

Activation of a plant plasma membrane Ca^{2+} channel by $\text{TG}\alpha 1$, a heterotrimeric G protein α -subunit homologue

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Abstract Wild-type and GTPase-deficient recombinant $\text{TG}\alpha 1$ were used along patch-clamp techniques to study the role of heterotrimeric G proteins in the regulation of the hyperpolarized active tomato plasma membrane Ca^{2+} channel. Recombinant α -subunits induced an increase in channel activity as shown by the increase in channel events and the mean open probability of the channel. Our results suggest a membrane-delimited pathway involving heterotrimeric G proteins in Ca^{2+} channel activation.

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Key words: Heterotrimeric G protein; Plasma membrane Ca^{2+} channel; $\text{TG}\alpha 1$; Signal transduction

1. Introduction

The existence and the possible function of G proteins in plants has been increasingly documented [1,2]. G proteins have been shown to be involved in the response of plants to pathogens [3–5], in the control of K^+ channel opening in guard cells [6–9], the transmission of red and blue light-induced signals [10,11], and secondary messenger regulation [12,13]. G proteins act as molecular signal transducers where active or inactive states depend on the binding of GTP or GDP, respectively. The G proteins include two major subfamilies, the heterotrimeric G proteins and the small G proteins. Whereas the heterotrimeric G proteins contain α -, β - and γ -subunits, the small G proteins appear to be similar to free α -subunits, operating without the $\beta\gamma$ heterodimer. Generally, it is the α -subunit of the G protein that has the receptor binding region and possesses a guanosine nucleotide binding site and GTPase activity [14,15]. Both classes of G proteins use the GTP/GDP cycle as a molecular switch for signal transduction. Interaction of the G protein with the activated receptor promotes the exchange of GDP, bound to the α -subunit, for GTP and the subsequent dissociation of the α -GTP complex from the $\beta\gamma$ heterodimer.

Genes encoding plant homologues α - and β -subunits of the heterotrimeric G protein family have been cloned. Ma et al. [16] reported a *GPA1* gene coding for an *Arabidopsis thaliana* α -subunit and several *GPA1* homologues have been also identified in tomato [17], rice [18] and other plants [1]. Genes encoding for a β -subunit have also been cloned from *Arabidopsis* and maize [19].

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Evidence supporting the participation of G proteins in signal transduction in plants is indirect and is based mainly on the effect of biochemical agents affecting G protein functions. These include non-hydrolysing GTP analogues and AlF_4^- that induce a conformational change in the α -subunit that promotes the dissociation of the trimeric complex and the association with the effector(s). Bacterial toxins, such as cholera and pertussis toxins, and mastoparan, isolated from wasp venom, have been also used to probe G protein function in plant cellular processes ([1,2], and references therein).

We have recently characterized a plasma membrane Ca^{2+} channel in tomato cells that was activated by fungal elicitors, and our results suggested that the activation of the channel by fungal elicitors could be modulated by G proteins [20,21]. Here we used wild-type as well as GTPase-deficient recombinant $\text{TG}\alpha 1$ and patch-clamp techniques to study the role of G proteins in the regulation of the tomato plasma membrane Ca^{2+} channel.

2. Materials and methods

2.1. Plant material

Growth of tomato cell suspensions and preparation of protoplasts were as previously described [21]. Cytosolic and subcellular membrane fractions were prepared as described previously [22].

2.2. Cloning and mutagenesis of the *TGAI* cDNA

Subcloning was carried out by PCR using Vent DNA polymerase (New England Biolab) and *TGAI* cDNA as a template. Two oligonucleotides: *tga1* (5'-TATGGATCCATGGGCTCGTTGTGC-3') and *tga2* (5'-GCTGAATTCTCATAGTAAACCTGC-3') were designed for the amplification of the *TGAI* coding sequence for in-frame cloning with GST into the *Bam*HI/*Eco*RI sites in the pGEX-2TK vector (Pharmacia). PCR-based point mutagenesis was carried out as described [23] with some modifications. An oligonucleotide containing the base substitution 5'-GTTGGAGGTCGAGAAATGAG-3' was used together with primer *tga2* to amplify a 506 bp fragment corresponding to the 3' region of *TGAI*. The product was purified from an agarose gel using the geneclean III kit (Bio101) and used as a primer in a second PCR reaction along with primer *tga1* to generate the full-length coding sequence of *TGAI* carrying the one base-pair substitution. The wild-type and mutagenized PCR products were blunt-end ligated into pGEM-7Zf(+) digested with *Sma*I. The inserts were subsequently digested using *Bam*HI/*Eco*RI and ligated into pGEX-2TK cut with those same enzymes. All constructs were sequenced to confirm mutagenesis and fidelity.

2.3. Overexpression and purification of wild-type and mutant *TGAI*

Wild-type and mutagenized constructs, cloned in pGEX-2TK, were used to transform *E. coli* BL21 (pLysS). An overnight culture grown in LB medium containing 50 $\mu\text{g}/\text{ml}$ ampicillin was diluted 1:100 into 800 ml of LB medium and grown at 30°C for 2 h. Isopropyl β -D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce recombinant protein expression. Three h after induction, cells were collected by centrifugation, and bacterial pellets were resuspended in a buffer containing 50 mM Na-HEPES pH 8.0, 200 mM NaCl and 0.1 mM PMSF. Following lysis of the bacterial

pellet and DNase I treatment, the fusion protein was purified from the bacterial extract using glutathione affinity chromatography as per manufacturer's instructions (Pharmacia). To isolate cleaved TG α 1, thrombin (Pharmacia) was used for the proteolytic release of TG α 1 from GST directly on the affinity column as per manufacturer's instructions (Pharmacia).

2.4. GTP hydrolysis assays

GTPase activity was determined as described by Graziano et al. [24]. The reaction mixture included 1 μ M [γ - 32 P]GTP (5000 Ci/mmol, Amersham) in a buffer containing 50 mM Na-HEPES pH 8.0, 2 mM MgSO $_4$, 1 mM DTT. Reactions were initiated by the addition of 20 pmol TG α 1. Release of [32 P]P $_i$ was measured using a 5% Norite buffer according to Brandt et al. [25]. For GTP binding and hydrolysis assays on nitrocellulose membranes, 2 μ g of purified GST, wild-type TG α 1, and mutant TG α 1-Q223L fusion proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Assays were performed as described elsewhere [26]. One μ Ci [γ - 32 P]GTP or [α - 32 P]GTP (5000 Ci/mmol, Amersham) per ml was used.

2.5. Production of polyclonal anti-TG α 1 antibodies

Polyclonal antibodies were generated by the conventional method from a New Zealand White rabbit injected with recombinant TG α 1 as an antigen. Antibodies were affinity-purified using immunoblots as described by Harlow and Lane [27].

2.6. Measurement of single channel currents in isolated protoplasts

For single channel measurements, protoplasts were kept in a glass bathing chamber containing 50 mM CaCl $_2$, 0.1 mM potassium glutamate, 1 mM MgSO $_4$, 5 mM Tris-MES buffer (pH 6.1) and sorbitol to give a final osmolarity of 450 mOsmol. Glass pipettes pulled with a vertical puller (Adams and List) from borosilicate glass capillaries (Kimax-51, VWR) coated with silicone (Sigmacote, Sigma) and fire polished, had a tip resistance of 5–10 M Ω when filled with 100 mM potassium glutamate, 1 mM MgSO $_4$, 3 mM Mg-ATP, 0.05 mM CaCl $_2$ +0.1 mM BAPTA to give a final free Ca $^{2+}$ concentration of 100 nM [28], 5 mM Tris-MES buffer (pH 7.3) and sorbitol with a final osmolarity of 475 mOsmol. GTP, and recombinant proteins were added at the concentrations indicated in the figure legends. Outside-out patches of plasma membranes were obtained after the whole-cell configuration mode [20] by quickly pulling the pipette away from the protoplasts. Single channel currents were measured at 23°C in voltage-clamp mode using a Dagan 3900 amplifier (Dagan Corporation) digitized on line (TL-1 DMA Interface; Axon Instruments), stored on a 386 based 33 MHz computer and acquired and analyzed with pClamp 6.0.2 software (Axon Instruments). Data were filtered with a four-pole Bessel filter at 200 Hz, digitized at 2 kHz and stored on disk. Open channel probabilities (P_o) were calculated with amplitude histograms obtained from single channel recordings as described previously [20].

3. Results

3.1. Overexpression and purification of recombinant TG α 1 proteins

It has been shown previously that the substitution of a conserved glutamine residue in the GTPase domain of different G proteins resulted in a constitutively active α -subunit [24]. A comparison of the amino acid sequences of TG α 1 with several α -subunits from other heterotrimeric G proteins revealed a glutamine at position 223 (Fig. 1, upper panel). This residue was substituted by a leucine by site-directed mutagenesis of the TG α 1 and the resultant clone designated as Q223L.

The putative open reading frame of the *TGAI* gene product and the Q223L mutant were cloned into the pGEX-2TK expression vector, and these vectors were used to transform *E. coli* BL21 pLysS cells. SDS-PAGE of bacterial proteins showed the presence of a \approx 66 kDa protein only in extracts from bacteria carrying the pGEX-*TGAI* and the pGEX-

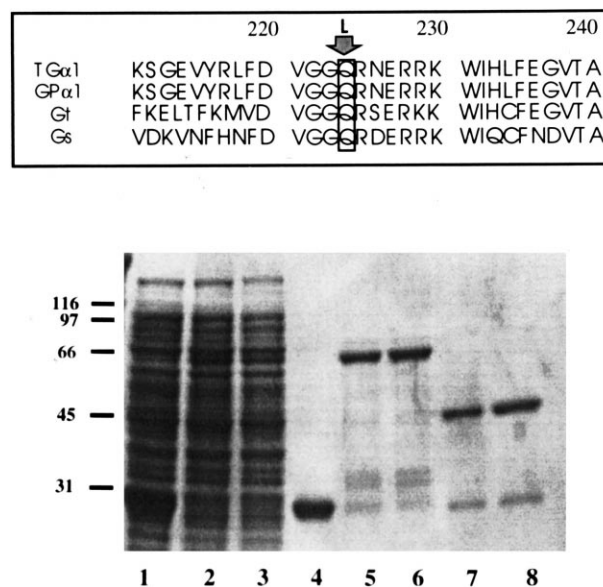


Fig. 1. Purification of recombinant TG α 1-WT and TG α 1-Q223L. Upper panel: Sequence alignment of the deduced TG α 1 amino acid sequence with other known α -subunits from *Arabidopsis thaliana* (GP α 1), stimulatory α -subunit (G $_s$) and transducin (G $_t$). The conserved Gln residue (223) was mutated to Leu in TG α 1 to produce the mutant TG α 1-Q223L. Numbers at the top of the panel refer to the amino acid positions in TG α 1. Lower panel: Coomassie blue-stained 10% SDS-PAGE showing the purification of the different gene products from total bacterial proteins. Lanes 1–3: bacterial lysates from pGEX (1), pGEX-2TK-TG α 1-WT (2), pGEX-2TK-TG α 1-Q223L (3); lanes 4–6: purified recombinant GST fusion proteins, GST alone (4), GST-TG α 1-WT (5), GST-TG α 1-Q223L (6); lanes 7–8: purified recombinant proteins after thrombin cleavage, TG α 1-WT (7) and TG α 1-Q223L (8).

TGAI-Q223L constructs (Fig. 1, lanes 1–3). Both pGEX-*TGAI* and the pGEX-*TGAI*-Q223L constructs were designed to express the proteins as a GST (glutathione S-transferase) fusion protein with GST at their N-terminal end. This allowed the purification of the GST fusion proteins by affinity chromatography on a glutathione column (Fig. 1, lanes 4–6). Furthermore, the pGEX-2TK vectors contain a thrombin-specific cleavage site between GST and the desired product, allowing the subsequent proteolytic release of the wild-type and *TGAI* gene products resulting in a 45 kDa TG α 1 recombinant protein (Fig. 1, lanes 7–8).

3.2. GTP hydrolysis and GTP binding by the recombinant TG α 1 proteins

The predicted TG α 1 sequence showed high homology to known α -subunits of heterotrimeric G proteins. GTP binding proteins have a characteristic high affinity to guanosine nucleotides and specifically bind and hydrolyze GTP. To assess the GTP hydrolytic activity of the recombinant proteins, enzyme-dependent GTP hydrolysis was measured using [γ - 32 P]GTP as a substrate (Fig. 2). The rate of GTP hydrolysis by TG α 1 was 1.5–3 pmoles Pi- μ g protein $^{-1}$ min $^{-1}$ and the K_{cat} of GTP hydrolysis was 0.08–0.13 mole Pi-mole protein $^{-1}$ min $^{-1}$. The rate of GTP hydrolysis by the TG α 1-Q223L mutant was negligible within the time course of the experiments (Fig. 2). No significant hydrolysis was detected when using [γ - 32 P]ATP as an alternative substrate (not shown).

The lack of GTP hydrolytic activity by the TG α 1-Q223L

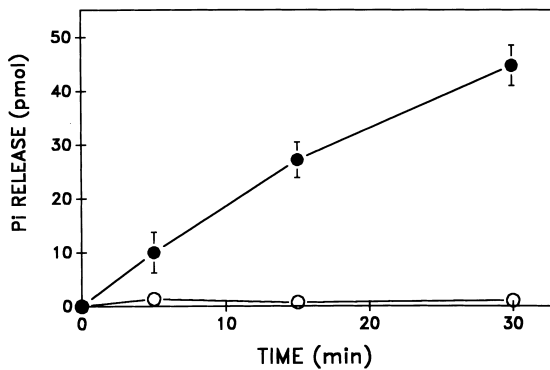


Fig. 2. GTP hydrolysis by recombinant TG α 1-WT and TG α 1-Q223L. Enzyme-dependent hydrolysis was monitored as described in Section 2. Twenty pmol of either TG α 1-WT (●) or TG α 1-Q223L (○) were used. Values are mean \pm S.D. ($n=3$).

mutant could be due to its inability to either hydrolyze GTP or a significantly reduced affinity for GTP. In order to characterize the mutation, GTP binding and GTP hydrolytic assays were carried out on nitrocellulose membranes to which the GST fusion proteins were transferred. The nitrocellulose membranes were incubated in the presence of either [α - 32 P]GTP or [γ - 32 P]GTP and the resulting autoradiogram allowed for the visualization of the assay (Fig. 3). Coomassie blue-stained gels after SDS-PAGE of GST, TG α 1-WT and TG α 1-Q223L GST fusions showed that equal amounts of protein were used in the experiments (Fig. 3a). The incubation of the proteins with [α - 32 P]GTP showed that both wild-type and mutant proteins were able to bind GTP (Fig. 3b). A 66 kDa radiolabeled protein of similar intensity was detected in both wild-type and mutant fusion proteins, suggesting a similar GTP binding capacity. Autoradiograms of nitrocellulose membranes incubated with [γ - 32 P]GTP showed a 66 kDa radiolabeled protein with a markedly increased intensity in the TG α 1-Q223L, indicating that GTP was still bound to the mutated enzyme (Fig. 3c).

3.3. Subcellular localization of TG α 1

A polyclonal antibody, affinity-purified from the serum of a rabbit injected with recombinant TG α 1, was used to determine the subcellular location of the protein. Western blot analysis, using the anti-TG α 1 antibody and different subcellular fractions from tomato cell suspensions, was performed

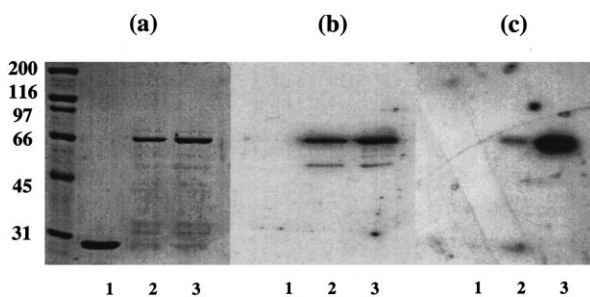


Fig. 3. GTP binding and hydrolysis by recombinant enzymes transferred to nitrocellulose membranes. a: Coomassie blue-stained 10% SDS-PAGE. b: Autoradiogram of proteins transferred to a nitrocellulose membrane and incubated with [α - 32 P]GTP for 30 min as described in Section 2. c: As in b but proteins were incubated with [γ - 32 P]GTP. Lane 1: molecular markers; lane 2: 2 μ g GST; lane 3: 2 μ g GST-TG α 1-WT; lane 4: 2 μ g TG α 1-Q223L.

(Fig. 4). A 45 kDa protein, corresponding to the size of the recombinant TG α 1 was detected in microsomal, ER and plasma membrane fractions (Fig. 4b). No immunoreactivity was observed in the cytosolic fraction and a faint immunoreactive protein was detected in the tonoplast fraction, probably due to contamination by other endomembranes.

3.4. Regulation of the tomato plasma membrane Ca $^{2+}$ channel by TG α 1

Our previous work has indicated that G proteins may play a role in the activation of tomato plasma membrane Ca $^{2+}$ channels by fungal elicitors [20,21]. These channels are activated by hyperpolarized membrane potentials [20] and the presence of the fungal elicitor resulted in a greater probability of channel opening [21]. The elicitor stimulation of the channel activity was abolished by GDP(β S), whereas GTP(γ S) and mastoparan produced a similar effect to that observed in the presence of the fungal elicitor [21]. To examine the effect of G proteins on the tomato plasma membrane Ca $^{2+}$ channel, single channel recordings were measured at a holding potential of -180 mV from outside-out patches of plasma membrane. The membranes were exposed to 50 mM Ca $^{2+}$ in the bathing solution with GTP and recombinant TG α 1-WT or TG α 1-Q223L in the pipette. Fig. 5 shows some original single channel recordings from isolated patches of membranes and the corresponding amplitude histograms. Under control conditions, channel activity was detected at membrane potentials more negative than -100 mV. We have shown previously that the downward deflections corresponded to the movement of Ca $^{2+}$ into the cytosol [20,21]. Amplitude histograms of the single channels revealed at least one channel in the patch of membrane with an amplitude of -1 pA (Fig. 5). In the presence of 20 μ M GTP, the channel events became more frequent

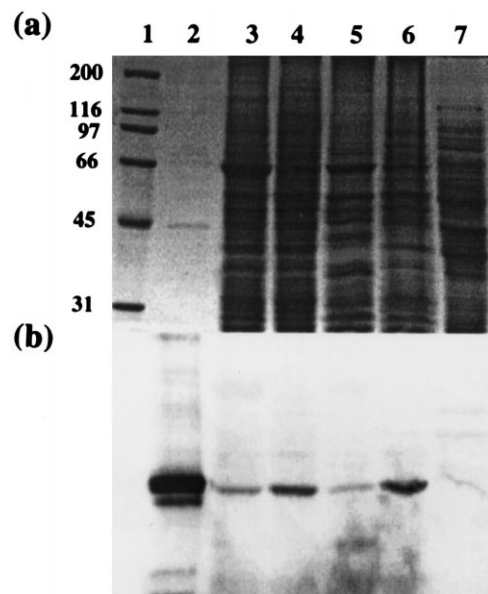


Fig. 4. Immunological detection of TG α 1 in different subcellular fractions. a: Coomassie blue-stained 10% SDS-PAGE of cytosolic and membrane fractions. Thirty μ g of each fraction were loaded. One hundred ng of purified TG α 1 was used as a positive control. b: Western immunoblot using an affinity-purified anti-TG α 1. Lane 1: molecular markers; lane 2: recombinant TG α 1; lane 3: microsomal fraction; lane 4: plasma membranes; lane 5: tonoplast; lane 6: ER; lane 7: cytosol.

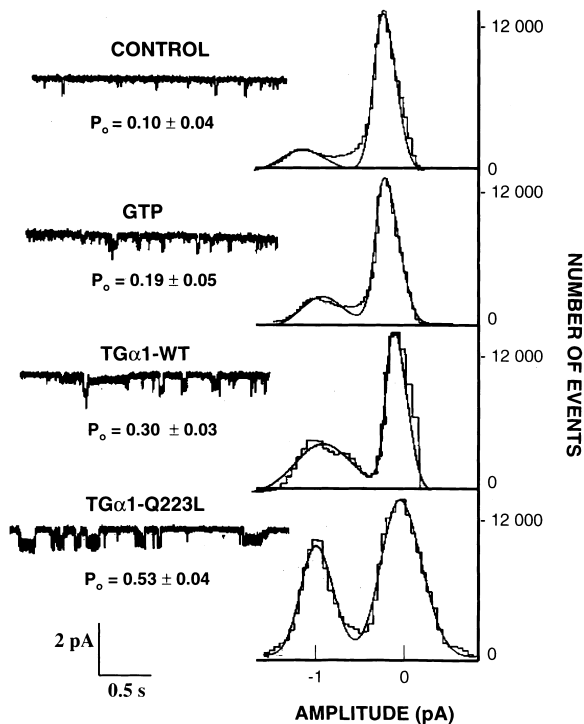


Fig. 5. Effect of GTP and recombinant TG α 1-WT and TG α 1-Q223L on plasma membrane Ca²⁺ channels. Single channel recordings from outside-out patches of plasma membrane maintained at -180 mV. Channel recordings were measured in the absence (control) or presence of $20 \mu\text{M}$ GTP (GTP) and in the presence of $20 \mu\text{M}$ GTP and 1 pmol/ml of wild-type (TG α 1-WT) or recombinant (TG α 1-Q223L) proteins in the pipette. Each downward deflection corresponds to the opening of one channel event allowing the influx of Ca²⁺ into the cytosol. Amplitude histograms were constructed from similar signal channel recordings and used to calculate P_o values. The area under the peak corresponding to 0 pA represents all the channels in the closed state while the area under the second peak (-1 pA) represents the total number of channels in the open state. P_o values under the signal channel recordings are the means \pm S.D. ($n=6$).

and a two-fold increase in the mean open probability (P_o) was observed. The presence of recombinant α -subunits in the pipette resulted in a significant increase in channel activity as shown by the increase in channel events and by the increase in the mean P_o , from 0.19 to 0.30 and 0.53 in the presence of TG α 1-WT and TG α 1-Q223L, respectively (Fig. 5). The presence of GST alone did not induce either an increase in channel events or P_o (not shown). The single channel conductance (11 pS) did not significantly change in the presence of the recombinant α -subunits; thus single channel current levels remained unchanged.

4. Discussion

The tomato *TGAI* cDNA has a predicted amino acid sequence showing significant homology to a G protein α -subunit. Its cloning into an expression vector facilitated its over-expression and purification from bacterial cells. The recombinant product, after thrombin proteolysis to release the GST fragment from the TG α 1 fusion protein, resulted in a 45 kDa protein (Fig. 1). This falls within the 35 – 45 kDa size range of known α -subunits from animals [1] and corresponds to the size of immunologically related polypep-

tides detected in plant membranes [3,5,19]. The K_{cat} of GTP hydrolysis is lower than the rate described for most of the mammalian α -subunits (2.2 – 3.5 min^{-1}) [24,29], but in the same range as those reported for two recombinant rice α -subunits (0.0075 and 0.44 min^{-1}) [30,31].

While the binding of GTP was equivalent in both TG α 1-WT and TG α 1-Q223L, no significant GTP hydrolysis could be detected in TG α 1-Q223L (Fig. 3). It has been proposed that the Q \rightarrow L substitution does not directly alter the rate of GTP hydrolysis, but it affects a 'switch' mechanism that mediates the conformational transition between the GTP- and GDP-bound forms of the enzyme [32]. The recent crystallization of G α in a heterotrimeric complex demonstrated the conformational changes in G α upon activation [33]. While the glutamine (Gln-223) was shown to stabilize the transition to a GTP hydrolysis mode, the conserved glycine (Gly-222) residue adjacent to the glutamine (Gln-223) in TG α 1 (Fig. 1) interacted with the γ phosphate of GTP, triggering the 'active' conformational change [33].

Western blot analysis of different subcellular fractions indicated that TG α 1 is predominantly associated with plasma membrane and ER (Fig. 4). These results are consistent with those reported for the *Arabidopsis* GP α 1 [34]. No signal was observed in the cytosolic fractions, indicating that TG α 1 is exclusively membrane associated, as would be expected for a heterotrimeric G protein.

We have shown previously the activation of tomato plasma membrane Ca²⁺ channels by GTP(γ)S and mastoparan [21]. Our previous work also showed that the activation of G proteins by GTP(γ)S and mastoparan mimicked the effect of fungal elicitors on the Ca²⁺ channel activity and that GDP(β)S abolished the effect of fungal elicitors [21]. These results provided indirect evidence suggesting the activation of the channel by G proteins. Our data now strongly support our initial observations. Although our results do not demonstrate a direct physical interaction between the plasma membrane Ca²⁺ channel and the heterotrimeric G protein α -subunit, they support the notion of a membrane-delimited pathway for the activation of the Ca²⁺ channel. In isolated patches of membranes, both recombinant TG α 1-WT and TG α 1-Q223L proteins activated the plasma membrane Ca²⁺ channel by affecting the P_o , but not the channel conductance. In conclusion, our current and previous findings demonstrate the regulation of tomato plasma membrane Ca²⁺ channels by G proteins and suggest their possible role in the response of tomato cells to fungal pathogens.

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