Expression of plant cyclic nucleotide-gated cation channels in yeast

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Abstract

The functional properties of inwardly conducting plant cyclic nucleotide-gated cation channels (CNGCs) have not been thoroughly characterized due in part to the recalcitrance of their functional expression in heterologous systems. Here, K⁺ uptake-deficient mutants of yeast (trk1,2) and Escherichia coli (LB650), as well as the Ca²⁺-uptake yeast mutant mid1,cch1, were used for functional characterization of Arabidopsis thaliana CNGCs, with the aim of identifying some of the cultural and physiological conditions that impact on plant CNGC function in heterologous systems. Use of the Ca²⁺-uptake yeast mutant provided the first evidence consistent with Ca²⁺ conduction by the A. thaliana CNGC AtCNGC1. Expression of AtCNGC1 in LB650 demonstrated that mutants of Escherichia coli (which has no endogenous calmodulin) can also be used to study functional properties of CNGCs. Expression of AtCNGC2 and AtCNGC4 enhanced growth of trk1,2 in the presence of hygromycin; AtCNGC1 has less of an effect. Deletion of the AtCNGC1 calmodulin-binding domain enhanced growth of trk1,2 at low external K⁺ but not of LB650, suggesting that yeast calmodulin may bind to, and down-regulate this plant channel. In vitro binding studies confirmed this physical interaction. Northern analysis, green fluorescent protein:AtCNGC1 fusion protein expression, as well as an antibody raised against a portion of AtCNGC1, were used to monitor expression of AtCNGC1 in LB650. In the presence of the activating ligand cAMP, expression of the AtCNGC1 channel with the calmodulin-binding domain deleted increased intracellular [K⁺] of trk1,2. Trk1,2 is hypersensitive to the toxic cations spermine, tetramethylammine, and NH₄⁺. These compounds, as well as amiloride, inhibited trk1,2 growth and thereby improved the efficacy of this yeast mutant as a heterologous expression system for CNGCs. In addition to characterizing mutants of yeast and E. coli as assay systems for plant CNGCs, work presented in this report demonstrates, for the first time, that a plant CNGC can retain ion channel function despite (partial) deletion of its calmodulin-binding domain and that yeast calmodulin can bind to and possibly down-regulate a plant CNGC.

Key words: Calmodulin, calmodulin-binding domain, CNGC, cyclic nucleotide binding domain, cyclic nucleotide-gated channel, plant Ca²⁺ transport, plant ion channel.

Introduction

Recent work by Lemtiri-Chlieh and Berkowitz (2004) documented the presence of inwardly conducting cation channels in the plasma membrane of plant (Arabidopsis thaliana) cells that are activated by the cytosolic secondary messenger cAMP. The Arabidopsis genome is known to encode 20 cyclic nucleotide-gated cation channels (CNGCs) (Mäser et al., 2001). Thus, this recent work with native membranes is consistent with the presence of functional CNGCs in planta. Plant CNGCs are inwardly rectified, ligand gated, non-selective cation channels; their selectivity profiles may differ (Hua et al., 2003a), but in all cases studied to date their conductance is increased in the presence...
of cyclic nucleotides (of all known plant channels, this feature is unique to CNGCs) and K⁺ permeates their pores.

Use of heterologous expression systems for functional analysis has led to the characterization of the translation products of many plant ion transport genes (Mäser et al., 2001). The expression of nucleotide coding sequences in frog (Xenopus laevis) oocytes, animal cell cultures, and/or appropriate yeast (Saccharomyces cerevisiae) mutants have contributed to our understanding of the properties of numerous plant ion channels. Characterization of plant CNGC channel properties in this manner has been hindered by the lack of progress with functional expression in these systems.

Plant CNGC expression in oocytes and animal cell cultures is problematic (Davenport, 2002; Leng et al., 2002; Balagué et al., 2003; Hua et al., 2003a). Escherichia coli K⁺-uptake mutants have been used to functionally characterize several plant ion channels (KAT1 and AKT2; for review see, Uozumi, 2001). However, no publication has yet examined the utility of this heterologous expression system for the functional study of plant CNGCs. Yeast lacks endogenous CNGCs, suggesting this model eukaryote as a possibility for functional analysis of plant CNGCs. However, recent reviews of the plant CNGC literature (Talke et al., 2003) note that ‘the phenotypes observed with the yeast mutants expressing plant CNGCs are weak and yeast might not be a suitable expression system for CNGCs’. As pointed out in a review of plant cation channels (Véry and Sentenac, 2002), functional analysis upon expression in heterologous systems could be hindered by interaction of the plant protein with regulatory systems/molecules present in the heterologous cell (even if the protein is properly expressed and inserted into the correct membrane). The work presented here focused on characterizing some of the culture and physiological conditions that impact on plant CNGC (focusing on the A. thaliana CNGC AtCNGC1) function in the K⁺ uptake-deficient yeast mutant trk1,2. In addition, the present work contributes to the plant CNGC literature by demonstrating, for the first time, functional complementation by a plant CNGC of an E. coli K⁺-uptake mutant as well as a yeast Ca²⁺-uptake mutant.

Materials and methods

Yeast strains and growth media

Unless otherwise noted, all reagents and chemicals used for all work in this report were purchased from Sigma (St Louis, MO, USA). The wild-type W303 and isogenic K⁺ (Mercier et al., 2004) as well as Ca²⁺ (Locke et al., 2000) uptake-deficient S. cerevisiae strains WD3 (trk1,2) and ELY151 (mid1,clch1), respectively, were used. The strains were transformed with a yeast empty vector (pSM1052, 2 μm origin, URA3 marker), or the plasmid containing AtCNGC1 (and carboxyl-terminal deletions of the AtCNGC1 coding sequence; see Fig. 3 in Results), AtCNGC2, or AtCNGC4. The plant cDNAs were expressed under the control of the yeast phosphoglycerate kinase promoter (Mondal and Roy, 1990). Transformation was done using a yeast transformation kit (Qiogen; Irvine, CA, USA). All yeast strains were grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or SC medium (minimal medium containing 2% glucose, 0.5% ammonium sulphate, and 0.17% Yeast Nitrogen Base without amino acids and without ammonium sulphate; Qbiogene). Histidine (30 μg ml⁻¹), leucine (0.1 mg ml⁻¹), uracil (30 μg ml⁻¹), methionine (0.1 mg ml⁻¹), and lysine (0.1 mg ml⁻¹) were added when needed. Growth assays of trk1,2 yeast were performed in APG medium (10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, trace minerals, and vitamins; pH 3.2) with additional K⁺ (added as the Cl⁻ salt in all cases) as noted. Growth assays were performed by inoculating 1 ml of liquid APG medium with overnight cultures grown in selective SC medium. Cells were washed 3× with sterile distilled deionized Milli Q water prior to inoculation. Growth was monitored by measuring OD600 nm after 72 h at 30 °C. Drop tests were performed on solid APG agar plates with additions as noted in the text and figure legends.

Generation of DNA constructs encoding channels

The cDNAs encoding plant AtCNGCs used in this study were subcloned into pSM1052 (a yeast 2 μm-based plasmid with amino-terminal His or green fluorescent protein (GFP) tags for expression and functional analysis in yeast. The subcloning was done using PCR and sequence-specific primers with MutI and NorI restriction sites at the 5' and 3' ends, respectively. Vent DNA polymerase (Stratagene; La Jolla, CA, USA), and AtCNGC cDNAs as template (Mercier et al., 2004). A similar PCR-based strategy was used to generate various carboxyl-terminal deletions of AtCNGC1 as shown in Fig. 3.

RNA extraction and northern analysis

K⁺ uptake-deficient yeast strain trk1,2 was transformed with plasmids as indicated in Fig. 4 (see Results), and grown in selective SC medium (-ura, his, leu and 100 mM KCl) at 30 °C to logarithmic phase. Cells were cooled on ice prior to harvesting by centrifugation and washed 2× with ice-cold sterilized Milli Q water. Total RNA was isolated and quantified as described by Sambrook et al. (1989). RNA was size-fractionated on gels containing 1.2% (w/v) agarose in 20 mM 3-[N-morpholino] propane sulphonic acid, 5 mM sodium acetate, 1 mM EDTA, and 1.8% (w/v) formaldehyde; pH 7. RNA was transferred from the agarose gel by capillary blotting to a Hybrid-N membrane (Amersham Biosciences, Piscataway, NJ, USA) using 10× SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0). Sequence-specific probes were labelled and detected using a non-radioactive digoxigenin labelling and detection kit (Roche Applied Science; Indianapolis, IN, USA) and autoradiography. The yeast actin gene (ACT1) was used as an internal reference.

Complementation of a K⁺-uptake mutant of E. coli

The K⁺ uptake-deficient E. coli strain LB650 (Stumpe and Bakker, 1997) with mutations in the TRK H and TRK G genes was used for complementation studies. The wild-type and carboxyl-terminal deletion mutant constructs of the AtCNGC1 cDNA under control of the phosphoglycerate kinase promoter were transformed into the E. coli LB650 strain. Cultures were grown in medium containing 10 g l⁻¹ tryptone, 5 g l⁻¹ yeast extract, 10 g l⁻¹ KCl, 100 μg ml⁻¹ ampicillin, 30 μg ml⁻¹ kanamycin, and 35 μg ml⁻¹ chloramphenicol. Cells were grown overnight, washed 4× with Milli Q water and resuspended to a final OD600 nm of 2. For the complementation assay, 15 μl of 2 OD cell suspension was diluted into 1 ml of minimal liquid medium (Ahn et al., 2004) with the desired concentrations of KCl in 24 micro-well plates. Alternatively, the suspension was serially diluted and 5 μl of each dilution was plated on minimal medium agar plates. Cells were grown for the indicated time periods at 37 °C.
Fluorescence microscopy

Trk1,2 yeast was grown in SC-dropout medium at 30°C to logarithmic phase. A 1 ml culture was briefly centrifuged to pellet the cells, which were then washed 2× with the same medium. After final resuspension, the cells were grown for 2 h before aliquots were taken for microscopy. Cell suspensions of 3 μl were dropped on poly-L-lysine-treated cover slips and placed on slides. Samples were viewed on an inverted Olympus IX70 microscope (Olympus, Melville, NY, USA) equipped with fluorescence and phase contrast optics and a 100× oil immersion lens. Digitized images (16-bit) were acquired using an Olympus CCD camera and MagnaFire software. GFP and bright field images were merged using Adobe Photoshop CS 8.0 (Adobe Systems, Inc., San Jose, CA, USA).

Intracellular cation determination

For measurement of intracellular levels of K+, trk1,2 yeast was grown in selective SC medium overnight to OD600 nm of 2–3, and then washed 4× with sterilized Milli Q water. The cells were starved for K+ by resuspending harvested cells in sterilized Milli Q water and incubating them on a rotary shaker for 2 h at 4°C. The K+-starved cells were grown for 2 h in APG medium with 50 mM KCl in the presence or absence of 100 μM dibutylryl-cAMP (Bt2-cAMP). Thirty minutes prior to harvesting, all cultures were brought to 100 mM amiloride [in all cases, the especially potent amiloride analogue S3226 (Schwark et al., 1998) was used]. Cultures were harvested according to the protocol described by Garcia et al. (1997). The ion concentration in the clarified extracts was determined with an atomic absorption spectrometer (Varian, Palo Alto, CA, USA) in the flame emission mode.

Complementation of cch1,mid1 response to mating pheromone

Cells were grown in selective SC medium (SC-trp, leu, ura) overnight, washed 3× with sterilized Milli Q water, and then resuspended in liquid YPD medium to an OD600 nm of 4. An aliquot (100 μl) of this resuspension was added to 4 ml of top agar (YPD with 0.7% agar), mixed, and overlaid on YPD agar plates. Immediately after solidification of the top agar-containing cells, sterile cellulose discs (0.6 cm; Schleicher & Schuell, Keene, NH, USA) with 45 μg or 60 μg of synthetic α-factor were placed on the nascent lawn. The plates were incubated at 30°C and photographed after 48 h.

AtCNGC–CaM binding assays

Protein–protein interactions were assayed by Förster resonance energy transfer (FRET) using fluorescent indicator proteins (FIPs) as described previously (Hua et al., 2003b). Multiple fusion proteins consisting of the CaM-binding domains of either AtCNGC1 (amino acids 601–625, FIP-AtCNGC1) or AtCNGC2 (amino acids 645–670, FIP-AtCNGC2) fused C-terminal to the coding sequence of blue consisting of the CaM-binding domains of either AtCNGC1 (amino acids 413–426 of AtCaM2, AtCaM8, and yeast CaM (CMD1p) were also expressed through Sephacryl S-200 (Amersham Biosciences). Recombinant agarose affinity chromatography and gel filtration chromatography plates were incubated at 30°C as described previously (Hua et al., 2003b).

Generation of an antibody immunoreactive with AtCNGC1 and use for immunoblot analysis

A 15 mer peptide corresponding to amino acids 413–426 of AtCNGC1 with a cysteine added at the N-terminus was generated, linked to the carrier protein keyhole limpet haemocyanin, and used to immunize New Zealand White rabbits. Anti-AtCNGC1 was affinity purified using the peptide and the SulfoLink kit (Pierce Biotech., Rockford, IL, USA) following the manufacturer’s protocol. Affinity-purified anti-AtCNGC1 was used for immunoblot analysis of SDS-PAGE fractionated protein using the ECL detection kit (Amersham Biosciences).

Results

Expression of plant CNGCs in the trk1,2 K+ uptake-deficient yeast mutant has yielded variable results. Growth enhancement of the mutant upon expression of plant CNGCs has been demonstrated (Köhler et al., 1999; Leng et al., 1999). However, in some cases (Schuurink et al., 1998) no suppression of the mutant phenotype occurred, and in other studies (Mercier et al., 2004) different plant CNGC isoforms displayed varying ability to promote growth of the mutant at low external K+ and/or in the presence of hygromycin (hyg). The trk1,2 mutant has a hyperpolarized (inside negative) cell membrane potential due to reduced uptake of K+; this results in hypersensitivity to the cationic antibiotic hyg and (partial) complementation of hyg sensitivity upon expression of heterologous cation transporters (Madrid et al., 1998).

Growth of the trk1,2 mutant transformed with several different Arabidopsis CNGCs in the presence of hyg on solid and liquid APG medium is shown in Fig. 1. In both experiments, it appears that the plant CNGCs have varied ability to complement the trk1,2 phenotype. On solid medium containing 50 mM K+ (i.e. ‘low’ K+ medium) and in the presence of hyg, drop assays of serial dilutions indicate that transformation with AtCNGC1 results in growth of trk1,2 to an extent no greater than when trk1,2 is transformed with the empty vector (Fig. 1A, panel 2). Alternatively, in this experiment, transformation with AtCNGC2 and AtCNGC4 significantly complemented trk1,2 growth sensitivity at low K+ in the presence of hyg.

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of the constructs shown here) did not grow at all when \([K^+]\) in the liquid
the experiments shown in (B) and (C).

Effect of plant CNGC expression on growth of a \(K^+\) uptake-

Fig. 1.

![Graph A](image1.png)

A

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(B) Effect of AtCNGCs on growth of yeast strain trk1,2

![Graph B](image2.png)

B

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(C) Effect of AtCNGCs on growth of yeast strain trk1,2

![Graph C](image3.png)

C

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Fig. 1. Effect of plant CNGC expression on growth of a \(K^+\) uptake-
deficient yeast. (A) Trk1,2 yeast with empty vector (EV), AtCNGC1, AtCNGC2, or AtCNGC4 were serially diluted (either no dilution (1:0) or

diluted 1:10 with Milli Q water) and plated on solid APG medium with

either 100 mM \(K^+\) (panel 1) or APG medium with 50 mM \(K^+\) and 10

gl\(^{-1}\) hyg (panel 2). Growth of the same strains was monitored in liquid

APG medium containing 50 mM \(K^+\) either with (B) or without (C) 5

gl\(^{-1}\) hyg. Growth of wild-type yeast isogenic to the trk1,2 mutant was

monitored in the experiments shown in (B) and (C). The wild-type yeast
strain grew to an OD\(_{600}\) nm of 3.69 \pm 0.04 and 3.86 \pm 0.09, respectively, in

the experiments shown in (B) and (C). Trk1,2 yeast (transformed with any

of the constructs shown here) did not grow at all when \([K^+]\) in the liquid

cultures was 25 mM or lower (data not shown). The solid-medium

experiment shown in (A) was repeated twice; growth was recorded after

72 h for this and all other experiments with trk1,2 yeast grown on solid

medium. Results shown in (B) are the means \(\pm SE\) (n=4) of one

experiment. This experiment was repeated three times. Results in (C) are

the compiled means \(\pm SE\) (n=6) of three experiments with two

replications per treatment in each experiment. For the liquid culture

experiments shown in (B) and (C), and all other liquid culture

experiments with trk1,2 yeast, growth measurements were recorded after

72 h. For the results shown in (B) and (C), and all other liquid culture

growth experiments (Figs 2, 6A), means separation was evaluated by

ANOVA analysis followed by Tukey–Kramer Multiple Comparison

tests. Based on this analysis, in (B) and (C), bars representing treatment

means underneath different letters were found to be significantly different

at \(P<0.05\).

Previous studies (Lesage et al., 1996; Sychrova et al.,

1999) suggested that addition of amiloride, an inhibitor of the yeast vacular \(Na^+(K^+)/H^+\) antiporter NHX1 (Schwark et al., 1998; Gaxiola et al., 1999; Darley et al., 2000), would increase \(K^+\) efflux from yeast and, thus, amiloride may be an especially strong inhibitor of the trk1,2 mutant. Using patch clamp analysis of \(K^+\) currents (data not shown), it was confirmed that amiloride increases an outward \(K^+\) current in this yeast mutant. Thus, it was speculated that addition of amiloride to the assay medium would reduce the ‘basal level’ of trk1,2 growth (i.e. growth of the mutant not expressing any recombinant channel protein) and concomitantly make growth that does occur more dependent on function of the heterologously expressed CNGC. Results testing this model are shown in Fig. 2. In the absence of amiloride, growth of trk1,2-expressing AtCNGC1 was 33% greater than growth of the mutant transformed with the empty vector; this difference was not statistically significant \((P>0.05)\). Addition of amiloride greatly reduced growth of

trk1,2 transformed with the empty vector (as well as the other treatments; Fig. 2). Growth of trk1,2-expressing AtCNGC1 was increased by 44% over yeast transformed with the empty vector when amiloride was included in the

(Fig. 1A, panel 2). Liquid culture assays (Fig. 1B) showed similar trends; trk1,2 growth was significantly enhanced (as compared with the empty vector control) upon expression of AtCNGC2 and AtCNGC4, while the effect of AtCNGC1 was not significant. Expression of the plant CNGCs was also found to suppress the \(K^+\) uptake-deficient phenotype of trk1,2; i.e. growth at low \(K^+\) in the absence of hyg (Fig. 1C). The growth enhancement (over that shown by yeast transformed with the empty vector) due to CNGC expression was, however, modest in this experiment.

Demonstration of the function of a ligand-gated channel such as a plant CNGC by enhancement of yeast mutant growth could potentially be affected by a number of factors. Proper expression and processing of the recombinant protein, as well as targeting and localization to the correct membrane, would be required. Selectivity of the recombinant channel protein for conductance of the ion required by the yeast mutant (and not other, competing ions) could also affect the functional assay. In addition, regulatory molecules present in the yeast could modulate the recombinant channel. In the case of CNGCs, which are activated by cytosolic cyclic nucleotides (Leng et al., 1999, 2002) (cAMP and/or cGMP), inhibited by calmodulin (CaM) (Hua et al., 2003b), and (at least in the case of animal CNGCs) modulated by phosphorylation/dephosphorylation (Kramer and Molokanova, 2001; Kaupp and Seifert, 2002), such regulatory systems/molecules in the yeast could impact on the function of the recombinant plant channel. Further work presented in this report investigated a number of these factors, in terms of optimizing the ability of yeast mutants to be used as a functional assay for these ligand-gated plant ion channels.
assay medium; the effect of AtCNGC1 on growth was not statistically significantly different from growth of yeast transformed with the empty vector in the presence of amiloride (Fig. 2). (Results with the AtCNGC1M2 construct shown in Fig. 2 will be discussed subsequently.)

Plant CNGCs are members of the superfamily of 'P-loop' ion channels that are typically composed of polypeptides having six membrane-spanning domains (S1–S6) with a pore-forming region (P-loop) between S5 and S6 (Fig. 3A). These polypeptides have, flanking the six membrane spanning domains, hydrophilic amino- and carboxyl termini that extend into the cell cytosol. Sequence analysis and protein–protein interaction studies indicate that the CaMBD of plant CNGCs lies adjacent to the cyclic nucleotide binding domain (CNBD) near the carboxyl end of the polypeptide (Köhler and Neuhaus, 2000). As portrayed in the model shown in Fig. 3A, AtCNGC1 has a hydrophilic carboxyl tail beginning after S6; Gln408 to Asp716. Within this region of the polypeptide resides a CNBD from Leu494 to Gln609, and a CaMBD from Phe602 to Trp624. Recent work has presented a three-dimensional structural projection of the plant CNGC CNBD (Hua et al., 2003b), providing some additional insight into how this region of the plant CNGC protein accommodates the dual regulatory functions of cyclic nucleotide binding and CaM inhibition of cyclic nucleotide binding/activation. This structural modelling predicts that a portion of the CNGC CaMBD could be removed without affecting cyclic nucleotide binding/activation of the channel, even though early sequence analyses of plant CNGCs suggested that the CaMBD entirely overlapped with the predicted CNBD (Köhler et al., 1999). Experiments shown in Fig. 3 tested this hypothesis.

Deletion mutations (AtCNGC1M1, M2, and M3) of the AtCNGC1 coding sequence were generated which left intact varying lengths of the carboxyl-terminus. A summary of these constructs is shown in Fig. 3A (below the topological model). AtCNGC1M1 corresponds to a deletion at the carboxyl-terminus that leaves both the CaMBD and the CNBD intact. AtCNGC1M2 retains the CNBD, but deletes a portion of the CaMBD (including the tryptophan residue Trp624 that is highly conserved in plant CNGCs) thought critical for CaM binding (Zielinski, 1998; Hua et al., 2003b). AtCNGC1M3 deletes the CaMBD and, in addition, a portion of the CNBD. Solid culture growth assays (at high and low K+) of trk1,2 transformed with various carboxyl-terminal deletion mutants of AtCNGC1 were evaluated (Fig. 3B); amiloride was present in all treatments. No growth occurred at low (50 mM) K+ when trk1,2 was transformed with the empty vector, and expression of (full-length) AtCNGC1 resulted in little to no suppression of this phenotype. Expression of AtCNGC1M1 appears to provide modest improvement of trk1,2 growth at low K+. Expression of AtCNGC1M2, which leaves intact the CNBD but removes a critical portion of the CaMBD resulted in the greatest growth of trk1,2 at low K+. Deleting a portion of the AtCNGC1 CNBD (i.e. the AtCNGC1M3 construct) reduces the positive effect of channel expression on trk1,2 growth at low K+; however, it is noted that this construct still provides growth enhancement over trk1,2 transformed with either the empty vector or AtCNGC1 (Fig. 3B). It is concluded from the results presented in Fig. 3 that deleting a portion of the AtCNGC1 CaMBD improves the ability of the channel to complement the inhibition of trk1,2 growth at low K+. Further support for this contention is shown in Fig. 2. In liquid culture experiments, growth of trk1,2 transformed with AtCNGC1M2 was greater than with yeast transformed with the full-length AtCNGC1 coding sequence, in the absence (36% increase) or presence (231% increase) of amiloride. Addition of amiloride also increased the difference between growth of trk1,2 transformed with AtCNGC1M2 as compared with yeast transformed with the empty vector; from 82% stimulation in the absence of amiloride to a 376% stimulation in the presence of amiloride. Results shown in Fig. 2 also indicate that amiloride improves the use of trk1,2 as a functional assay for CNGCs (i.e. in the case of AtCNGC1M2 in these experiments).

Expression of (full-length) AtCNGC1 and the deletion construct AtCNGC1M2 in yeast was monitored in the work shown in Fig. 4. Northern analysis (Fig. 4A) indicated that AtCNGC1 and AtCNGC1M2 message was present in yeast transformed with the corresponding cDNAs. Fluorescence patterns of yeast expressing GFP, (full-length) AtCNGC1: GFP, and AtCNGC1M2:GFP fusion proteins were monitored

Fig. 2. Addition of amiloride to liquid APG medium reduces growth of the trk1,2 mutant, but improves its use as a functional assay of plant CNGCs. Growth of trk1,2 transformed with either empty vector, full-length AtCNGC1, or a carboxyl-terminal deletion mutant of AtCNGC1 (‘AtCNGC1M2’; see Fig. 3) in liquid APG medium (containing 50 mM K+) either without (solid bars) or with (open bars) 100 μM amiloride. Results are presented as the compiled means ± SE (n=10) of five experiments with two treatment replications per experiment. ANOVA analysis of means separation was undertaken separately for growth data recorded in the absence and presence of amiloride. Bars representing treatment means underneath different letters are significantly different. Average growth of wild-type yeast in these experiments in the absence of amiloride was 3.82±0.13; addition of amiloride had no significant effect on growth of wild-type yeast (not shown).
Carboxyl-terminal deletions of the AtCNGC1 coding sequence affect the ability of the channel to complement growth inhibition of trk1,2 at low K⁺. (A) Topological model depicting functional regions of the AtCNGC1 polypeptide. This model is similar to that presented in a recent review (Talke et al., 2003), and is based on studies of the physical interaction of CaM with the CAMBD (Arazi et al., 2000; Köhler and Neuhaus, 2000), as well as site-directed mutagenesis analysis of the pore region (Hua et al., 2003a) and protein modelling analysis of the CNBD based on threading this portion of the CNGC through the known crystal structure of the cAMP-dependent protein kinase A CNBD (Hua et al., 2003b). For clarity of presentation, single-letter abbreviations for amino acids are used; Q (glutamine), L (leucine), F (phenylalanine), W (tryptophan), and D (aspartic acid). The six membrane-spanning domains (S1–S6) along with the P-loop pore are shown. The region of the polypeptide downstream from the membrane-spanning domains (starting at Q408, to the carboxyl-terminus at D716) extends into the cytosol and includes several regulatory domains; this region of the polypeptide is shown surrounded by a broken line. Also identified in this cartoon are the amino acids at the beginning and end of the cyclic nucleotide (L494 and Q609, respectively) and CaM (F602 and W624, respectively) binding domains. Various carboxyl-terminal deletion mutations were generated from the AtCNGC1 coding sequence. Lines underneath the cartoon represent the varying lengths of the carboxyl-termini of these deletion mutation constructs. The full-length carboxyl-terminal region (i.e. the amino acids downstream from the membrane spanning domains) of wild-type AtCNGC1 extends from amino acid 408 to amino acid 716. The deletion construct 'AtCNGC1M1' (see construct names to the left of panel 1 in B) extends only to amino acid 657, AtCNGC1M2 includes the carboxyl-terminus to amino acid 621 only, AtCNGC1M3 includes the carboxyl-terminus to amino acid 601 only. (B) Growth (serial dilutions of drop tests, as in Fig. 1) of trk1,2 yeast transformed with either the empty vector (EV), wild-type AtCNGC1, AtCNGC1M1, AtCNGC1M2, or AtCNGC1M3 on solid APG medium containing 100 μM amiloride and with either 100 mM (panel 1) or 50 mM (panel 2) K⁺. The numbers directly to the right of panel 2 indicate the amino acid (aa) length of the carboxyl-terminus (i.e. the portion of the polypeptide downstream from the membrane-spanning domains) of each of the AtCNGC1 constructs. Growth of the yeast was recorded after 72 h. This experiment was repeated twice. Similar results were obtained with both experiments; results from one experiment are shown.

in the work shown in Fig. 4B–J. GFP expressed alone resulted in accumulation in the yeast cell cytosol (Fig. 4B–D), as shown by fluorescence in the area of the cell surrounding the vacuole (Fig. 4D). By contrast, the AtCNGC1-GFP fusion protein appears to be present in the plasma membrane (Fig. 4E–G). The fluorescent ring at the cell membrane in Fig. 4F appears punctate, similar to the pattern found by Zeng et al. (2004). This pattern is consistent with channel localization in lipid rafts—animal CNGCs have been shown to be associated with lipid rafts (Brady et al., 2004). Expression of the AtCNGC1 deletion construct as a fusion protein (AtCNGC1M2:GFP), however, appears varied (Fig. 4H–J). Although there is some fluorescent protein in the plasma membrane, accumulation of AtCNGC1M2:GFP at the cell membrane appears to be less than with AtCNGC1:GFP. In addition, GFP fluorescence is observed in the lumen of the yeast vacuole and perhaps the endomembrane trafficking system in yeast expressing AtCNGC1M2:GFP. This finding is consistent with the possibility that the AtCNGC1M2 protein, due to removal of 94 amino acids from the carboxyl end of the protein, may be more rapidly turned over than the full-length polypeptide in yeast. Nonetheless, results shown in Fig. 4 indicate that AtCNGC1 and AtCNGC1M2 are expressed in trk1,2 yeast. Even though a significant proportion of the deletion, mutant channel protein may be degraded in yeast. It is noted that its ability to complement trk1,2 mutant phenotypes is greater than the full-length channel (Figs 2, 3).

One possible explanation for the improved performance of AtCNGC1M2 over AtCNGC1 in the present functional assays using trk1,2 yeast (Figs 2, 3) could be altered interaction of the recombinant plant channel protein with yeast CaM. This hypothesis is supported by the following points: activation of plant CNGCs by cyclic nucleotide is blocked by CaM (Hua et al., 2003b), plant CNGCs show varying ability to bind to plant CaM isoforms (Köhler and Neuhaus, 2000), and plant CaMs are similar enough in tertiary structure to yeast CaM that they can functionally replace yeast CaM (Zielinski, 2002). Current reviews note that CaM’s flexible tertiary structure leads to promiscuous interactions with target proteins and, in particular, yeast, and plant CaMs seem to group functionally together (Yamniuk and Vogel, 2004). These aforementioned points,
along with the finding that deletion of part of the CaMBD from the AtCNGC1 coding sequence improves the ability of the channel to complement trk1,2 yeast phenotypes, suggest that in the present functional assays in yeast, endogenous yeast CaM protein may bind to, and down-regulate, AtCNGC1. By contrast to AtCNGC1, it is found that AtCNGC2 expression consistently enhances growth of trk1,2 yeast (Fig. 1A–C). Therefore, it is speculated that the CaMBD of AtCNGC1 and AtCNGC2 may have different binding affinities for yeast CaM. Studies were undertaken to examine this model further.

A FIP designed to report CaM binding to the CNGC CaMBDs was constructed and expressed. For comparison, studies were undertaken with the CaMBD of AtCNGC1 and AtCNGC2, and binding of the CaMBDs to yeast CaM as well as A. thaliana CaM2 and CaM8 was evaluated (Fig. 5). Results shown in Fig. 5 are consistent with the aforementioned model. Interaction of CaM with the FIP in these assays is inferred by the ability of CaM proteins to reduce the efficiency of FRET between BFP and GFP; the FRET efficiency change is indicated by a decrease in GFP emission (emission peak at 510 nm) following the addition of recombinant CaM (Hua et al., 2003b). Yeast CaM decreased FRET (i.e. GFP fluorescence emission with a peak at 510 nm) of FIP-AtCNGC1, denoting binding to the AtCNGC1 CaMBD (Fig. 5A, top pair of traces), while no physical interaction of yeast CaM with FIP-AtCNGC2 was noted (Fig. 5B, the top two traces, which are superimposed). Different results were noted when the FIP proteins were tested for binding to A. thaliana CaM proteins. Of the 10 A. thaliana isoforms, CaM2 represents a conserved sequence, while CaM8 is divergent. Arabidopsis thaliana CaM2 as well as CaM8 reduced FRET of both FIP-AtCNGC1 (Fig. 5A) and FIP-AtCNGC2 (Fig. 5B). As reported previously (Hua et al., 2003b), only minor changes in FRET in the absence of Ca\(^{2+}\) in these assays were observed (data not shown); the interaction of CaM with the CaMBD of plant CNGCs is Ca\(^{2+}\)-dependent (also see Arazi et al., 2000; Köhler and Neuhaus, 2000). Thus, it is concluded that AtCNGC1 and AtCNGC2 bind to A. thaliana CaM proteins, but only AtCNGC1 physically interacts with yeast CaM.

BLAST searches of the E. coli genome indicate the absence of CaM in this prokaryote; CNGCs as well are absent (Talke et al., 2003). Therefore, it is reasoned that E. coli could provide an alternative heterologous assay system (albeit with limitations inherent when a membrane protein native to eukaryotes is expressed in a prokaryote host (Uozumi, 2001) for plant CNGCs without the possible influence of any endogenous CaM. The series of experiments shown in Fig. 6 focused on functional analysis of AtCNGC1 and AtCNGC1M2 in the K\(^{+}\) uptake-deficient (i.e. trkG,H) E. coli strain LB650 (Schlosser et al., 1995). Results of liquid culture (Fig. 6A), serial dilutions of a drop assay on solid medium (Fig. 6B), and solid culture on streaked plates (Fig. 6D) are shown. Interestingly, the
relative effect of (full-length) AtCNGC1 versus the mutant construct AtCNGC1M2 that lacks a portion of the CaM binding domain on growth of this *E. coli* K⁺-uptake mutant is different from that shown for *trk1,2*. In these experiments, *E. coli* transformed with the full-length channel consistently grew better than when the *E. coli* was transformed with either the mutant channel AtCNGC1M2 or the empty vector. These results indicate that when expressed in a heterologous system lacking endogenous CaM, the full-length AtCNGC1 plant channel can function as well or better than a mutated construct with the CaMBD removed. These results are consistent with results shown in Figs 3 and 5, and support the hypothesis that, in the *trk1,2* yeast mutant, AtCNGC1 function may be inhibited by an endogenous factor such as yeast CaM. An antibody generated against a peptide corresponding to a presumed cytoplasmic portion of the AtCNGC1 protein was used to monitor expression of the channel in *E. coli* (Fig. 6C). A polypeptide at the predicted molecular weight of AtCNGC1 (83 kDa) that was immunoreactive with this antibody was noted in SDS-PAGE-fractionated protein from *E. coli* transformed with *AtCNGC1* that was not present in *E. coli* transformed with the empty vector (Fig. 6C). This result confirms expression of AtCNGC1 in this prokaryote. It should be noted that an immunoreactive protein corresponding to AtCNGC1M2 was not identified in extracts prepared from *E. coli* transformed with *AtCNGC1M2* (data not shown). It is speculated that the mutant channel AtCNGC1M2 was present at such low levels (perhaps because it was degraded at a relatively high rate) that it was below the detection level in these experiments. Even if the mutant channel AtCNGC1M2 was present at low levels (compared with AtCNGC1) in *E. coli*, it appears that it may be functional in this prokaryote. In liquid culture assays (Fig. 6A), as well as on streaked solid medium plates (Fig. 6D), *E. coli* transformed with *AtCNGC1M2* grew better than *E. coli* transformed with the empty vector [but see the drop assays (Fig. 6B, where no differences were noted between empty vector and *AtCNGC1M2*).

Attempts were made to test further the hypothesis that yeast CaM binds to AtCNGC1 by demonstrating protein–protein interactions between the AtCNGC1 CaMBD and yeast CaM using several different commercially and/or publicly available yeast two-hybrid assays (data not shown). Overexpression of yeast CaM (i.e. as the ‘bait’ in the two-hybrid assay) was found to inhibit growth of the yeast. These results are interpreted to suggest that overexpression of CaM perturbed metabolism in the yeast strains used for the two-hybrid assay; this system could not be used to support the protein–protein interactions between AtCNGCs and yeast CaM demonstrated in the work shown in Fig. 5.

To date, functional characterizations of plant CNGCs as ligand-gated cation channels relied on K⁺ conductance as a transport assay (Köhler *et al.*, 1999; Leng *et al.*, 1999, 2002; Balagué *et al.*, 2003; Hua *et al.*, 2003a, b; Mercier *et al.*, 2004). However, phenotypes of *Arabidopsis* plants with mutations in CNGC genes appear to be related to their possible role *in planta* as inwardly conducting Ca²⁺ transporters (Clough *et al.*, 2000; Sunkar *et al.*, 2000; Balagué *et al.*, 2003; Chan *et al.*, 2003). Recent work by Lemtiri-Chlieh and Berkowitz (2004) has suggested a role for CNGCs in * planta* as inward rectified Ca²⁺ channels. To date, only one report has demonstrated inward Ca²⁺ conductance by a plant CNGC (i.e. AtCNGC2) upon expression in a heterologous system (human embryonic kidney cells) (Leng *et al.*, 1999). Here, the utility of yeast as an assay for inward Ca²⁺ conduction by plant channels such as CNGCs is demonstrated.

Haploid yeast cells generate cytosolic Ca²⁺ signals in response to mating pheromone (i.e. either the ‘α’ or the ‘α’ factor) (Muller *et al.*, 2001). Yeast mutants with translational arrest of the endogenous Ca²⁺ transporters CCH1 and MID1 fail to generate such Ca²⁺ signals and thus exposure of this genotype to α factor results in growth arrest due to inactivation of cyclin-dependent kinase and reduced expression of G₁ cyclins (Ferrando *et al.*, 1995). Cells that cannot generate and/or perceive a cytosolic Ca²⁺ signal fail to grow on solid medium plates in the ‘diffusion halo’ surrounding a filter disc containing α factor (Ferrando *et al.*, 1995).
Growth of the cch1,mid1 mutant in the halo surrounding a filter disc containing \( \alpha \) factor was used as a functional assay of plant CNGCs as inwardly Ca\(^{2+} \)-conducting channels. Results are shown in Fig. 7. In this experiment, \( \alpha \) factor diffuses into the growth medium from the filter disc, preventing growth of yeast that cannot generate and/or perceive a cytosolic Ca\(^{2+} \) signal; the ‘dead zone’ lacking yeast colonies appears as an empty halo around the filter disc. At two different concentrations of \( \alpha \) factor, no colonies were evident around the filter disc when mid1,cch1 was transformed with either the empty vector or full-length AtCNGC1. Expression of AtCNGC1M2 in this yeast mutant, however, resulted in the growth of colonies in the halo around the filter disc. These results suggest Ca\(^{2+} \) permeates the AtCNGC1 pore. Further, these results support the contention that an endogenous factor in yeast inhibits the full-length AtCNGC1 protein while the AtCNGC1M2 channel is less affected. Whether the present CNGC functional assay using yeast monitors K\(^{+} \) (Figs 1C, 2, 3) or Ca\(^{2+} \) conduction, the full-length channel does not function well but, when a portion of the CaMBD is removed, the channel conducts K\(^{+} \) or, in this experiment, Ca\(^{2+} \). These results are the first demonstration (albeit indirectly) of Ca\(^{2+} \) permeability of the plant channel AtCNGC1.

In addition to hyg (Fig. 1A, B), trk1,2 yeast is hypersensitive to a number of other toxic cations such as spermine, tetramethylamine (TMA), and NH\(^{4+} \) (Mulet et al., 1999; Plant et al., 1999; Bihler et al., 2002; Erez and Kahana, 2002; Forment et al., 2002). It was reasoned that inclusion of these cations in the assay medium would reduce the basal level of trk1,2 growth (i.e. growth of trk1,2 transformed with the empty vector). It is suspected that the channels were not present at levels sufficient for immunodetection using the present antibody in these additional studies; the antibody raised against a short peptide sequence of AtCNGC1 may not be strongly immunogenic with the full-length protein. (D) Experimental protocol was similar to that for the work shown in (B) except that the overnight cultures were streaked on solid minimal medium containing 2 mM K\(^{+} \). Experiments shown in (A–D) were repeated twice (each) with similar results.
amiloride (Fig. 2), hyg (Fig. 1) or other toxic cations may improve the efficacy of such yeast mutants as heterologous assay systems for functional characterization of cloned plant CNGCs.

Depending on the assay medium used, prior studies of plant CNGC function using trk1,2 yeast have shown positive results either without (Köhler et al., 1999) or with (Mercier et al., 2004) addition of cyclic nucleotide to the assay medium. In a set of experiments, the effect of cyclic nucleotide (Bt2-cAMP, a lipophilic analogue of cAMP), was tested on trk1,2 yeast under the assay conditions used in the present studies. For these experiments, amiloride was present in the growth medium. It was found that addition of 100 μM Bt2-cAMP had no significant effect on growth or K⁺ concentration of trk1,2 transformed with either the empty vector or AtCNGC1 (data not shown). However, it was found that addition of Bt2-cAMP increased growth of trk1,2 yeast transformed with AtCNGC1M2 by 14% [from an OD of 1.80±0.11 (mean±SE) to an OD of 2.06±0.08]. For comparison, the OD of the empty vector trk1,2 in the presence of Bt2-cAMP was 0.44±0.13. Addition of Bt2-cAMP was also found to increase K⁺ concentration of AtCNGC1M2 yeast by 19% (i.e. from 197±3.8 mM to 223±6.1 mM; means±SE). This level of K⁺ found in AtCNGC1M2 yeast in the presence of Bt2-cAMP (223 mM) was also 28% higher than the K⁺ concentration of trk1,2 transformed with the empty vector (174±7.0) under similar assay conditions (i.e. in the presence of Bt2-cAMP). Analysis of variance indicates that the differences between

K⁺ concentration of AtCNGC1M2 yeast in the presence of Bt2-cAMP as compared with that of (i) AtCNGC1M2 yeast in the absence of Bt2-cAMP, (ii) empty vector yeast in the absence of Bt2-cAMP, and (iii) empty vector yeast in the presence of Bt2-cAMP were all statistically significant (P <0.01). These results are consistent with the possibility that the mutant construct (AtCNGC1M2) of the plant AtCNGC1 channel, which has a portion of the CaMBD deleted, still binds cAMP and that this mutant channel’s ability to complement the trk1,2 growth phenotype and increase K⁺ influx into trk1,2 yeast is enhanced (although only modestly) by addition of exogenous activating ligand cAMP.

Discussion

In the experiments included in this report, growth of trk1,2 yeast in APG medium with 50 mM K⁺ was used as a functional assay of plant CNGCs. It should be noted that prior studies of plant CNGCs using trk1,2 yeast evaluated growth on media with much lower K⁺ contents (Schuurink et al., 1998; Köhler et al., 1999; Leng et al., 1999; Mercier et al., 2004). However, the present APG medium has a much lower pH (~3.5) than that used in these prior studies. Trk1,2 yeast growth is sensitive to such a low pH in the growth medium (Mulet et al., 1999; Bilhler et al., 2002). In one prior study, growth of trk1,2 yeast at pH 3.5 was still not maximal even in the presence of 100 mM K⁺ (Calero et al., 2000). Thus, it is concluded that the present assay conditions (APG medium at pH 3.5 with 50 mM K⁺) were appropriate for evaluating function of plant CNGCs as inwardly conducting K⁺-permeable channels.

The present results indicate that the use of the trk1,2 mutant as a functional assay for plant K⁺-conducting channels can be improved by the addition of amiloride, an inhibitor of the yeast vacuolar Na⁺(K⁺)/H⁺ antiporter (see results with empty vector and AtCNGC1M2 yeast shown in Fig. 2). This finding suggests that after K⁺ enters the yeast cytosol through the heterologously expressed plant channel, movement of K⁺ (i.e. into the yeast vacuole through the action of the amiloride-sensitive vacuolar Na⁺(K⁺)/H⁺ antiporter, or alternatively out of the cell via endogenous yeast plasma membrane K⁺ efflux transporters) may impact on the efficacy of trk1,2 yeast as a functional assay of such K⁺ transport proteins. This point has not been considered in prior studies using this yeast mutant as a functional assay of plant K⁺-conducting channels. It is speculated that, in the presence of amiloride, a greater proportion of the K⁺ entering the cytoplasm in the trk1,2 yeast (either in the presence or absence of the heterologously expressed plant CNGC) would be available for efflux from the cell. The present studies do not rule out the possibility that amiloride may affect other yeast proteins besides NHX1, the vacuolar Na⁺(K⁺)/H⁺ antiporter. However, as shown in Fig. 2, inclusion of amiloride in the trk1,2 growth medium reduces
the growth of the yeast mutant transformed with the empty vector and increases the percentage stimulation of growth due to expression of plant CNGCs as compared with the empty vector control.

In addition to the effect of hyg on trk1,2 growth and use of the mutant for functional analysis of plant CNGCs (Fig. 1; also see Mercier et al., 2004), it is found that the presence of toxic cations such as spermine, TMA, or ammonium in the assay medium reduces growth of trk1,2 yeast transformed with an empty vector and increases the relative difference between growth of empty vector trk1,2 and the yeast mutant transformed with plant CNGCs (results mentioned in text). A large body of work (Mulet et al., 1999; Bihler et al., 2002; Erez and Kahana, 2002; Forment et al., 2002) demonstrates that trk1,2 yeast is hypersensitive to the aforementioned toxic cations. Thus, the present work suggests that inclusion of these compounds in the culture medium may improve the use of trk1,2 yeast as an assay of heterologously expressed, inwardly conducting, cation channels such as plant CNGCs. The present results with these toxic cations (as well as amiloride) are consistent with previous studies, indicating that they should reduce the basal level of trk1,2 (transformed with the empty plasmid) growth. However, as is the case with any inhibitor, interpretation of their effect on this assay system should be made with due caution, as these compounds may be having unknown affects on the yeast, as well as the plant, CNGCs.

In the report by Mercier et al. (2004), it was noted anecdotally that expression of AtCNGC1 did not complement hyg sensitivity of trk1,2 yeast as strongly as AtCNGC2 and AtCNGC4. Results presented here (Fig. 1) are consistent with this assertion. One possible explanation for the relatively weak complementation of trk1,2 hypersensitivity by AtCNGC1, as compared with AtCNGC2, is that yeast CaM binds to AtCNGC1, inhibiting channel activation. This hypothesis is supported by the results of experiments shown in Figs 2, 3, 5, 6, and 7. It is acknowledged that the possibility cannot be ruled out, from work presented here, that differences in growth of yeast transformed with AtCNGC1, AtCNGC2, and AtCNGC4 are due to a variable expression level of functional CNGC proteins. Prior studies have documented that CNGC mRNA is present in yeast transformed with AtCNGC1, AtCNGC2, and AtCNGC4 (Mercier et al., 2004). However, the level of message and/or functional CNGC protein could be different; hence the variation in extent of suppression of the trk1,2 growth phenotype could be affected by this factor.

Whether assayed for function using the K⁺ uptake-deficient trk1,2 yeast mutant (Figs 2, 3) or the Ca²⁺ uptake-deficient cch1,mid1 mutant (Fig. 7), deletion of a portion of the AtCNGC1 protein that binds CaM improved yeast performance, suggesting that endogenous CaM in yeast interacts with AtCNGC1. Consistent with this possibility, it was also found that addition of exogenous activating ligand (a lipophilic analogue of cAMP), beyond that already present in yeast, increased the growth and K⁺ concentration of trk1,2 yeast expressing the AtCNGC1 channel with the CaMBD removed (results mentioned in text), while the addition of the cAMP analogue had no significant effect on growth or K⁺ concentration of the yeast mutant transformed with either the empty vector or the wild-type channel AtCNGC1 (data not shown). Improvement of AtCNGC1 channel function by deletion of the CaMBD did not occur using an assay system devoid of endogenous CaM (i.e. expression in E. coli; Fig. 6). Protein–protein interaction studies (Fig. 5) confirmed binding of yeast CaM to AtCNGC1, while yeast CaM was not found to bind to AtCNGC2. When yeast mutants are used for functional analysis of plant proteins that are modulated by CaM, the possible confounding effect of yeast CaM on plant protein function should be considered. The results presented here with AtCNGC1M2 represent the first report that a plant CNGC can retain function as a channel protein with the CaM binding domain deleted. This interesting finding can provide the basis for future studies of the role CaM may play in regulating these channels in planta.

It should be noted that Köhler and Neuhaus (2000) reported results different from those shown here (Fig. 5) regarding A. thaliana CaM binding to AtCNGC1 and AtCNGC2. Using a yeast two-hybrid approach to evaluate protein–protein interactions, they found that CaM8 did not bind to either AtCNGC1 or AtCNGC2, and that CaM2 affinity for AtCNGC2 was far greater than for AtCNGC1. Other than the different assay systems employed, there is no explanation for their differing results regarding A. thaliana CaM isoform binding to AtCNGC1 and AtCNGC2. However, in their work employing the yeast two-hybrid system, the binding of A. thaliana CaMs to CNGCs was done in a ‘background’ of endogenous yeast CaM. It is interesting to note that they report far greater binding of A. thaliana CaMs to AtCNGC2 than AtCNGC1; in the case of CaM2, there is ~40-fold greater binding to AtCNGC2. Perhaps, endogenous yeast CaM bound to AtCNGC1 in their work and influenced the extent of binding of A. thaliana CaMs to AtCNGC1. In the present case, the focus is on differences in yeast CaM binding to AtCNGC1 and AtCNGC2; Köhler and Neuhaus (2000) did not evaluate yeast CaM binding to the CNGCs.

Numerous experiments presented in this report demonstrate that the AtCNGC1M2 mutant channel is functional (in yeast as well as E. coli). However, an interesting finding with a different mutant construct of AtCNGC1 is noted. Although the greatest stimulation in trk1,2 growth (compared with both the empty vector and AtCNGC1 wild-type channel) was found from the AtCNGC1M2 deletion construct, positive effects were also found from AtCNGC1M3 (Fig. 3), suggesting that even with more of the CNBD deleted, the channel is still functional in the present yeast assay. This finding contrasts with work by Sunkar et al.
(2000) with the CNGC NtCBP4 in tobacco. NtCBP4 is a tobacco homologue of AtCNGC1. They expressed (using the 35S promoter) a carboxyl-terminal deletion construct of NtCBP4 in tobacco. Their NtCBP4 deletion was nearly identical to the AtCNGC1M3 mutant construct in this study; their construct had one less amino acid, corresponding to the Gln601 in AtCNGC1—the present AtCNGC1M3 has this residue. They found that tobacco plants expressing the NtCBP4 carboxyl-terminal deletion had a Pb$^{2+}$ uptake-related phenotype similar to Arabidopsis plants that had a mutation in the AtCNGC1 gene. They interpreted their results as suggesting that the NtCBP4 carboxyl-terminal deletion was not functional, and in planta, formed tetrameric channels with native NtCBP4, rendering the channel inactive. Thus, if their conclusion is correct, the present result with AtCNGC1M3 expression in yeast differs from their findings with the tobacco homologue. One possible explanation for this difference is that the native channels in tobacco could be composed of more than one CNGC subunit, and further, that the high-level expression of their NtCBP4 carboxyl-terminal deletion driven by the 35S promoter affected channels formed by other CNGC polypeptides. They did not directly test whether or not their NtCBP4 carboxyl-terminal deletion was functional when forming homomeric channel complexes, as must be the case in the present experiments with AtCNGC1M3 in yeast. Nonetheless, this difference is noted.

The present results with expression of AtCNGC1 (i.e. the AtCNGC1M2 construct with the CaMBD deleted) in mid1,cch1 yeast are the first demonstration of this plant channel conducting Ca$^{2+}$. The possibility is acknowledged that deletion of the CaMBD from the carboxyl-terminal region of AtCNGC1 could somehow affect the geometry of the channel pore selectivity filter, thus altering the selectivity of the channel for ions. However, mutational analysis of both animal (Flynn et al., 2001) and plant (Hua et al., 2004a) CNGCs indicates that selectivity for different cations is due to the amino acids that comprise the selectivity filter in the pore region, and not by the cytosolic carboxyl-terminal region of the protein. Many of the published phenotypes of plants with mutations preventing expression of CNGC proteins are related to their possible function in the plant as Ca$^{2+}$-conducting channels (Clough et al., 2000; Sunkar et al., 2000; Balague et al., 2003; Chan et al., 2003). However, there is currently little direct evidence in the literature demonstrating Ca$^{2+}$ conductance by the translation product of a plasma membrane-localized plant ion channel gene and linking the Ca$^{2+}$-conducting function of the specific channel with a Ca$^{2+}$-related phenotype of a plant mutated in the corresponding gene. In the case of AtCNGC1, Sunkar et al. (2000) noted that Pb$^{2+}$ is currently hypothesized to move into plants through Ca$^{2+}$-conducting channels; they demonstrated that A. thaliana AtCNGC1 mutant plants had reduced Pb$^{2+}$ uptake. The demonstration here that AtCNGC1 conducts Ca$^{2+}$ using a heterologous system (Fig. 7) provides experimental support for this model. White and colleagues (White et al., 2002; White and Broadley, 2003) have reviewed the evidence identifying ‘VICCs’ (voltage-independent non-selective cation channels) as contributing to Ca$^{2+}$ influx into plant cells, as well as work pointing to CNGCs as possible genes that encode these channels. Results presented here are consistent with the possibility that AtCNGC1 may be an inwardly conducting non-selective cation channel permeable to Ca$^{2+}$. In summary, the work presented in this report identifies a number of factors in the yeast cell, and conditions in the assay medium, that could affect the efficacy of the trk1,2 mutant as an assay system for plant K$^{+}$-conducting channels such as CNGCs. Conditions that reduce growth of the trk1,2 mutant when growth is dependent on endogenous channels improve the assay in terms of the relative effect of the plant channel on growth. These conditions include addition of toxic cations (hyg, spermine, TMA, and NH$^{+}_4$), as well as amiloride (which may block K$^{+}$ uptake in the yeast vacuole). All of these additions reduce growth of the trk1,2 mutant. In addition, it appears that there are factors in the yeast cell which also affect the efficacy of this assay system. Endogenous yeast CaM, and perhaps cyclic nucleotides in the yeast cytosol, may affect activity of the plant CNGC. It is concluded that plant CNGCs can be functionally characterized using yeast mutants as demonstrated in this work.

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