each category is given in parentheses). Therefore, the result of our phenotypic analysis matches that from our genotypic analysis, indicating that transmission of the mutant allele is normal.

cngc2-2 plants dropped even more drastically when they were treated with distilled water supplemented with 20 mM $CaCl_2$ (*c*. one seed per silique on average), and they produced no seeds at all when the $CaCl_2$ concentration was increased to 40 mM.

To determine whether the failure in seed development in *cngc2* grown in high-Ca^{2+} conditions was the result of a defect in embryogenesis (i.e. whether embryos homozygous for the *cngc2* mutation are lethal or substantially less viable), we examined the seed yield of two self-fertilized *CNGC2/cngc2-2* plants watered with distilled water supplemented with 20 mM $CaCl_2$. We observed that siliques derived from *CNGC2/cngc2-2* were full of healthy seeds and, indeed, bore the same number of seeds as siliques from wild-type plants grown in parallel (Fig. 1s). This suggests that no significant defect in embryogenesis is associated with the *cngc2-2* mutation.

To evaluate possible male and female gametophytic defects in *cngc2-2*, we examined the transmission efficiency of the mutant allele when parental plants were treated with elevated levels of Ca^{2+}. *CNGC2/cngc2-2* plants were watered with distilled water supplemented with 20 mM $CaCl_2$, and their progenies from self-fertilization were genotyped by PCR using standard methods (Chan *et al.*, 2003). An expected ratio of 1 : 2 : 1 between the three possible genotypes (*CNGC2/CNGC2*, *CNGC2/cngc2-2* and *cngc2-2/cngc2-2*) was obtained (Table 1a). This result was also confirmed by phenotypic segregation analysis of the progenies from self-fertilized *CNGC2/cngc2-2* plants that were watered with distilled water supplemented with 20 mM $CaCl_2$. These progenies were also grown under the same high-Ca^{2+} condition. Tall plants with normal siliques were classified as the wild-type phenotype, and small plants with very short, empty siliques were classified as the mutant phenotype. A ratio of wild-type to mutant phenotypes of 3 : 1 was observed (Table 1b). Treating parental *CNGC2/cngc2-2* plants with distilled water yielded essentially the same result (data not shown). Our data indicate normal Mendelian segregation of the *cngc2-2* allele, regardless of the external Ca^{2+} concentration. Therefore, the undeveloped ovules in *cngc2-2* plants are not likely to be the result of a severe defect in male or female gametophyte development.

To evaluate a potentially more subtle gametophytic defect(s) in *cngc2-2*, reciprocal crosses between *cngc2-2* and wild-type plants were performed (Fig. 2a). The pollen and ovule donors were treated with distilled water or distilled water plus 20 mM $CaCl_2$. Success of the crosses was confirmed by PCR genotyping of the progenies and, as expected, all were heterozygous for *cngc2-2* (data not shown). When pollen from *cngc2-2* was deposited onto wild-type stigma, regardless of whether the *cngc2-2* plants were treated with distilled water or distilled water supplemented with 20 mM $CaCl_2$, full siliques of normal length were formed (Fig. 2a, bottom left panels in each quadrant; Fig. 2b, left panels). The resulting siliques were indistinguishable from those obtained from parallel crosses using wild-type pollen and stigmas (Fig. 2a, bottom right panels in each quadrant). This suggests that *cngc2-2* pollen is normal and fertile. To confirm this result, we examined pollen tube growth *in vitro*. Pollen from both wild-type and *cngc2-2* anthers, taken from plants treated with distilled water or distilled water plus 20 mM $CaCl_2$, resulted in normal pollen tube growth in all cases (Fig. S2a–d, see Supporting Information). We also checked *cngc2-2* pollen with Alexander staining (Alexander, 1969), and the results showed that mutant pollen was viable (data not shown). Therefore, *cngc2-2* pollen is viable and capable of forming pollen tubes *in vitro* as well as *in vivo*, and is unlikely to be a major contributor to the observed decrease in fertility in the mutant.

Results were very different when *cngc2-2* was used as the ovule donor. When *cngc2-2* plants were treated with distilled water and the resulting stigmas were deposited with wild-type pollen, shorter siliques containing gaps between developing seeds were formed, similar to those obtained from parallel crosses using *cngc2-2* pollen and pistils (Fig. 2a, top panels in left quadrant; Fig. 2b, top right panel). Using stigmas derived from *cngc2-2* plants that were treated with distilled water supplemented with 20 mM $CaCl_2$ resulted in the formation of very short and empty siliques, regardless of the source of pollen (Fig. 2a, top panels in right quadrant; Fig. 2b, bottom right panel). Results of this series of crosses suggest a possible defect(s) in the *cngc2-2* pistil. However, our current data indicate that the *cngc2-2* female gamete is viable (Table 1a,b). Therefore, we focused our investigation on other possible defects in the female reproductive organ that could result in reduced fertility of *cngc2-2* in the presence of elevated Ca^{2+}.

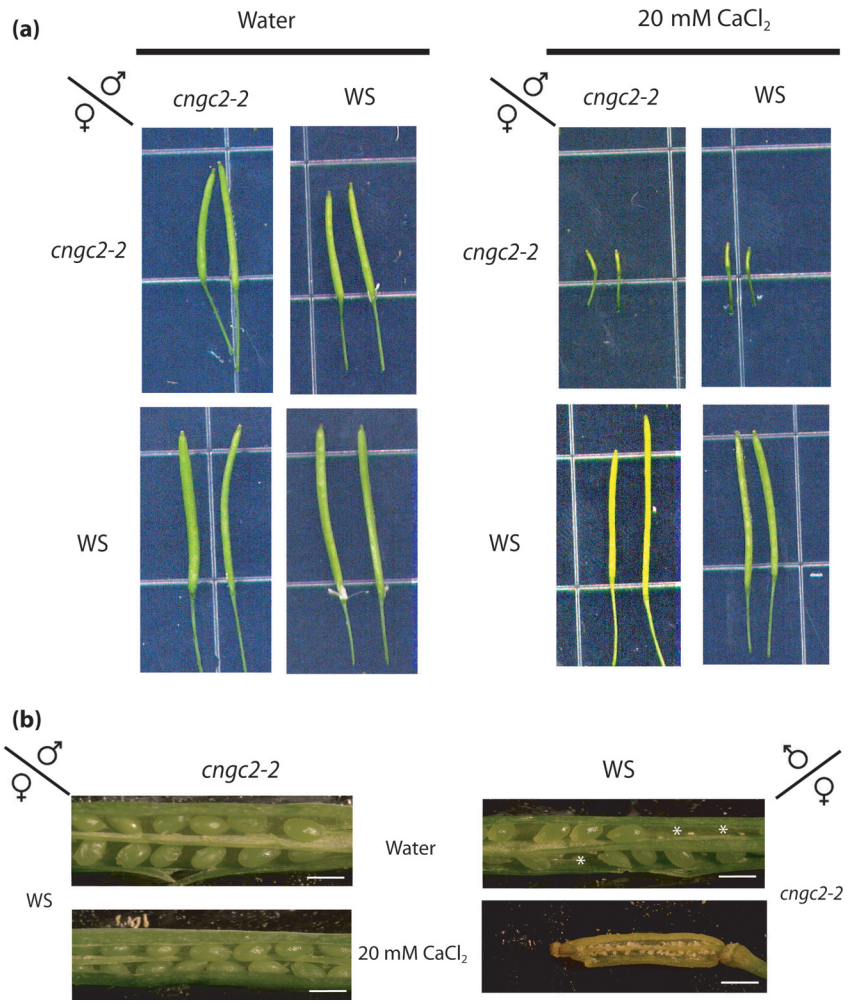


Fig. 2 *cngc2* pistils do not support cross-fertilization. Arabidopsis wild-type and *cngc2-2* plants watered with distilled water or distilled water supplemented with 20 mM $CaCl_2$ were used as pollen and ovule donors in self- and reciprocal crosses. Representative siliques are shown in (a). Opened siliques from reciprocal crosses as described above are shown in (b) (bar, 2 mm). When the ovule donor was from *cngc2-2* plants treated with distilled water, the resulting silique contained missing seeds (denoted by asterisks). When the mutant was treated with high Ca^{2+} and used as the ovule donor, the resulting cross always failed and did not yield any viable seeds.

Elevated Ca^{2+} results in suboptimal pollen deposition and pollen tube growth on *cngc2-2* pistils

To further analyze the fertility defect in *cngc2-2*, we examined pollen tube growth on pistils *in vivo*. Plants were treated with distilled water or distilled water supplemented with 20 mM $CaCl_2$, and developing siliques were collected 1 d after the flowers opened. They were then fixed and stained with aniline blue to observe the presence of pollen tubes. Wild-type and *cngc2-2* plants in distilled water, and wild-type plants in distilled water plus 20 mM $CaCl_2$, all developed long pollen tubes that reached ovules (Fig. 3a–c). However, *cngc2-2* plants in distilled water with 20 mM $CaCl_2$ did not exhibit any substantial pollen tube growth (Fig. 3d). The degree of pollen tube extension correlated with the amount of pollen grains interacting with stigmas in the mutant. When grown under the control condition (watered with distilled water), *cngc2-2* stigmas displayed pollen grain adhesion, as shown by both light microscopy and ESEM (Fig. 3e,f). When grown under an elevated Ca^{2+} condition (distilled water with 20 mM $CaCl_2$), very few, if any, pollen grains were deposited on *cngc2-2* stigmas (Fig. 3g,h). Therefore, under high-Ca^{2+} conditions, the mutant displayed two different defects: suboptimal pollen deposition and a decrease in pollen tube growth after successful pollen adhesion.

We then asked whether possible alterations in the timing of pistil maturation in *cngc2-2* could contribute to a failure in pollen deposition and subsequent growth. As *cngc2* mutants show changes in the regulation of programmed cell death (Yu *et al.*, 1998; Clough *et al.*, 2000, Chan *et al.*, 2003), we postulated that mutant flowers might exhibit early programmed cell death in their pistils that leads to an inhibition of pollen interaction and growth. If our hypothesis is true, younger flowers may be expected to be more receptive than older flowers in supporting pollen adhesion and pollen tube growth. To test this hypothesis, we crossed wild-type pollen with wild-type and *cngc2-2* emasculated flowers at different stages: the youngest open flower and two stages of progressively more immature flowers. We avoided using older flowers for this experiment, especially those from wild-type plants, as pollen deposition from mature stamens of the same flower may already have occurred and could confound our experimental results. All plants used were treated with distilled water supplemented with 20 mM

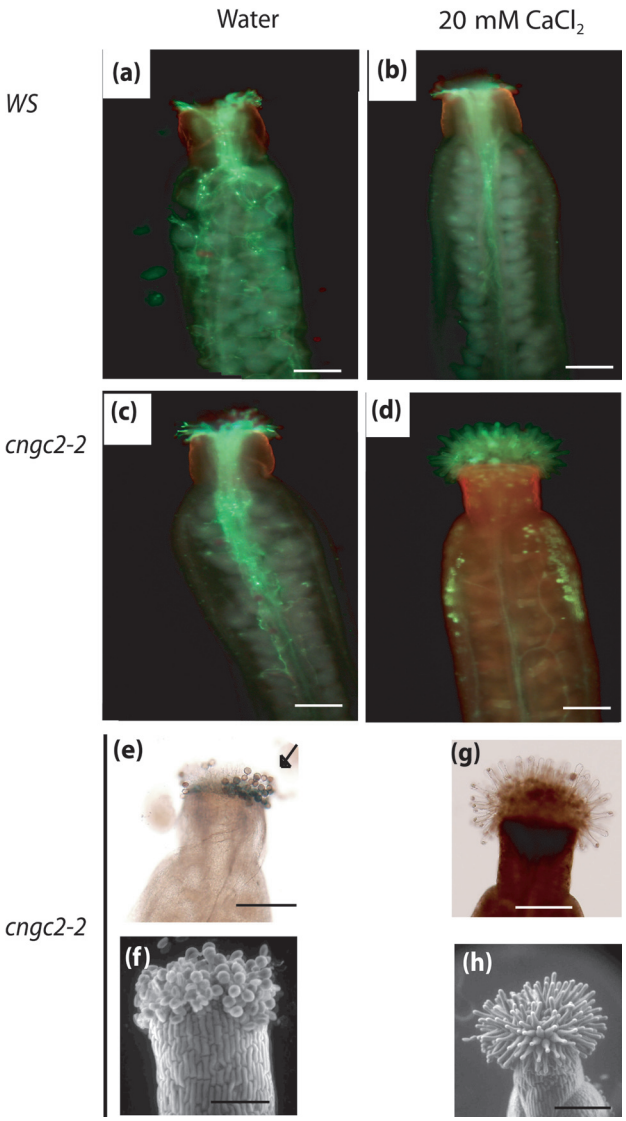


Fig. 3 *cngc2* plants show little pollen deposition and growth. Growth of pollen tubes in Arabidopsis wild-type (a, b) and *cngc2-2* (c, d) plants is shown. Pistils were obtained from plants watered with either distilled water (a, c) or distilled water supplemented with 20 mM $CaCl_2$ (b, d) (bars, 100 μm). One day after the flowers opened, the pistils were collected and stained with aniline blue. In these fluorescence micrographs, aniline blue-stained tissues, mainly pollen tubes, emitted strong fluorescence signals (bright green). Background signals were a result of autofluorescence (dull-red) from other tissues. These results indicated that *cngc2* plants grown in a high-Ca^{2+} environment sustained very little pollen tube growth. We also examined the stigmas of *cngc2-2* plants for pollen adhesion with light microscopy (e, g) and environmental scanning electron microscopy (ESEM; f, h) (bar, 100 μm). Pollen grains stained with aniline blue appeared blue in light micrographs (indicated by the arrow in e) and as small spheres on the stigmatic surface in ESEM images. Both techniques indicated that there was very little pollen deposition on stigmas when mutant plants were treated with elevated levels of Ca^{2+}.

$CaCl_2$ throughout the course of the experiment. Twenty-four hours after pollen was deposited, pistils were stained with aniline blue to visualize pollen tubes. We observed that, in crosses between wild-type pollen and pistils, proper pollen tubes were formed that extended into the ovules, regardless of the stage at which pistils were pollinated (Fig. 4a–c). In crosses using wild-type pollen and *cngc2-2* pistils of various stages, pollen deposition and growth of pollen tubes were observed. However, none of the pollen tubes grew sufficiently to reach the ovules (Fig. 4d–i). This was true even when pistils from more mature mutant flowers were used with wild-type pollen (data not shown). These results suggest that wild-type pollen can successfully interact with mutant stigmas and initiate growth, but later stages of pollen tube extension are severely curtailed. This is not likely to be a result of early initiation of programmed cell death or changes in developmental timing of *cngc2-2* pistils. Instead, conditions in the *cngc2-2* pistils seem to be unfavorable for pollen tube growth.

Elevated Ca^{2+} causes abnormal flower structures in *cngc2-2*

To ascertain whether other factors contributed to the decreased fertility in *cngc2-2*, we examined the structure of mutant flowers grown under control and elevated Ca^{2+} conditions. In normal flower development in *Arabidopsis*, an opened flower contains stamens that are generally slightly longer than the pistil. This allows the anther to release pollen onto the stigma, resulting in optimal pollination efficiency (Edlund *et al.*, 2004). When treated with distilled water, *cngc2-2* plants displayed relatively normal petals, pistils and stamens when compared with the wild-type (Fig. 5a,b; left panels). However, in distilled water supplemented with 20 mM $CaCl_2$, *cngc2-2* petals were shorter, causing the pistil to protrude out of a closed flower (Fig. 5a, bottom right panel). No such effects were observed in wild-type flowers grown in parallel (Fig. 5a, top right panel). Images obtained from ESEM showed that cells in *cngc2-2* petals were shorter than those in the wild-type (data not shown). However, *cngc2-2* pistils and the cells therein in both mature (open) and young (closed) flowers, regardless of external Ca^{2+} concentrations, were comparable in length with those from the wild-type (Fig. 5c, top panels in each quadrant and data not shown).

We next examined the length of stamens. When treated with distilled water, mature *cngc2-2* flowers contained stamens that were about as long or slightly longer than the pistils (Fig. 5b, bottom left panel), and the length of stamens was very similar to that of the wild-type (Fig. 5b, left panels; Fig. 5c, bottom panels in upper quadrant). However, in distilled water supplemented with 20 mM $CaCl_2$, *cngc2-2* flowers had stamens that were distinctly shorter than the pistils. This was particularly evident when we compared mature *cngc2-2* flowers with those from the wild-type (Fig. 5b, right panels; Fig. 5c, bottom panels in lower quadrant). ESEM images showed that the epidermal cells that make up the filaments in *cngc2-2* stamens were shorter

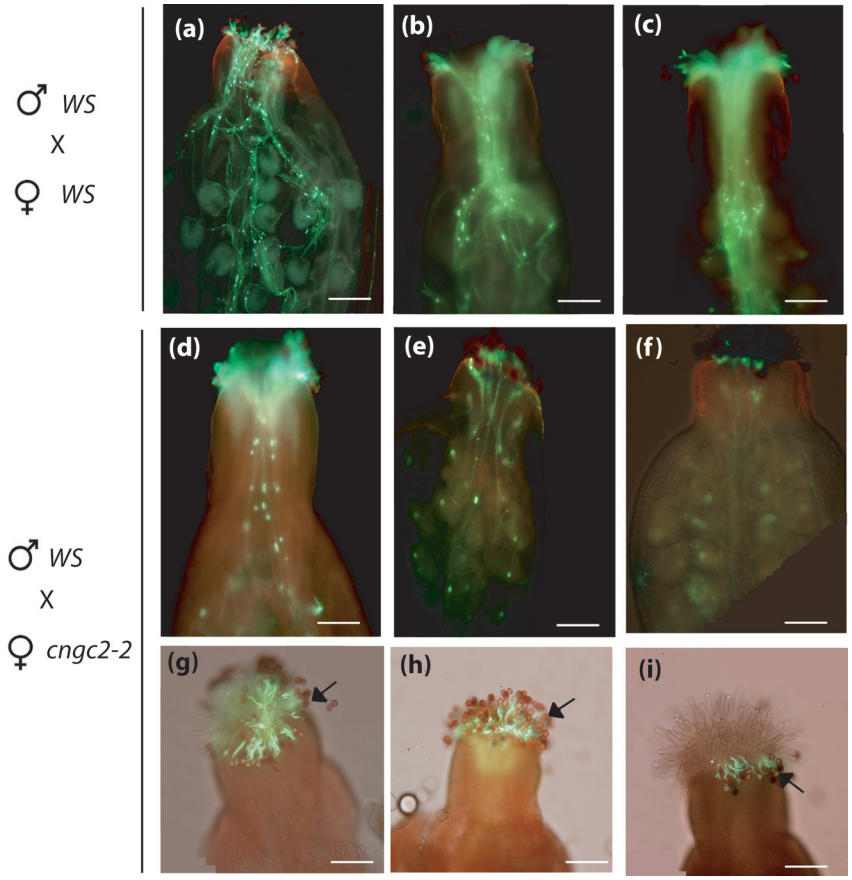


Fig. 4 *cngc2* pistils do not support pollen tube growth. We examined the growth of Arabidopsis wild-type pollen on wild-type and *cngc2-2* pistils. Pistils were from plants watered with distilled water supplemented with 20 mM $CaCl_2$. Wild-type pollen grains were transferred onto stigmas of different developmental stages: (1) flowers that had just opened (a, d, g); (2) flowers slightly younger than (1) (b, e, h); and (3) flowers substantially younger than (1) (c, f, i). Twenty-four hours after pollen transfer, flowers were fixed and stained with aniline blue. Fluorescence images showed that wild-type pollen tubes grew well on wild-type pistils of all stages examined (a–c, strong fluorescence signals came from pollen tubes; background signals were from autofluorescence of other plant tissues). By contrast, wild-type pollen tubes grew poorly on *cngc2-2* pistils and did not reach the ovules (d–f). Merged light and fluorescence microscopy images (g–i) showed that at least some wild-type pollen (highlighted by arrows) was capable of interacting with stigmas from *cngc2-2*, and limited pollen tube growth occurred. Bar, 200 μm. These results indicate that, even when *cngc2-2* stigmas received pollen grains, pollen tube growth was stymied and pollen tubes did not extend sufficiently to reach ovules to enable fertilization.

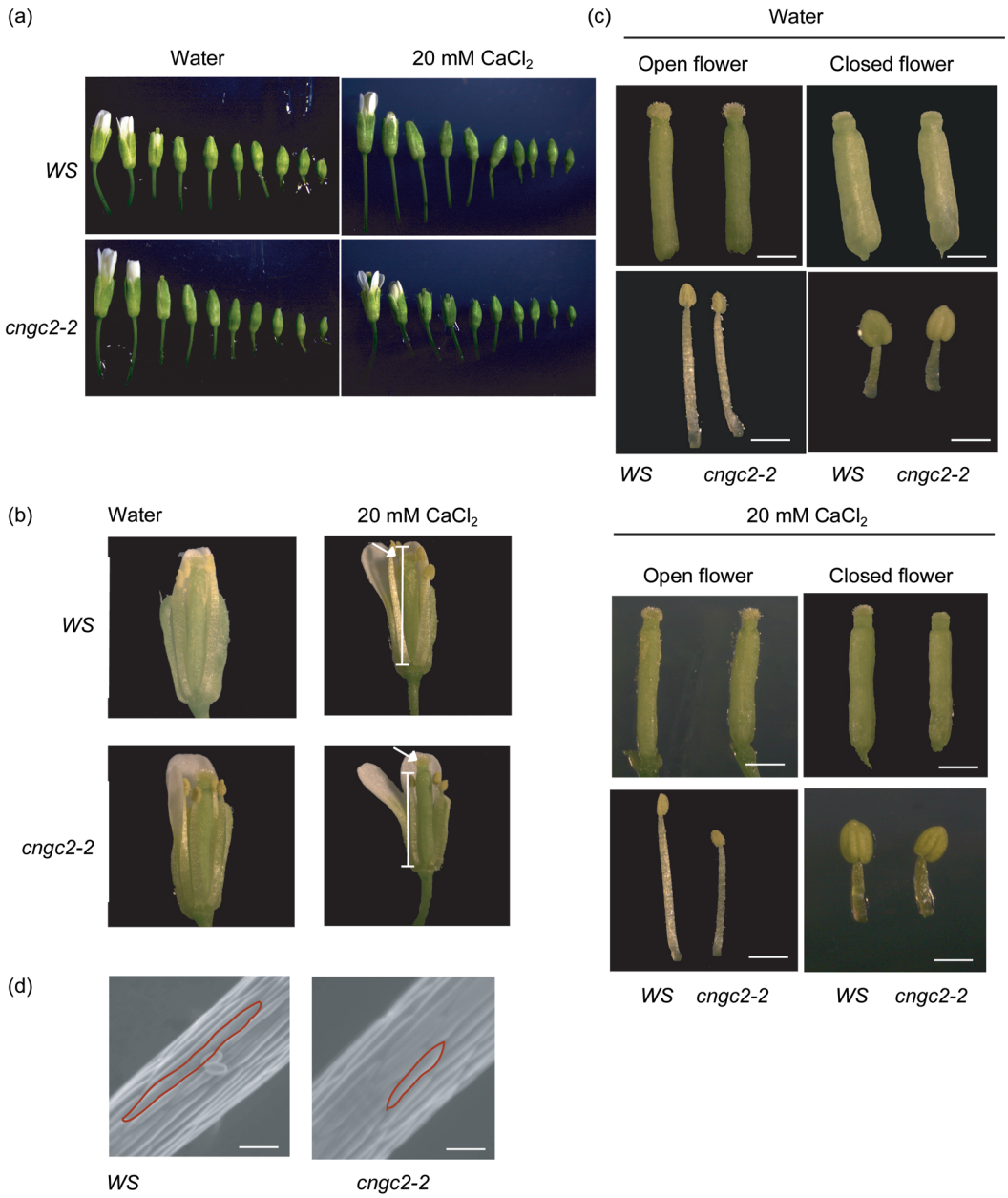
than those from the wild-type (Fig. 5d). A careful examination of mutant and wild-type flowers from plants exposed to high external Ca^{2+} revealed that a significant proportion (57%) of mature *cngc2-2* flowers contained short stamens, whereas no wild-type flowers exhibited this phenotype (Fig. 5e). As short stamens limit the amount of pollen that can interact with stigmas, this probably contributes to the reduction in seed yield in *cngc2-2*.

Discussion

CNGC2 is required for optimal plant growth at physiologically relevant levels of Ca^{2+}, ranging from low millimolar values to low tens of millimolar values. The growth phenotype of the *cngc2* mutant (reduction in plant size, silique length and seed yield) is seen in as low as ~1 mM external Ca^{2+} (concentration in our soil mix), and its severity increases as the Ca^{2+} concentration is progressively elevated to 40 mM. Although most soil solutions contain less than 2.5 mM Ca^{2+}, some reach much higher levels (< 0.4% has soil Ca^{2+} higher than 25 mM according to the work by Epstein, 1972). Importantly, *cngc2* mutants are specifically hypersensitive to environmental Ca^{2+}, but not other commonly encountered ions, such as K^+, Na^+, Mg^{2+} or Cl^- (Chan *et al.*, 2003). In this report, we also showed that *cngc2-2* plants did not display any hypersensitivity towards KCl up to 80 mM (Figs 1r, S1g,h), although this level of Cl^- may be toxic for some plant species (White & Broadley, 2001). Our working model is that CNGC2 is required for one or more Ca^{2+}-dependent signaling pathways that contribute to normal growth in high-Ca^{2+} environments (Chan *et al.*, 2003, 2008). This report extends our previous work and focuses on exploring the underlying reason(s) for the defect in fertility in *cngc2*.

Our data suggest that sporophytic, but not gametophytic, factors contribute to the reduction in seed yield in *cngc2-2*. The main defects are short stamens and a suboptimal pistil environment, which become progressively more severe as the concentration of Ca^{2+} in the medium increases. When *cngc2-2* plants were treated with distilled water only, we observed a small but noticeable decrease in stamen length and in the number of long pollen tubes in their pistils. These factors probably contribute to reduced seed yield in the mutant under control (i.e. low-Ca^{2+}) growth conditions. However, because of technical constraints, we did not quantify this observation. When *cngc2-2* plants were exposed to high-Ca^{2+} conditions, the reduction in stamen length (which most probably led to a reduction in the amount of pollen deposited) and the decrease in the number of long pollen tubes were very apparent (Figs 3–5).

Short stamens, relative to the length of the pistil, limit the amount of pollen that can be deposited onto the stigma of the



(e)

Genotype	Flowers with normal stamens	Flowers with short stamens
WS	100	0
cngc2-2	43	57

Fig. 5 *cngc2* plants have shorter petals and stamens in high external Ca^{2+}. Arabidopsis plants were treated with distilled water or distilled water supplemented with 20 mM $CaCl_2$, and various flower organs were examined. In (a), developmental series of flower buds, each taken from a single and comparable flower shoot of mutant and wild-type plants, are presented. It should be noted that pistils from *cngc2-2* plants treated with distilled water plus 20 mM $CaCl_2$ protruded out of closed flowers. Some sepals and petals were removed to reveal the height of stamens (white bar) relative to pistils (tip highlighted by arrow) in (b). In (c), we carefully compared the stamen and pistil length from wild-type and *cngc2-2* flowers of the same stage (bars, 0.25 mm). Our result clearly showed that, although the length of mutant pistils was relatively normal regardless of the growth condition, mutant stamens were substantially shorter when plants were exposed to high external Ca^{2+}. In (d), ESEM images of epidermal cells from the filaments of stamens isolated from plants treated with high Ca^{2+} are presented. For ease of comparison, representative cells from the wild-type and *cngc2-2* filament are outlined (bar, 50 μm). Our result suggests that mutant stamens are shorter because of the smaller cell size. A careful comparison between the stamen lengths of wild-type and *cngc2-2* plants treated with elevated levels of Ca^{2+} in (e) indicated that 57% of mutant flowers possessed short stamens, whereas no wild-type flowers exhibited this phenotype.

same flower, thereby reducing fertility in a self-fertilizing plant. *cngc2-2* plants grown in high-Ca^{2+} conditions have shorter cells in various tissues, including stamens, petals, leaf and stem epidermal cells (this report; A. Trow *et al.*, University of Wisconsin-Whitewater, Whitewater, WI, USA, unpublished), suggesting a defect in cell elongation in the mutant. Microarray data indicate that *CNGC2* mRNA is present at moderate to moderately high levels in the aforementioned tissues (Arabidopsis eFP browser, Winter *et al.*, 2007. This database only contains information on mature stamens but not their elongating cells, but it is possible that CNGC2 is localized there as well.). This raises the intriguing possibility that, in tissues in which CNGC2 expression is relatively high, one of its functions may be to regulate cell elongation. Correct Ca^{2+} signaling is known to be necessary for cell elongation. For example, cell elongation requires re-structuring of the actin-based cytoskeleton, and this process is regulated by the Ca^{2+}-dependent phosphorylation of actin-depolymerization factor protein (Allwood *et al.*, 2001). Perhaps in the aforementioned *Arabidopsis* tissues, Ca^{2+} influx through CNGC2, either directly or indirectly, helps to establish the prerequisite signal for cell elongation.

The reduction in fertility of *cngc2-2* is also a result of a suboptimal pistil environment for pollen tube growth. Independent of the source of pollen, using *cngc2-2* pistils in *in vivo* pollen growth assays, especially when plants were grown in high-Ca^{2+} environments, resulted in short pollen tubes. It should be noted that *cngc2-2* pollen is fundamentally normal, as indicated by results from *in vitro* pollen tube growth assays (Fig. S2) and genetic crosses with *WS* pistils (Fig. 2). One possibility is that mutant pistils lack (or are low in) growth-promoting factors for optimal pollen tube extension. Results from an initial semi *in vivo* pollen tube growth assay (Palanivelu & Preuss, 2006), where *WS* pollen was deposited on stigmas from *WS* or *cncg2-2* plants treated with 20 mM $CaCl_2$ before the stigmas were dissected off and placed in an agar pollen growth medium, showed that long pollen tubes were able to form on both *WS* and *cncg2-2* stigmas (Fig. S3a, see Supporting Information). This result suggests that *cngc2-2* pistils may indeed be deficient in pollen tube growth-promoting factor(s).

The exact identity of the pollen tube growth-promoting factor(s) suggested above is not known, but one possible candidate is sucrose, which is present in a high concentration in the pollen growth medium and can serve as an energy source for rapid pollen tube growth. The medium also contains a substantial amount of Ca^{2+}, which has been implicated as a chemotropic factor for pollen tubes. For example, successful pollination and pollen tube elongation have been associated with increases in cytoplasmic Ca^{2+} concentrations in the papilla cells at the site of pollen tube penetration in *Arabidopsis* (Iwano *et al.*, 2004), and Ca^{2+} in the pistil may be important in guiding and supporting pollen tube growth in various plant species (Lord, 2003). Although there is no evidence to show whether or how the loss of CNGC2 affects the level of sugar, Ca^{2+} or other pollen tube growth factors in pistils, microarray data support the expression of *CNGC2* in mature pistils and early silique development (Arabidopsis eFP browser, Winter *et al.*, 2007).

The result of the semi *in vivo* pollen growth experiment can also be explained by an alternative but nonmutually exclusive interpretation: *cngc2-2* pistils may contain an inhibitory factor that suppresses pollen tube extension. Instead of supplying a missing pollen tube growth-promoting factor(s), the synthetic medium may serve to remove or greatly dilute the putative inhibitory factor from the dissected stigma, and hence pollen tube growth is normal. Results from a preliminary experiment suggest that extract from *cngc2-2* pistils can indeed inhibit the growth of wild-type pollen (Fig. S3b), and lend support to the above hypothesis.

Apart from Ca^{2+}, cyclic adenosine monophosphate-dependent signaling has been shown to be important in pollen tube growth and reorientation (Moutinho *et al.*, 2001; Rato *et al.*, 2004). Several CNGCs also have exclusive or preferential expression in pollen (Bock *et al.*, 2006). Among them is CNGC18, a Ca^{2+}-permeable channel which is essential for polarized pollen tube growth (Frietsch *et al.*, 2007). By contrast, CNGC2 does not show high expression levels in pollen, a result supported by our reverse transcription PCR experiments (data not shown) and microarray data (Honys & Twell, 2004; Bock *et al.*, 2006), and *cngc2* mutant pollen is largely normal. Interestingly, if we examine the transcriptional profile of *cngc2-2* plants (Chan *et al.*, 2008), the expression of selected proteins specific or preferential to pollen is clearly impacted (PR1, Honys & Twell, 2003; WRKY46, Honys & Twell, 2004; ACA13, Bock *et al.*, 2006). The precise roles of these proteins in pollen tube growth are not clear, and we do not know whether their altered expression contributes to the observed defect in pollen tube growth in *cngc2-2*.

The *cngc2* mutant has constitutively high levels of SA that may explain some of its growth phenotypes. Previous work has shown that dwarfism of the mutant is partially, but not completely, a result of high concentrations of SA (Clough *et al.*, 2000). We observed that the defect in seed production was independent of SA. Experiments on transgenic lines (*cngc2-1* homozygotes/*nahG*$^+$; Clough *et al.*, 2000) and genetic mutants (*cngc2-1*/*sid2* double homozygotes; see Materials and Methods) indicated that, despite manipulations that effectively reduced SA in the mutant to near-normal or low levels, the reduction in seed yield under high-Ca^{2+} growth conditions persisted (Fig. S4, see Supporting Information). Therefore, functional CNGC2, but not SA level, is correlated with optimum seed development in calcified environments.

In this report, we focused our discussion on the relationship between CNGC2, Ca^{2+} signaling and effects on *Arabidopsis* fertility. However, CNGC2 can also mediate K^+ influx in several heterologous systems (Leng *et al.*, 1999, 2002). We cannot completely exclude the possibility that the reduction in seed production in *cngc2* is a result of defects in K^+ uptake that are induced/exacerbated by high external Ca^{2+}. However, current data strongly support a link between Ca^{2+} flux, CNGC2 function

and various physiological processes (see Introduction). Furthermore, there is no evidence for K^+ deficiency in *cngc2* in any published work (Hampton *et al.*, 2005; see also Discussion in Chan *et al.*, 2008). Consistent with previous transcriptional data on *cngc2-2* (Chan *et al.*, 2008), which suggest changes in the expression of various signal transduction genes in the mutant, our current working model is that alterations in a CNGC2-dependent signaling pathway(s), either directly or indirectly, lead to the fertility defects reported here.

In summary, the function of CNGC2 is required for optimum seed yield, especially in high-Ca^{2+} growth environments. Our results suggest the potential convergence and interplay between cyclic nucleotide and Ca^{2+} signaling through a Ca^{2+}-permeable CNGC, and their importance in regulating plant growth and reproduction. Future studies clarifying the localization of CNGC2 by an *in situ* hybridization technique, and characterizing potential alterations in internal Ca^{2+} concentrations of the *cngc2* mutant using fluorescent calcium reporters targeted to different intracellular compartments and tissues (Palmer & Tsien, 2006), may help to elucidate how CNGC2 affects plant development and fertility.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 *cngc2* plants are smaller than the wild-type when grown in high-Ca^{2+} conditions.

Fig. S2 Pollen from *cngc2* plants displays normal germination and growth *in vitro*.

Fig. S3 *cngc2* pistils are suboptimal for pollen tube growth.

Fig. S4 *cngc2* plants with normal/low salicylic acid (SA) concentrations still have poor seed yield.

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