A Cyclic Nucleotide-Gated Channel (CNGC16) in Pollen Is Critical for Stress Tolerance in Pollen Reproductive Development[1][W][OA]

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Cyclic nucleotide-gated channels (CNGCs) have been implicated in diverse aspects of plant growth and development, including responses to biotic and abiotic stress, as well as pollen tube growth and fertility. Here, genetic evidence identifies CNGC16 in Arabidopsis (*Arabidopsis thaliana*) as critical for pollen fertility under conditions of heat stress and drought. Two independent transfer DNA disruptions of *cngc16* resulted in a greater than 10-fold stress-dependent reduction in pollen fitness and seed set. This phenotype was fully rescued through pollen expression of a *CNGC16* transgene, indicating that *cngc16-1* and *16-2* were both loss-of-function null alleles. The most stress-sensitive period for *cngc16* pollen was during germination and the initiation of pollen tube tip growth. Pollen viability assays indicate that mutant pollen are also hypersensitive to external calcium chloride, a phenomenon analogous to calcium chloride hypersensitivities observed in other *cngc* mutants. A heat stress was found to increase concentrations of 3′,5′-cyclic guanyl monophosphate in both pollen and leaves, as detected using an antibody-binding assay. A quantitative PCR analysis indicates that *cngc16* mutant pollen have attenuated expression of several heat-stress response genes, including two heat shock transcription factor genes, *HsfA2* and *HsfB1*. Together, these results provide evidence for a heat stress response pathway in pollen that connects a cyclic nucleotide signal, a Ca^{2+}-permeable ion channel, and a signaling network that activates a downstream transcriptional heat shock response.

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The reproductive phase in flowering plants can be highly sensitive to hot or cold temperature stresses. Even a single hot day or cold night can sometimes be fatal to reproductive success. Pollen development and fertilization are often the most temperature-sensitive part of reproductive development (Zinn et al., 2010). A heat stress response in pollen, like vegetative tissues, involves changes in gene expression, including increased mRNA levels for heat shock transcription factors (e.g. HsfA2) and heat shock proteins (e.g. Heat Shock Protein17-CII and Thermosensitive Male Sterile1; Frank et al., 2009; Yang et al., 2009; Giorno et al., 2010). Nevertheless, the signaling pathways underlying these responses remain poorly understood, especially in pollen development.

Cyclic nucleotide monophosphates (cNMPs) 3′,5′-cyclic guanyl monophosphate (cGMP) and cAMP play key roles in the regulation of diverse cellular processes in eukaryotes and prokaryotes (Jammes et al., 2011), including biotic and abiotic stresses. In plants, cNMPs have been implicated in pathogen responses and salt and osmotic stresses (Jammes et al., 2011; Li et al., 2011; Ma and Berkowitz, 2011; Moeder et al., 2011).

Recently, cNMPs have also been linked to heat stress responses in vegetative tissues from Arabidopsis (*Arabidopsis thaliana*) and the moss *Physcomitrella patens* (Finka et al., 2012; Gao et al., 2012). In these cases, genetic and electrophysiological evidence suggest that cNMPs activate cyclic nucleotide-gated ion channels (CNGCs).

CNGCs are Ca^{2+}-permeable cation transport channels that are activated by cNMPs and deactivated by binding Ca^{2+}/calmodulin (Cukkemane et al., 2011; Ma and Berkowitz, 2011; Spalding and Harper, 2011). They have been identified in both plant and animal systems (Schuurink et al., 1998; Köhler and Neuhaus, 2000; Becchetti et al., 2009; Zelman et al., 2012). In plants, CNGCs have a binding site for calmodulin that overlaps with a site for cNMP (Köhler et al., 1999). Thus, CNGCs have the potential for integrating signals from cyclic nucleotide and Ca^{2+} signaling pathways (Newton and Smith, 2004).

Arabidopsis contains 20 *CNGC* family members (Mäser et al., 2001) that are differentially expressed in all tissues (Talke et al., 2003). CNGC18 has been shown to provide an essential function in pollen tube tip growth (Frietsch et al., 2007). This is consistent with pharmacological evidence that cyclic nucleotide signals in pollen can trigger growth-altering Ca^{2+} signals (Moutinho et al., 2001; Rato et al., 2004; Wu et al., 2011). Here, we show that CNGC16, another pollen expressing CNGC, is critical for heat stress tolerance, providing a link between a stress-triggered cNMP signal and a downstream transcriptional heat shock response.

RESULTS

cngc16 Disruptions Show a Stress-Dependent Segregation Distortion

To identify genes involved in stress tolerance, we initiated a screen for transfer DNA (T-DNA) mutations that showed a stress-dependent segregation distortion under conditions of hot days and cold nights (Fig. 1B). For in vivo pollen tube growth in Arabidopsis, approximately 2 h is required for the first pollen tubes to grow through the stigma and enter the ovary and about 4 to 5 h for tubes to traverse 50% of the distance to the bottom of the ovary (Schiøtt et al., 2004; Crawford et al., 2007). The stress conditions chosen here were designed to provide only a small window of optimal growth conditions to force pollen to cope with a stress condition in order to complete a fertilization event. The daily cycling between hot and cold conditions allowed plants to be grown under the same stress regime for their entire life cycle, whereas a continuous exposure to either the hot or cold stress would have been lethal. The stress cycle used was found to reduce seed set in wild-type siliques by nearly 2-fold (Fig. 1C; Supplemental Fig. S1), providing an optimal stress condition in which to screen for mutants that either increased or decreased reproductive stress tolerance.

A stress-dependent decrease in transmission was observed (Table I) for two independent T-DNA insertions in a gene encoding cyclic nucleotide-gated channel16 (*CNGC16*; Fig. 1A).

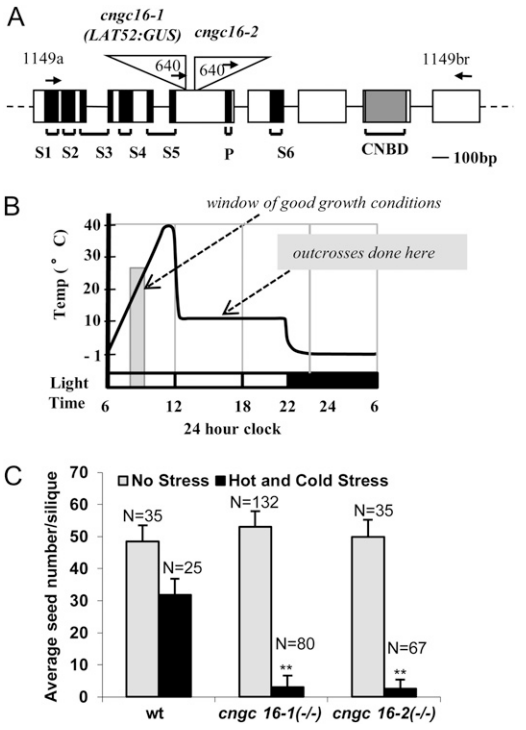


Figure 1. Knockout mutations for *cngc16* and corresponding stress-dependent seed set phenotype. A, Schematic diagram of *CNGC16* gene model (AT3G48010) and T-DNA insertion sites in *cngc16-1* and *cngc16-2*. Positions are shown for T-DNA insertions (triangles), exons (rectangles), introns (lines), and primers (arrows). Black regions represent transmembranes (S1–S6) and pore (P) domains in the corresponding protein. Gray shading represents the cyclic nucleotide binding domain (CNBD). B, Schematic diagram of the hot day and cold night stress cycling from −1°C to 40°C and forcing the period of pollen tube growth and fertilization to overlap with suboptimal temperatures. C, Seed set analysis of *cngc16* shows a near-sterile phenotype under the hot/cold stress conditions diagrammed in B. *n*, Number of siliques counted. Student's *t* test was performed to detect significant differences between *cngc16* mutants and the wild type (wt) under hot and cold stress. **Student's *t* test significant at $P < 0.01$.

Both insertions were associated with a T-DNA harboring a Basta resistance marker (Sessions et al., 2002). When plants heterozygous for *cngc16-1*(−/+) and *16-2* (−/+) were allowed to self-fertilize under conditions of hot days and cold nights, the Basta marker showed an approximately 50% transmission instead of an expected 75% ($n = 720$, $P < 0.001$; Table I). This distortion was confirmed in a subset of progeny by PCR genotyping of *cngc16-1* and *cngc16-2* T-DNA insertion site borders ($n = 61$, $P < 0.01$). In contrast, plants grown under standard nonstress conditions showed segregation very close to the expected 75% (e.g. 69%–73%, $n = 536$).

To determine if a heat stress alone was sufficient to reduce transmission of the *cngc16* T-DNA insertions, heterozygous mutants were transferred into a heat stress chamber (32°C or 37°C) for 4 to 6 d while flowering and then returned to normal conditions for seed maturation. Flower meristems were pruned to

Table I. *Segregation analysis showing non-Mendelian transmission of the T-DNA in cngc16 mutants under various stresses*

cngc16-1(+/−) and *cncg 16-2*(+/−) were self-pollinated under no stress, hot/cold (hot days and cold nights; Fig. 1B), 32°C, 37°C, or drought stresses. Statistical significance was determined by Pearson's χ^2 test.

Allele (−/+)	Selfing Condition	F1Total	Segregation of Basta Resistance Marker		
			Expected	Observed	*P* Value
			%		
cngc16-1	No Stress	282	75	73	<0.700
cngc16-2	No Stress	254	75	69	<0.300
cngc16-1	Hot/Cold	380	75	49	<0.001
cngc16-2	Hot/Cold	340	75	52	<0.001
cngc16-1	32°C	323	75	53	<0.001
cngc16-2	32°C	945	75	52	<0.001
cngc16-1	37°C	369	75	52	<0.001
cngc16-2	37°C	213	75	48	<0.001
cngc16-1	Drought	104	75	48	<0.001
cngc16-2	Drought	390	75	48	<0.001

leave only siliques that resulted from self-fertilization events that occurred during the stress period. Seed from these siliques were then harvested and used to determine the transmission frequency of T-DNA mutations to F1 progeny conceived only during the limited stress period. The heat stress alone resulted in an equivalent segregation distortion to that observed under a hot day/cold night stress regime (Table I).

To determine if a drought stress could also result in a segregation distortion, heterozygous plants were subjected to a period of severe drought and then rescued through restored watering. As the drought stress became more severe, the resulting siliques became shorter and contained fewer seeds. To focus only on F1 progeny whose reproductive origins were actually impacted by the period of drought stress, seeds were selectively harvested from the shortest siliques, which contained progeny conceived during the most severe stages of the drought stress. These progeny showed a segregation distortion equivalent to that observed with a hot day/cold night stress regime (Table I).

cngc16 Mutations Result in a Stress-Dependent Pollen Defect

To determine whether the observed stress-dependent segregation distortion in self-fertilized plants was due to a defect in either the male or female gametophytes, we conducted reciprocal crosses between *cngc16*(+/−) mutants and plants that were either wild type or male sterile (*ms1-1*). When *cngc16* heterozygotes were used as females, a normal 50% transmission frequency was observed with or without stress conditions (Table II, groups A and E). In contrast, *cngc16* pollen transmission under stress conditions was reduced to 1% to 2.4% (Table II, groups B and D). This indicated that *cngc16* pollen alone had a stress-dependent defect in transmission.

To determine which aspects of *cngc16* pollen are stress sensitive, plants were exposed to stress either before and/or after a cross. When pollen were allowed to develop under a stress of hot days and cold nights and then outcrossed and allowed to fertilize under nonstress conditions, transmission efficiency dropped approximately 1.5-fold from 31% observed for control conditions down to 21% (Table II, group C). For these crosses the expected transmission efficiency was considered to be 31%, based on our nonstress control conditions. This slight decrease in transmission for the nonstress control (i.e. from 50% down to 31%) is likely due to manual pollination resulting in multiple stresses, such as wounding from brushing pollen onto the stigma, in addition to a low humidity stress that is common in dry climates when a growth chamber door is opened to perform a cross.

The largest relative decrease in *cngc16* transmission efficiency occurred when pollen from a heterozygous *cngc16* mutant (grown without stress) was used to pollinate an *ms1-1* female, and the outcrossed plant moved into the stress chamber within 30 min. Under these conditions, the *cngc16* transmission efficiency was reduced more than 10-fold, down to 1.2% to 2.4% (31% expected; Table II, group B). However, if the stress exposure was delayed by 1 to 2 h, the transmission efficiency was less severe, with only a 1.5-fold decrease similar to that observed when pollen were stressed only during maturation (Table II, group G). This bracketing indicates that while mutant pollen were still sensitive to stress during grain maturation or tube growth, they were most sensitive during the first 1 to 2 h postpollination, corresponding to germination and early tube growth into the stigma (Crawford et al., 2007).

Homozygous *cngc16* Mutants Have Reduced Seed Set under Conditions of a Hot/Cold Stress

Two possible types of pollen defects could explain a stress-dependent segregation distortion: (1) A reduction

Table II. *Segregation analysis showing a stress-dependent defect in cngc16 pollen transmission*

Transmission efficiencies are shown for reciprocal crosses between *cngc16-1*(–/+), *cngc16-2*(–/+), and wild-type (WT) plants or *ms1-1* plants under various conditions. All outcrosses in which pollinated plants were moved to a stress chamber were done between 2 and 5 PM (10°C) on the stress cycle shown in Figure 1. "Pre" and "Post" refer to application of stress to the female recipient or pollen before or after the manual cross. All post-cross stress treatments occurred within 30 min of the cross unless otherwise indicated. Statistical significance was determined by Pearson's χ^2 test.

Group	Hot/Cold Stress Regime		Female × Male	F1 Total	Segregation of Basta Resistance Marker		
	Pre	Post			Expected	Observed	*P* Value
					%		
A							
Female	−	−	*cngc16-1*(–/+) × WT	285	50	48	<0.7
			cngc16-2(–/+) × WT	156	50	50	1
Pollen	−	−	WT × *cngc16-1*(–/+)	490	50	33	<0.001
			WT × *cngc16-2*(–/+)	792	50	31	<0.001
B							
Female	−	+	*ms1-1* × *cngc16-1*(–/+)	602	50	2.4	<0.001
Pollen	−	+	*ms1-1* × *cngc16-2*(–/+)	162	50	1.2	<0.001
C							
Female	−	−	WT × *cngc16-1*(–/+)	170	50	21	<0.001
Pollen	+	−	WT × *cngc16-2*(–/+)	40	50	20	<0.01
D							
Female	−	+	*ms1-1* × *cngc16-2*(–/+)	96	50	1	<0.001
Pollen	+	+					
E							
Female	+	+	*cngc 16-1*(–/+) × WT	97	50	48.4	<0.95
Pollen	−	+					
F							
Female	+	+	*ms1-1* × *cngc16-2*(–/+)	97	50	2.1	<0.001
Pollen	+	+					
G							
Female	−	+[a]	*ms1-1* × *cngc16-1*(–/+)	458	50	20.6	<0.001
Pollen	−	+[a]	*ms1-1* × *cngc16-2*(–/+)	427	50	22.6	<0.001

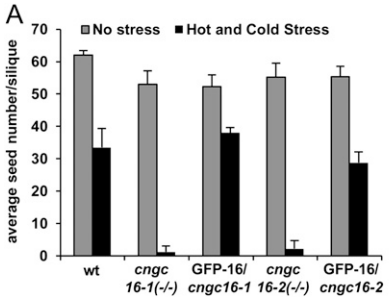
[a]Indicates a 1-h delay before crossed plants were transferred to the stress chamber.

in competitiveness compared with the wild type, for example, from a slightly slower rate of pollen grain germination or tube growth; or (2) complete sterility, for example, from death or the inability to grow or discharge sperm cells. To help distinguish between these two alternatives, we grew homozygous mutants under conditions of hot days and cold nights and quantified the number of seeds per silique (Fig. 1C; Supplemental Fig. S1). For both *cngc16-1*(−/−) and *cngc16-2*(−/−) mutants, we observed a stress-dependent 10-fold decrease in seed set. This indicates that there is stress-dependent transmission defect even in the absence of competition with wild-type pollen. This is consistent with a model in which the mutant pollen show a stress-dependent lethality, as opposed to slightly slower rate of pollen grain germination or tube growth.

Expression of GFP-CNGC16 Rescues the *cngc16* Stress-Dependent Phenotype

To determine if pollen expression of a GFP-tagged CNGC16 could rescue the stress dependent phenotypes, a transgene encoding a GFP-CNGC16 was stably transformed into homozygous *cngc16-1* and *cngc16-2* backgrounds. The *CNGC18* promoter was used for relatively weak levels of pollen expression (Frietsch et al., 2007), whereas an *ACA9* promoter was used for strong expression (Schiøtt et al., 2004). The *ACA9* promoter was previously used with a parallel construct to drive the expression of a GFP-CNGC18, which provided sufficiently high expression levels to allow the GFP-GNCG18 to be visualized in pollen (Frietsch et al., 2007). However, for both GFP-CNGC16 rescue constructs, we were unable to detect a GFP signal, suggesting that mRNA or protein expression levels were kept very low, despite the use of a relatively strong *ACA9* promoter. Nevertheless, both rescue constructs were observed to restore seed set to levels equivalent to the wild type (approximately 35 seeds) grown under parallel stress conditions (Fig. 2A).

In addition to rescuing the seed set phenotype, the *CNGC16* transgene also rescued the stress-dependent segregation distortion phenotype. This was observed in a competition assay in which 50% of the mutant pollen harbored a rescue construct (i.e. *cngc16*−/− with a hemizygous transgene). The transmission frequency of the rescue construct was assayed using the associated hygromycin marker. In the absence of stress, a normal 50% transmission efficiency was observed for a pollen outcross, indicating that under control conditions, all pollen were equally competitive (Fig. 2B). In contrast, under conditions of a hot/cold



B

Female X Male	F1 Total	Segregation of Basta resistance marker: Exp (%)	Obs (%)	p value
No Stress				
GFP-16(+/-)/16-1(-/-) **X** 16-1(-/-)	308	50	48	<0.7
GFP-16(+/-)/16-2(-/-) **X** 16-2(-/-)	279	50	52	<0.7
Hot and Cold Stress				
ms1-1 **X** GFP-16(+/-) / 16-1 (-/-)	17	50	100	<0.001
ms1-1 **X** GFP-16(+/-) / 16-2 (-/-)	91	50	100	<0.001

Figure 2. Seed set and segregation analysis showing the rescue of the male sterile phenotype. A, Seed set analyses of *cngc16* knockouts rescued with a *CNGC18p(i)-GFP-CNGC16* construct [seed stock nos. 1646 and 1647 for *cngc16-1*(−/−) and *cngc16-2*(−/−) backgrounds, respectively] showing seed set levels equivalent to the wild type (wt) under a hot and cold stress regime (siliques counted = 5). B, Outcrosses with representative rescue lines seed stock numbers 1646 and 1647 [for *cngc16-1* (−/−) and *cngc16-2*(−/−) backgrounds, respectively]. The rescue construct *CNGC18p(i)-GFP-CNGC16* was hemizygous in all crosses. A rescue experiment was repeated with similar results using two additional lines harboring the same GFP-CNGC16 under the control of the *ACA9* pollen promoter (seed stock nos. 1648 and 1649; data not shown). For crosses done with a hot/cold stress, the female had a *ms1-1* phenotype. After a manual fertilization, the plants were moved to a hot day/cold night stress chamber, with the entry time at approximately 3 PM and temperature at 10°C (see Figure 1B). Statistical significance was determined by Pearson's χ^2 test.

stress, the only pollen transmission observed was for mutant pollen harboring *GFP-CNGC16* ($n = 108$, $P < 0.001$). Together, the observation that the stress-dependent segregation distortion and reduced seed set phenotypes could be rescued using a pollen-expressed GFP-CNGC16 corroborates that *cngc16-1* and *cngc16-2* represent loss-of-function null mutations.

The Viability of *cngc16* Pollen Can Be Reduced by Temperature Stress or Elevated $CaCl_2$

Alexander staining was used as an assay to evaluate pollen viability in response to environmental conditions. In pollen isolated from plants grown under a stress of hot days and cold nights, pollen from homozygous *cngc16* mutants were twice as likely to be dead compared with wild-type controls (Fig. 3; Supplemental Fig. S2).

In an attempt to use an in vitro pollen growth assay to study the *cngc16* phenotype, we observed that less than 1% of *cngc16* mutant pollen grew tubes longer than 200 μm. Instead, more than 80% of the pollen grains either failed to germinate, arrested as short pollen tubes (i.e. <30 μm), or ruptured (Fig. 4).

To determine if a specific component of the pollen germination medium was responsible for the observed developmental block, we examined the effects of varying concentrations of $CaCl_2$ from 0 to 10 mM in a standard liquid germination medium. After 3 h, the viability of pollen grains was assayed using Alexander's stain (Fig. 5). At 10 mM $CaCl_2$, *cngc16* pollen showed a more than 2-fold increase in pollen death compared with the wild type. This increase in lethality also occurred with 10 mM $CaCl_2$ in a Tris-MES buffer, suggesting that additional nutrient components normally present in our standard germination medium were not necessary for the hypersensitivity to $CaCl_2$.

The Induction of Key Thermotolerance Genes Is Impaired in *cngc16* Pollen

To elucidate the underlying cause of the *cngc16* stress sensitivity, we performed real-time quantitative reverse transcription (RT)-PCR analyses on a subset of genes previously identified as stress markers (Fig. 6). As an example of a nonresponsive marker, *Zat12* failed to show any temperature-dependent changes in mutants or the wild type. In contrast, stress-induced changes were observed for two HSF genes, *HsfA2* and *HsfB1*. While both *HSF*s still showed a weak stress-induced increase in *cngc16-1* and *cngc16-2* backgrounds, their induction was 2- to 4-fold lower compared with wild-type controls. Similarly, a downstream gene under the control of *HsfA2* was also assayed (*BCL-2-associated athanogene6* [*Bag6*], At2g46240; Nishizawa-Yokoi et al., 2009) and found to have a 2- to 7-fold lower induction

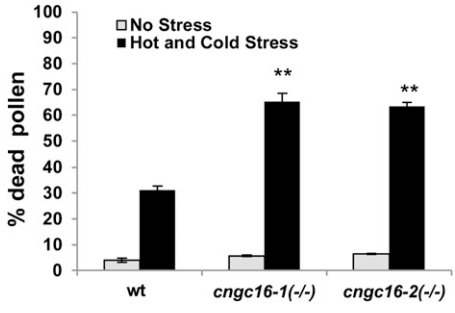


Figure 3. Viability staining showing *cngc16* mutants are hypersensitive to stress. Viability was assayed using Alexander's reagent. Pollen were harvested from plants growing under conditions of hot days and cold nights (see Figure 1B). Values represent means ± SD of three independent experiments (n = 50–100 pollen grains for each experiment). Student's t test was done to compare the pollen viability of *cngc16* mutants with wild-type (wt) plants grown under a hot and cold stress regime. **Student's t test significant at $P < 0.01$.

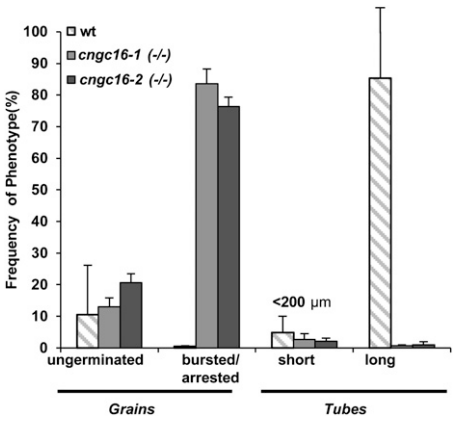


Figure 4. *cngc16* pollen show poor growth and bursting when germinated in vitro. Pollen grains were harvested from plants grown under normal conditions. Germination and growth were allowed to proceed for approximately 12 h on a standard in vitro agar-based growth medium containing 1 mM $CaCl_2$. Values represent means ± SD of three to five independent experiments, each with approximately 200 pollen grains. wt, Wild type.

compared with the wild type. It is noteworthy that the *BAG6* induction was more impaired in the *cngc16-1* mutant background than *cngc16-2*, which might indicate that lines harboring *cngc16-1* and *cngc16-2* have functional differences. At a minimum, the *cngc16-1* line includes an additional quartet mutation that was included in subset of lines generated for the Syngenta Arabidopsis Insertion Library T-DNA knockout collection (Alonso et al., 2003). The quartet mutant has a defect that alters the pollen cell wall and keeps the four meiotic products physically linked together in a tetrad (Rhee and Somerville, 1998). Regardless of potential modifiers in *cngc16-1*, both mutants show an impaired stress-dependent transcriptional response.

Heat Stress Increases cGMP Levels

To determine if a heat stress can trigger a rise or fall in the level of a cyclic nucleotide, we attempted to quantify levels of cAMP and cGMP in pollen with and without a heat stress. To detect cNMPs, we used antibodies that could distinguish between cAMP and cGMP. Unfortunately, we were unable to detect cAMP above background levels, leaving us to estimate that its concentration in pollen grains and leaves is less than 74 pmol g^{-1} dry weight. However, we were able to detect cGMP, at 13.65 pmol g^{-1} dry weight in leaves and 0.019 pmol g^{-1} dry weight in pollen and observed a small but statistically significant heat stress-dependent increase in both leaves and pollen (Fig. 7).

DISCUSSION

Genetic evidence presented here identifies CNGC16 in Arabidopsis as critical to reproductive success under conditions of heat stress or drought (Table I). Two independent T-DNA disruptions of *cngc16* (Fig. 1A) resulted in stress-dependent reductions in pollen fitness (Table II) and seed set (Fig. 1C). This phenotype was fully rescued through pollen expression of a *CNGC16* transgene (Fig. 2), indicating that *cngc16-1* and *cngc16-2* were both loss-of-function null alleles. *CNGC16* is expressed primarily in pollen (Fig. 8), which is consistent with a stress-dependent phenotype associated with pollen transmission. In contrast, there was no detectable transmission deficiency through the female gametophyte (Table II, group E).

Germination of *cngc16* Pollen Grains Is Highly Sensitive to Environmental Conditions

The most stress-sensitive period for *cngc16* pollen was observed as a 10-fold decrease in transmission efficiency when the stress environment was introduced just after a manual pollination (Table II). When the stress exposure was limited to prepollination, there was only a 1.5- to 2.5-fold decrease in transmission efficiency. This is consistent with a viability assay showing that when pollen grains developed under

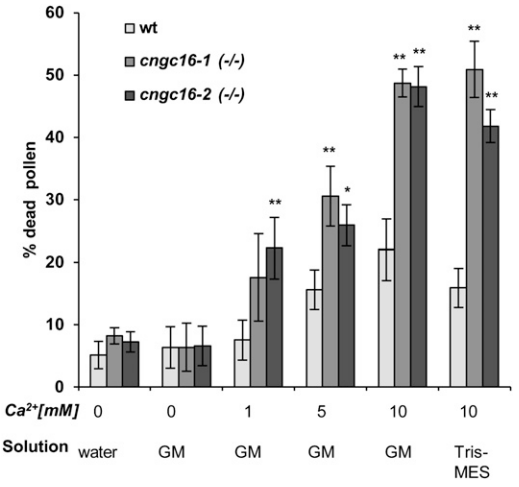


Figure 5. Alexander viability staining demonstrates that *cngc16* pollen are hypersensitive to elevated $CaCl_2$ concentrations. Pollen grains were harvested into a water suspension from plants grown under normal conditions. Aliquots were modified as indicated and incubated for 3 h at 20°C in parallel. Incubations were done in solutions corresponding to water only, standard liquid in vitro germination medium, pH 7.5 (GM), or Tris-MES buffer (pH 7.5). Solutions were amended as indicated with Ca^{2+} using $CaCl_2$. Alexander staining was done after 3 h by pelleting pollen and resuspending pellets in 1 mL of Alexander stain for 30 min. Within the relatively short post hydration time frame assayed, wild-type (wt) controls for each solution showed less than 0.5% pollen grain germination. Viability counts were done with a digital camera mounted on a Leica DM IRE2 microscope. Values represent means ± SD of three independent experiments, each with approximately 50 pollen grains. Student's *t* test was done to compare the pollen viability of *cngc16* mutants to wild-type plants incubated at the same condition. *Student's *t* test significant at $P < 0.05$. **Student's *t* test significant at $P < 0.01$.

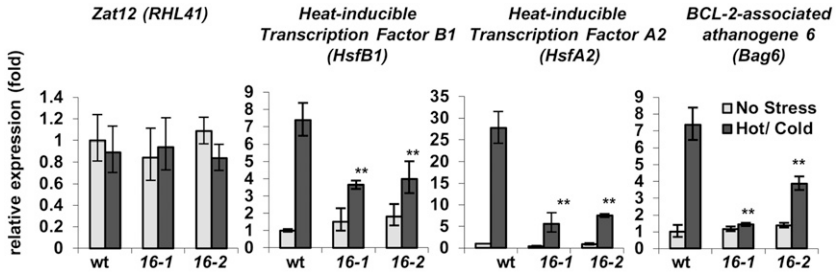


Figure 6. Quantitative PCR indicates *cngc16* pollen have attenuated expression of key stress response genes. Four stress-related genes (*Zat12* [AT5G59820], *HsfB1* [At4g36990], *HsfA2* [At2g26150], and *Bag6* [At2g46240]) were tested for their steady-state transcript levels in pollen collected after the peak heat stress (Figure 1B) from hot- and cold-stressed Arabidopsis plants. Values represent means ± SD of three to five independent experiments. Student's *t* tests were conducted to compare the relative fold change in mRNA abundance of the related gene in *cngc16* plants with wild-type (wt) plants. **Student's *t* test significant at $P < 0.01$.

stress conditions, the *cngc16* pollen were twice as likely to die compared with the wild type (Fig. 3). When the stress environment was applied 2 h postpollination, a similar 1.5- to 2.5-fold decrease in transmission efficiency was observed. This bracketing suggests that *cngc16* mutant pollen are most sensitive to stress conditions at the time of germination and/or growth into the stigma surface.

In vitro pollen growth assays corroborate that *cngc16* pollen grains are highly sensitive to environmental conditions. In comparison with wild-type pollen germinated under a standard in vitro growth condition (with 1 mM $CaCl_2$), less than 1% of the pollen grains produced tubes and more than 80% arrested as short tubes (<30 μm) or ruptured. This phenotype shows similarities to that observed for knockouts of *cngc18* (Frietsch et al., 2007) and double knockouts of *cngc7* and *cngc8* (J.F. Harper, unpublished data). However, a major difference between *cngc18*, *cngc7*, *cngc8*, and *cngc16* was that at least some of *cngc16* pollen grains produced long tubes. In addition, *cngc16* mutant pollen showed normal Mendelian transmission under nonstress conditions, whereas *cngc18*, *cngc7*, and *cngc8* are male sterile.

The sensitivity of *cngc16* pollen to in vitro growth conditions was at least partially due to an increased sensitivity to external $CaCl_2$ (Fig. 5). Using a viability assay based on Alexander staining, nearly 50% of *cngc16* pollen were found to be dead after a 3-h incubation in a buffered solution of 10 mM $CaCl_2$. This level of lethality is 2- to 3-fold higher than a wild-type control. An analogous $CaCl_2$ hypersensitivity has been reported for two other *cngc* mutant phenotypes (Chaiwongsar et al., 2009; Urquhart et al., 2011). For example, the growth reduction phenotype associated with a knockout of *cngc2* in Arabidopsis was further decreased by approximately 2-fold by supplementing soil with 10 mM $CaCl_2$.

While it is not clear why the loss of a Ca^{2+}-permeable CNGC would cause pollen or a whole plant to become more sensitive to $CaCl_2$, a recent patch clamp study on a *cngc-b* knockout mutant in the moss *P. patens* showed evidence for an increased open probability for two of three detectable Ca^{2+} permeable channel activities, consistent with a model in which the loss of one CNGC subunit might leave behind a CNGC complex with altered kinetic properties (Finka et al., 2012).

Heat Stress Can Trigger a Rise in cGMP

Results here provide evidence that a heat stress in either leaf tissue or pollen can trigger an increase in cGMP (Fig. 7). It is not clear if there was an additional increase or decrease in cAMP, as we were unable to detect cAMP using a similar immuno-based assay. In plant tissues, cGMP has been detected at levels ranging from 0.05 to 90 pmol g^{-1} fresh weight, with various stimuli triggering as much as a 9-fold increase (Penson et al., 1996; Durner et al., 1998). The levels of cGMP detected here lie

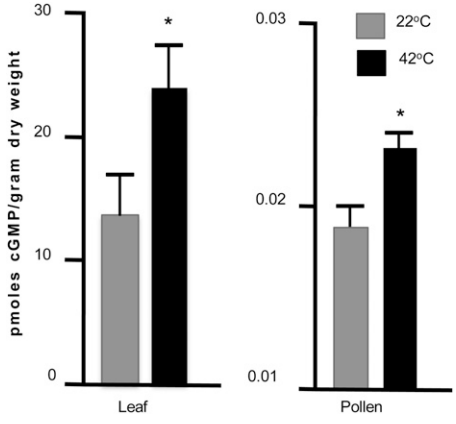


Figure 7. Heat stress increases the average cGMP levels in leaves and pollen. Tissues were harvested from plants before or after a 30-min heat stress at 42°C. cGMP concentrations were assayed by competitive binding assay using cGMP-horseradish peroxidase and cGMP-specific antibody. Stress-dependent increases were measured in eight independent experiments for leaf and four independent experiments for pollen. Wilcoxon rank test was used to evaluate significance (asterisk). Means are shown with SE.

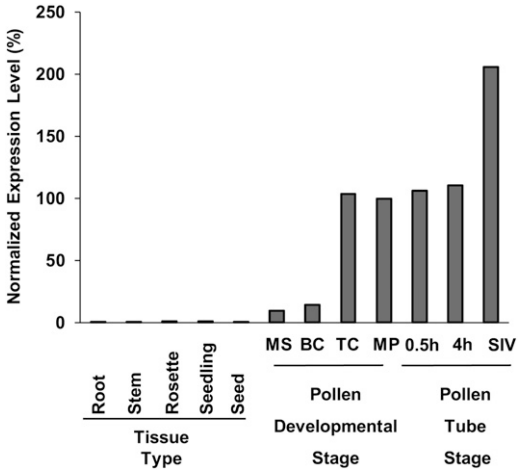


Figure 8. The expression profiles of *CNGC16* show preferential expression in pollen. Plant tissue types on the graph are as follows: 7-d-old wild-type roots, 7-d-old wild-type stem, 10-d-old wild-type rosette, 7-d-old wild-type seedling and wild-type seeds. Stages of male gametophyte development are as follows: MS, microspore; BC, bicellular; TC, tricellular; MP, mature pollen; 0.5h, pollen tube germinated in vitro for 30 min; 4h, pollen tube germinated in vitro for 4 h; and SIV, pollen tube germinated semi in vivo. The expression level for mature pollen was set to 100%. Expression data were extracted from the AtGenExpress database (http://jsp.weigelworld.org/expviz/expviz.jsp; Schmid et al., 2005) and the Pollen Transcriptome Navigator (http://pollen.umd.edu/), which uses Honys and Twell (2004) data for developmental stages of pollen expression profile and Qin et al. (2009) data for the in vitro- and semi in vivo-grown pollen tubes.

within this range, with pollen showing approximately 700-fold less than leaves. Despite this variation, the detected concentrations are consistent with the potential for localized levels of cGMP that are sufficient for activating enzymes such as a CNGC (Gehring, 2010).

While a small stress-dependent increase in cGMP was observed here in both leaf and pollen, there are two alternative physiological interpretations to consider. It is possible that the observed increase results from a small percentage of cells with a transient stimulation of cGMP production, consistent with a model in which heat triggers a cNMP signal that activates a CNGC channel. The observed increase in cGMP might appear small because only a small percentage of cells are actively generating a cGMP transient at any given moment. Alternatively, the increase in cGMP might reflect a new steady-state level, generated as a response to a continuous exposure to a cellular stress. In this model, the higher cGMP levels represent an adaptation to the stress response as opposed to being the signal that triggers the response. Regardless, these results indicate that a heat stress can alter the dynamics or steady-state levels of cGMP in pollen.

A CNGC16-Dependent Transcriptional Response

Pollen from *cngc16* mutants exposed to a heat stress showed an attenuated expression of three genes known to be associated with a heat stress response (Fig. 6). Two of these marker genes encode heat shock transcription factors HsfA2 and HsfB1, which are required for thermotolerance in vegetative tissues (Charng et al., 2007; Ikeda et al., 2011). The third was *Bag6*, which is one of the targets under the control of *HsfA2* (Nishizawa-Yokoi et al., 2009) and implicated in pollen germination and tube growth in Arabidopsis (Cartagena et al., 2008). The inability to fully activate a transcriptional stress response provides a mechanistic explanation for the stress-sensitive pollen phenotype observed for *cngc16* mutants.

A General Model for a cNMP-Triggered Heat Stress Response

There are two types of CNGC signaling pathways that are well characterized in animal systems (Kaupp and Seifert, 2002; Pifferi et al., 2006). In the first, the channel is gated open when a cNMP signal is produced, as occurs in the olfactory pathway. In the second, the channel is normally open due to a steady basal level of cNMP and closes when a phosphodiesterase is activated to reduce cNMP levels below the activation threshold, as occurs during vision. It is not clear if plants utilize one or both of these different pathways. The observation here that heat stress increases in cGMP can currently be explained in the context of both paradigms. In one model, heat stress triggers a cGMP signal, which activates CNGC16, similar to what happens in the animal olfactory response pathway (Leinders-Zufall et al., 1995). However, as discussed above, an increase in cGMP under stress conditions might alternatively reflect a new steady-state basal level, similar to what happens in animal vision when rod cells adapt to darkness (Fain, 2011).

There are several examples in animal systems for a potential role of cNMP in a heat stress response. In mammalian cells, a heat stress has been reported to increase cNMP levels (Kornbluth et al., 1977; Ewing et al., 1994; Zensho et al., 1998). Increases in cNMP concentrations have also been linked to the expression of heat stress response genes, such as those encoding heat shock proteins such as HSP-70 and HSP-90 in capillary endothelial cells (Martínez et al., 2006) or neuroblastoma cells (Zensho et al., 1998). However, the role of a CNGC in these pathways has not been established, and it should be noted that animals (and plants) have other potential cNMP-activated enzymes (Bridges et al., 2005). Nevertheless, a genetic knockout of a *CNGC* in *Caenorhabditis elegans* provides evidence for an animal CNGC pathway being involved in thermosensing (Cho et al., 2004; Hellman and Shen, 2011; Liu et al., 2012).

In plants, there are three recent examples linking CNGCs to a heat stress response. In the first, a knockout of *AtCNGC6* in Arabidopsis resulted in plants with vegetative tissues showing a decreased tolerance to heat stress (Gao et al., 2012). This appears

analogous to the increased heat stress sensitivity observed here for *cngc16* pollen. However, a comparable disruption of a heat stress response was not observed in knockouts of a *CNGC-b* in the moss *P. patens* and its putative ortholog in Arabidopsis (*CNGC2*; Finka et al., 2012). Rather, these mutant plants showed a hyperactivation of a heat stress response at lower temperatures. One possible explanation is that the plant CNGCs form heteromultimers, and the loss of one subunit may leave behind a new CNGC complex with altered channel properties, potentially making some channel activities hyperresponsive and others nonresponsive. In Arabidopsis pollen, it is not clear which of the other five pollen expressed CNGCs might have functional interactions with CNGC16. Regardless, a knockout of *cngc16* alone results in a disrupted stress response, providing genetic evidence for a signaling pathway that links cNMP signals to a transcriptional response that is critical for heat or drought stress tolerance during reproductive development.

MATERIALS AND METHODS

Plant Material and Control Growth Conditions

Metadata for *CNGC16* can be found at The Arabidopsis Information Resource (http://www.arabidopsis.org/) under Arabidopsis Genome Initiative accession number At3g48010. Both *cngc16-1 and cngc16-2* alleles are in the background of Arabidopsis (*Arabidopsis thaliana*) accession Columbia and were derived from the Syngenta Arabidopsis Insertion Library collection (Sessions et al., 2002). The *cngc16-1* (SAIL_232_B12, seed stock no. 95) and *cngc16-2* (SAIL_726_B04, seed stock no. 91) mutants carry a glufosinate (Basta) resistance marker in the T-DNA. In addition, the *cngc16-1* T-DNA insertion has a GUS marker under the control of the pollen-specific *LAT52* promoter. PCR-based genotyping of *cngc16* mutants was done using the following primers. To detect the wild-type gene, primers used were 1149a, 5′-GGACTAGTCGGCGCGCCCGGCGGAATGAGCAACCTCCACCTCTACACCTC-3′, and 1149b, 5′-CCCCGCGGCCGCGCCTAGGCCTCAGAAGAATCCTGGATCCTCTGGTTTAA-3′. To detect the T-DNA borders, primers 1149a and 1149b were used in combination with 640 (LB3), 5′-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3′.

Seeds were germinated and grown on 0.5× Murashige and Skoog medium containing 1% agar with or without Basta (10 μg mL^{-1}). Two-week-old seedlings were transferred to soil (MetroMix200; Hummert). Unless otherwise stated, all plants were grown under control conditions (8 h of light and 16 h of darkness, a light intensity of approximately 110 μmol m^{-2} s^{-1}, 40% humidity, and 22°C temperature) in growth chambers (Percival Scientific).

For experiments involving a hot/cold stress regime, flowering plants were grown for 2 to 4 weeks under conditions of hot days and cold nights (Zinn et al., 2010; Fig. 1B). Plants were grown in a growth chamber with 16 h of light, with the daybreak starting temperature at 10°C, rising to a peak 40°C for 1 h, and falling back to 10°C. For the 8-h night, the temperature was reduced to −1°C. For the heat-stress-only assays, *cngc16* heterozygous mutants were transferred into chamber at 32°C or 37°C for 4 to 6 d while flowering and then returned to normal conditions for seed maturation. For drought-stress experiments, plants were grown in a greenhouse with limited watering.

Plasmid Constructs

Plant expression constructs were made in a modified pGreenII vector system (Hellens et al., 2000) with a kanamycin selection marker for bacteria and a hygromycin marker for plants. The DNA sequence of each construct is provided as a supplemental file (Supplemental Data S1). CNGC16 is encoded by a complementary DNA (cDNA) that was PCR amplified and sequenced to ensure the absence of PCR mistakes. Plasmid 1610, *CNGC18p(i)-GFP-CNGC16*, encodes a GFP-tagged CNGC16 under the control of a *CNGC18* promoter (Frietsch et al., 2007). Representative transgenic plants rescued with plasmid construct 1610 are seed stock numbers 1646 and 1647 [*cngc16-1*(−/−) and *cngc16-2*(−/−) backgrounds, respectively]. Plasmid 1183, *ACA9p(i)-GFP-CNGC16*, is the same except the promoter is from *ACA9* (Schiøtt et al., 2004). Representative transgenic plants rescued with this construct were seed stock numbers 1648 and 1649 [*cngc16-1*(−/−) and *cngc16-2*(−/−) backgrounds, respectively].

In Vitro Germination of Pollen

In vitro germination of pollen was done with a slightly modified protocol from Boavida and McCormick (2007). In brief, pollen from open flowers was spread on the surface of a slide with a solid medium containing 1% low-melting agarose with 0.01% H_3BO_3, 1 mM $CaCl_2$, 5 mM KCl, and 10% Suc, pH 7.5. Slides were placed into a petri dish with a wet paper towel to humidify the chamber. Pollen were incubated at 22°C.

RNA Isolation and Real-Time Quantitative RT-PCR

For gene expression analysis, pollen from open flowers (stage 14; Nasrallah et al., 2002) were collected by vortexing cut flowers in water for approximately 10 s, allowing pollen to pass through a cell strainer with a 70-μm nylon mesh (Becton Dickinson and Company) and centrifuging pollen into a pellet at 14,000 rpm in a microfuge for 30 s. The supernatant was removed and pollen pellets frozen with liquid nitrogen. Pollen were harvested at midday for all experiments. For stress treatments, the midday harvest was preceded by 40°C for 1 h.

Total RNA was isolated from a pollen pellet (about 100 μL) using the RNeasy Plant Mini Kit (Qiagen). RNase-free DNase was used to eliminate genomic DNA. First-strand cDNA was synthesized using 2 μg of total RNA, GeneRacer oligo(dT) primer, and Superscript II RNase H$^-$ reverse transcriptase (Invitrogen). A fraction (0.14 μg) of the cDNA was used as the template in 20-μL reaction mixture per well in real-time PCR. TaqMan assays were purchased for *HsfA2*, *HSF4*, *Bag6*, and *Zat12* (assay ID nos. At02302989_g1, At02332920_g1, At02256657_g1 At02263674_g1, and At02269070_s1, respectively) from Applied Biosystems. Target gene data were normalized against *elongation factor1-α* (*EF1-α*) RNA levels (assay ID no. At02337969_g1; Applied Biosystems; Fig. 6). Supplemental Figure S3 includes results for five additional genes that showed similar patterns in the wild type and *cngc16* mutants: *Cation/H$^+$ exchanger9* (AT5G22910), *Catalase2* (AT4G35090), *GRAM domain-containing protein/ABA-responsive protein-related* (AT5G13200), *L-ascorbate peroxidase6* (AT4G32320), and *Cold Regulated78* (AT5G52310; assay ID nos. At02302989_g1, At02255530_g1, At02198567_g1, At02248433_g1, At02320471_g1, and At02320471_g1, respectively, from Applied Biosystems). Gene expression levels were quantified by real-time quantitative RT-PCR (CFX96; Bio-Rad). The thermal profile used for amplification was 95°C for 2 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed by using the comparative cycle threshold method. All quantitative PCR assays were validated in compliance with the "minimum information for the publication of real-time quantitative PCR experiments" guidelines (Bustin et al., 2009). This included validating probe efficiencies with a standard curve method (Larionov et al., 2005). Amplification efficiencies, which were calculated from the slope of the standard curve done with serial dilutions of cDNA (ranging from 0.1 to 1 μg), were 106.7%, 103%, 100%, 99.1%, and 90.6%, for *EF-1-α*, *HSF4*, *HsfA2*, *Bag6*, and *Zat12*, respectively. In addition, a "no template control" reaction was included to rule out cross contamination of reagents and surfaces. For each sample, a control was done without reverse transcriptase to test for genomic DNA contamination.

Pollen Viability Assay

Alexander's stain (Alexander, 1969), modified by Johnson-Brousseau and McCormick (2004), was used to score pollen viability. Pollen grains were incubated in 1-mL Alexander stain buffer diluted 1:50 in water (stock solution: 10 mL 95% ethanol, 5 mL 1% malachite green in 95% ethanol, 5 g of phenol, 5 mL 1% acid fuschin in water, 0.5 mL 1% orange G dye in water, 2 mL glacial acetic acid, 25 mL glycerol, and 50 mL of water) for 30 to 40 min. Stained pollen grains were imaged with a digital camera mounted on a Leica DM IRE2 microscope. Viable pollen grains were identified with a reddish color, while dead pollen grains were a pale turquoise blue (Supplemental Fig. S2).

ELISA Assay for Quantification of cGMP

Leaves (approximately 1.5 cm across) were collected from young plants before bolting and immediately pulverized in 500 μL extraction buffer (1 M HCl

in 100% ethanol). Samples were collected before and after a 30-min heat stress of 42°C. After spinning at 14,000 rpm in a microfuge for 10 min, the supernatant was transferred to a new tube, dried overnight, and resuspended in 250 μL of water. The debris pellet was dried and weighed and used for normalization of cNMP concentrations.

Pollen was collected at midday (as described above for RNA extractions) from approximately 200 mature wild-type plants. Each technical replicate had an approximately 0.2-mg wet weight of pollen (approximately 22,600 grains) in 0.5 mM 3-isobutyl-1-methylxanthine, a competitive nonselective phosphodiesterase inhibitor (Sigma-Aldrich), in 100 μL of water. Pollen samples were incubated for 30 min at room temperature (control conditions) or at 42°C (heat stress). After the incubation period, 500 μL extraction buffer was added and the samples were pulverized, vortexed, and spun at 14,000 rpm in a microfuge for 10 min. The supernatant was transferred to a new tube, dried overnight, and resuspended in 250 μL of water.

Plates were prepared and processed with a GenScript ELISA kit as described by the manufacturer (catalog no. L00253). Anti-cGMP (GenScript; catalog no. A01508) was used as the antigen specific antibody and was diluted to 0.05 μg mL^{-1} with binding buffer. The antigen (20 μL), which was either the extraction sample or a cGMP standard (Sigma-Aldrich), was loaded into each well and incubated 30 min at room temperature. The enzyme-linked secondary antibody cGMP-horseradish peroxidase (GenScript; M01058) was added to each well and incubated at room temperature for 1 h or 4°C overnight and then washed with 1× wash solution. The plate was developed with 100 μL of SureBlue Reserve TMB peroxidase substrate (Kirkegaard & Perry Laboratories; catalog no. 120283) and incubated at room temperature for 15 min with gentle agitation. The absorbance was measured at 640 nm. In this competitive ELISA assay, the lower the optical density, the higher the cGMP concentration. The concentration of extraction samples was determined using a standard curve of cGMP (0.061 pm/mL to 400 pmol mL^{-1}). A linear function was used to determine the concentration of samples. A Wilcoxon two-sample test was done to determine the statistical significance of the data.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The near-sterile phenotype of homozygous *cngc16* mutants under hot/cold stress regime.

Supplemental Figure S2. Viability of pollen as detected by Alexander's stain and light microscopy.

Supplemental Figure S3. Control quantitative PCR reactions showing examples of gene expression patterns that are similar between the wild type and *cngc16* mutant.

Supplemental Data S1. DNA sequences of plasmid constructs 1610, *CNGC18p(i)::GFP-CNGC16*, and 1183, *ACA9p(i)::GFP-CNGC16*.

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