Determination of Structural Regions Important for Ca\(^{2+}\) Uptake Activity in Arabidopsis MCA1 and MCA2 Expressed in Yeast

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(Received July 5, 2011; Accepted August 30, 2011)

MCA1 is a plasma membrane protein that correlates Ca\(^{2+}\) influx and mechanosensing in Arabidopsis. MCA2 is a paralog of MCA1, and both share 72.7% amino acid sequence identity and several common structural features, including putative transmembrane (TM) segments, an EF hand-like region in the N-terminal half, a coiled-coil motif in the middle and a PLAC8 motif in the C-terminal half. To determine structural regions important for Ca\(^{2+}\) uptake activity, the activity of truncated forms of MCA1 and MCA2 was assessed using yeast expression assays. The N-terminal half of MCA1 with a coiled-coil motif (MCA1\(^{1-237}\)) did not have Ca\(^{2+}\) uptake activity, while MCA2\(^{1-237}\) did. The N-terminal half of MCA1 without the coiled-coil motif (MCA1\(^{1-185}\)) showed Ca\(^{2+}\) uptake activity, as did MCA2\(^{1-186}\). Both MCA1\(^{1-173}\) and MCA2\(^{1-173}\) having the EF hand-like region had Ca\(^{2+}\) uptake activity. Deletion of a putative TM segment (Ile11–Ala33) and the Asp21 to asparagine mutation in MCA1 and MCA2 abolished Ca\(^{2+}\) uptake activity. Finally, MCA1\(^{173-421}\) and MCA2\(^{173-416}\) lacking the N-terminal half had no Ca\(^{2+}\) uptake activity. These results suggest that the N-terminal half of both proteins with the EF hand-like region is necessary and sufficient for Ca\(^{2+}\) uptake and that the coiled-coil motif regulates MCA1 negatively and MCA2 positively.

**Keywords:** Arabidopsis thaliana • Ca\(^{2+}\) channel • Ca\(^{2+}\) transport • Heterologous expression • Saccharomyces cerevisiae • Structure–function relationship.

**Abbreviations:** DTT, dithiothreitol; GFP, green fluorescent protein; HA, hemagglutinin; MCA, mid1-complementing activity; β-ME, β-mercaptoethanol; MS, mechanosensitive; ORF, open reading frame; TM, transmembrane; TRP, transient receptor potential.

**Introduction**

Ca\(^{2+}\)-permeable mechanosensitive (MS) channels or stretch-activated channels open to incorporate or mobilize Ca\(^{2+}\) into the cytoplasm in response to mechanical stimuli, such as touch, bending and gravity, and are thus postulated to be key components of mechanosensors in plants (Pickard and Ding 1993, Trewavas and Knight 1994, Perbal and Driss-Ecole 2003, Braam 2005, Teleswki 2006, Humphrey et al. 2007, Monshausen and Gilroy 2009). For example, touch and gravity induce an immediate and transient increase in \([Ca^{2+}]_{cyt}\) (Knight et al. 1991, Plieth and Trewavas 2002), and gadolinium, a blocker of ion channels, including MS channels, diminishes the gravity-induced \([Ca^{2+}]_{cyt}\) increase (Toyota et al. 2008). Cytoplasmic Ca\(^{2+}\) activates Ca\(^{2+}\)-binding proteins that eventually induce the expression of a set of genes to respond to a respective stimulus (Chehab et al. 2009). In contrast to bacterial and animal MS channels, including MscL, MscS and transient receptor potential (TRP) channels (Kung 2005), plant MS channels have long been poorly characterized; however, Msc-like proteins (MSLs) were found recently in Arabidopsis (Arabidopsis thaliana) (Haswell and Meyerowitz 2006, Haswell et al. 2008). In addition, MCA1 and MCA2 (locus names, At4g35920 and At2g17780, respectively) were found that might be related to MS channel activity in Arabidopsis (Nakagawa et al. 2007, Yamanaka et al. 2010).

MCA1 and MCA2 share 72.7% identity and a total of 89.4% similarity in amino acid sequence (see Fig. 1) and have the following same structural features. The N-terminal half has a region similar to the putative regulatory region of many rice putative protein kinases and an EF hand-like region. The C-terminal half possesses 2–4 putative transmembrane (TM) segments and a cysteine-rich domain of unknown function,
called the PLAC8 or DUF614 motif (Galaviz-Hernandez et al. 2003) (Fig. 1). In addition, there is a coiled-coil motif between the N- and C-terminal halves. Both proteins are localized in the Arabidopsis plasma membrane, as revealed by fluorescence microscopy with green fluorescent protein (GFP)-fused constructs (Nakagawa et al. 2007, Yamanaka et al. 2010). Irrespective of these structural similarities, tissue-specific expression and physiological function are not necessarily the same between the two proteins (Yamanaka et al. 2010). While MCA1 and MCA2 are expressed commonly in vascular tissues of cotyledons, leaves and the primary root, as well as at the center of rosettes in a region corresponding to the shoot apical meristem, MCA1 is not expressed in mesophyll cells of leaves and cotyledons where MCA2 is expressed. In addition, MCA1 is expressed in the promeristem and adjacent elongation zone of the primary root where MCA2 is not expressed. Functionally, MCA1, but not MCA2, is responsible for sensing and/or responding to the hardness of agar in the primary root, while MCA2, but not MCA1, is responsible for Ca\textsuperscript{2+} uptake in the root. Since MCA1 and MCA2 have no similarity in amino acid sequence to known peripheral membrane proteins, such as NaCl, Na\textsubscript{2}CO\textsubscript{3} and urea, the non-ionic detergent Triton X-100 and the ionic detergent SDS, and then subjected to centrifugation to separate the sample into soluble and membrane fractions. As shown in Fig. 3, in control experiments with distilled water, a significant amount of MCA2 was present in the membrane fraction, while Pma1 was predominantly present in the membrane fraction, and a cytoplasmic marker protein, enolase, in the soluble fraction. Considering the relative amounts between MCA2 and the two marker proteins in each fraction after each treatment, MCA2 in the membrane fraction appeared not to be solubilized by treatments with NaCl, Na\textsubscript{2}CO\textsubscript{3} and urea. Furthermore, MCA2 was not completely solubilized by Triton X-100, while it was thoroughly solubilized by SDS. These results suggest that a significant amount of MCA2 is present as an integral membrane protein in yeast cells, while a considerable amount of this protein is present in the soluble fraction. These results are not necessarily consistent with those of MCA1: the majority of MCA1 is present in the plasma membrane fraction as an integral membrane protein in yeast cells (Nakagawa et al. 2007). This result with MCA2 would explain why our fluorescent microscopic analysis failed to localize MCA2–GFP to yeast cellular membranes: it is conceivable that a considerable amount of MCA2–GFP was present as a soluble form. 

**Results**

**Membrane localization of MCA2 in yeast cells**

We determined previously that MCA1 expressed under the control of the yeast constitutive TDH3 promoter was localized in the yeast plasma membrane (Nakagawa et al. 2007). In the present study, we first determined the subcellular localization of MCA2 in yeast cells to compare the function of MCA1 and MCA2 using the same yeast expression system as the previous one, although our previous study has shown that MCA2 is localized in the Arabidopsis plasma membrane and is required for Ca\textsuperscript{2+} uptake from the root (Yamanaka et al. 2010). The MCA2 open reading frame (ORF) was placed under the control of the TDH3 promoter and expressed in yeast cch1 mid1 double mutant defective in the high affinity Ca\textsuperscript{2+} influx system, like the MCA1 ORF (Yamanaka et al. 2010). Total cell extracts were subjected to membrane fractionation using stepwise sucrose density gradient centrifugation. After centrifugation, the gradient was fractionated and the presence of MCA2 in each fraction was examined by SDS–PAGE followed by Western blotting. Fig. 2 shows that part of the MCA2 proteins expressed was present in the fractions where the plasma membrane marker protein Pma1 was included, while the majority of the MCA2 proteins appeared to be co-fractionated with the vacuolar marker protein Pho8. This result is not completely consistent with the previous observation that MCA2–GFP is present in the Arabidopsis plasma membrane (Yamanaka et al. 2010) and suggests that ectopic expression results in heterologous distribution of MCA2 in yeast cellular membranes. We also examined the subcellular localization of MCA2–GFP expressed under the control of the TDH3 promoter using a confocal fluorescence microscope. However, we failed to localize MCA2–GFP in yeast cellular membranes because GFP fluorescence from the cytoplasm interfered with that from cellular membranes (data not shown).

We next examined whether MCA2 is present as an integral membrane protein in yeast cells. Total cell extracts were treated with distilled water (control), ionic reagents that solubilize peripheral membrane proteins, such as NaCl, Na\textsubscript{2}CO\textsubscript{3} and urea, and non-ionic detergent Triton X-100 and the ionic detergent SDS, and MCA2 proteins appeared to be co-fractionated with the vacuolar marker protein Pho8. This result is not completely consistent with the previous observation that MCA2–GFP is present in the membrane fraction, while Pma1 was predominantly present in the membrane fraction, and a cytoplasmic marker protein, enolase, in the soluble fraction. Considering the relative amounts between MCA2 and the two marker proteins in each fraction after each treatment, MCA2 in the membrane fraction appeared not to be solubilized by treatments with NaCl, Na\textsubscript{2}CO\textsubscript{3} and urea. Furthermore, MCA2 was not completely solubilized by Triton X-100, while it was thoroughly solubilized by SDS. These results suggest that a significant amount of MCA2 is present as an integral membrane protein in yeast cells, while a considerable amount of this protein is present in the soluble fraction. These results are not necessarily consistent with those of MCA1: the majority of MCA1 is present in the plasma membrane fraction as an integral membrane protein in yeast cells (Nakagawa et al. 2007). This result with MCA2 would explain why our fluorescent microscopic analysis failed to localize MCA2–GFP to yeast cellular membranes: it is conceivable that a considerable amount of MCA2–GFP was present as a soluble form.

**MCA1 and MCA2 form homo-oligomers**

Since MCA1 and MCA2 have 19 and 20 cysteine residues, respectively, it is possible that both proteins are oligomerized homologously and/or heterologously by disulfide bonding. To examine this possibility, we performed non-reducing SDS–PAGE followed by Western blotting of total cell extracts prepared from yeast cells expressing MCA1 and MCA2 separately or together.

When expressed separately, MCA1 and MCA2 appeared as a monomer (45 kDa), dimer (90 kDa) and tetramer...
Fig. 1 Structural features of MCA1 and MCA2. (A) Scheme of MCA proteins. MCA1 and MCA2 are composed of 421 and 416 amino acid (aa) residues, respectively. The ARPK domain (Nakagawa et al. 2007) has homology to the regulatory domain of rice putative kinases, but its function is unknown. A C-terminal, cysteine-rich region similar to the PLAC8 motif found in plant and animal proteins is also of unknown function. Two thick lines connected by a thin line above the sequence represent a helix-loop-helix structure similar to the EF hand motif, as predicted by the Network Protein Sequence Analysis (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html). The shaded box represents the coiled-coil motif. Bars indicate the position of the potential transmembrane (TM) segments predicted mainly by the online program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/). (B) Sequence alignment of MCA1 and MCA2 with ClustalW (version 1.83). Amino acid sequence identity (and total similarity) between MCA1 and MCA2 is 72.7% (89.4%). Asterisks indicate identical amino acids; colons indicate amino acids with strong similarity; dots indicate amino acids with weak similarity. (C) Helical wheel models of residues Ile11–Ala33 of MCA1 and MCA2, based on the online program Helical Wheel Projections (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi). Black circles represent highly hydrophilic residues.
A trimer was not detected in this experiment. MCA1 generated an additional 110 kDa band, which was smaller than the predicted trimer (135 kDa). This could be a complex of MCA1 with a yeast cellular protein. Under reducing conditions, the vast majority of both proteins appeared as a monomer and a very small amount appeared as a dimer. Similar results were obtained with stepwise diluted dithiothreitol (0–1.0 mM DTT) as a reducing reagent (Supplementary Fig. S1).

To examine possible hetero-oligomer formation between MCA1 and MCA2, each was tagged with four tandemly arrayed hemagglutinin (HA) antigens to produce MCA1-HA and MCA2-HA, which were then co-expressed with MCA2 and MCA1, respectively, in yeast cells, so that a hetero-oligomer of different molecular size could be detected on Western blots. Both proteins without the HA tag were 45 kDa and those with the tag were 60 kDa. Then, if only a homo-dimer is formed, bands of 90 and 120 kDa must be detected. On the other hand, if a hetero-dimer is formed, a band of 105 kDa must be detected. The results showed that for a dimer, 90 and 120 kDa bands were detected, but a 105 kDa band was not (Supplementary Fig. S2), suggesting that MCA1 and MCA2 form a homo-oligomer but not a hetero-oligomer in yeast cells.

Relative Ca\(^{2+}\) uptake activity between MCA1 and MCA2

In our previous study, we noticed that MCA2 mediates greater Ca\(^{2+}\) uptake than MCA1 in yeast cells, when expressed under the control of the TDH3 promoter on a multicopy plasmid (Yamanaka et al. 2010). It is possible that this difference is due to a difference in the activity or amount of proteins in yeast cells. To distinguish between the two possibilities, we used MCA1-HA and MCA2-HA because the antibody against HA should detect the two proteins at the same sensitivity. Fig. 5A shows that addition of the HA tag did not seriously interfere with MCA1 and MCA2 activities mediating Ca\(^{2+}\) uptake (*P = 0.78; **P = 0.13, respectively). Although the average activity of MCA2-HA appeared to be approximately 1.4 times as high as that of MCA1-HA, this difference was not significant (**P = 0.25). On the other hand, Western blotting showed that the amount of MCA1-HA was approximately
Fig. 4 Immunodetection of oligomer formation of MCA1 and MCA2. Total cell extracts containing MCA1 or MCA2 were treated at 95°C with or without 2% β-mercaptoethanol in SDS sample buffer and analyzed by SDS–PAGE using 5–15% polyacrylamide gradient gels followed by Western blotting. (A) Detection of MCA1 homo-oligomers with the antibody Apep3. (B) Detection of MCA2 homo-oligomers with the antibody Bpep4.

Fig. 5 Expression and activity levels of MCA1 and MCA2. (A) MCA1 and MCA2 have the ability to increase Ca^{2+} accumulation when expressed on a multicopy plasmid. MCA1 and MCA2 cDNAs on a multicopy plasmid were expressed under the control of the TDH3 promoter in the mid1 mutant. Ca^{2+} accumulation was measured for 2 h under the conditions described in the Materials and Methods. Data are the mean ± SD for the three or four independent experiments. *P = 0.78, MCA1-HA vs. MCA1; **P = 0.13, MCA2-HA vs. MCA2; ***P = 0.25, MCA1-HA vs. MCA2-HA; ****P = 0.004, MCA1 vs. MCA2. (B) Expression level of MCA1-HA and MCA2-HA. Both proteins were expressed from a multicopy plasmid and detected with the monoclonal anti-HA antibody 12CA5. The intensity of the bands was quantified using Image J and the relative level was normalized to 1 in MCA2-HA. Data are the mean ± SD for the three independent experiments. *P = 0.03.
2.2 times as high as that of MCA2-HA (Fig. 5B; P = 0.03). Therefore, the specific activity of MCA2-HA can be estimated to be higher than that of MCA1-HA in yeast cells. It is noted that the average activity of untagged MCA2 was approximately 2.0 times as high as that of untagged MCA1 (Fig. 5A, ****P = 0.0004), although the amount of the two proteins cannot be compared because there is no antibody that reacts with the two proteins with the same reactivity. Hereafter, HA-tagged constructs were generally used for the convenience of detecting truncated forms of MCA1 and MCA2.

**Truncation analyses of MCA1 and MCA2**

To elucidate the function of each domain of the MCA proteins, we made a series of C-terminally HA-tagged mutants lacking the N-terminal or C-terminal region and expressed them under the control of the TDH3 promoter on multicopy plasmids in the yeast cch1 mid1 mutant (Fig. 6A). Note that, to avoid lengthy names of the constructs, the name of the tag, HA, was not added to the constructs. MCA1-135 (amino acids 1–135) and MCA2-135 (amino acids 1–135) have the ARPK domain only. MCA1-173 (amino acids 1–173) and MCA2-173 (amino acids 1–173) have the ARPK domain and the EF hand-like region. MCA1-185 (amino acids 1–185) and MCA2-186 (amino acids 1–186) also have the ARPK domain and the EF hand-like region. MCA1-237 (amino acids 1–237) and MCA2-237 (amino acids 1–237) have the ARPK domain, the EF hand-like region and the coiled-coil motif. Finally, MCA1-342 (amino acids 173–421) and MCA2-342 (amino acids 173–416) have the coiled-coil and PLAC8 motifs. Plasmids carrying these constructs were then introduced into cells of the cch1 mid1 mutant and the transformants were incubated for 3 d. Fig. 6B shows that no transformant was obtained when the MCA1-173 and MCA2-173 plasmids were used to transform the host cells, while plasmids carrying the other truncation produced transformants at a reasonable transformation efficiency. This result suggests that MCA1-173 and MCA2-173 are toxic to yeast cells, while the other mutant proteins are not.

To confirm the toxicity of MCA1-173 and MCA2-173, we established yeast cells expressing each protein under the control of the GAL1 promoter, which can be repressed by glucose and induced by galactose. For this purpose, we used a GAL1 strain as a host, in which the GAL1 promoter is inducible by galactose. The toxicity of MCA1-173 and MCA2-173 was examined by growing yeast cells overnight in glucose medium and spot testing on galactose plates. Fig. 6C shows that the growth of cells expressing MCA1-173 and MCA2-173 was restricted on galactose plates compared with glucose plates. The degree of growth restriction was more severe in MCA2-173-expressing cells than in MCA1-173-expressing cells.

To examine whether MCA1-173 and MCA2-173 kill cells or arrest cell proliferation, we confirmed the expression of both proteins (Fig. 7A) and then measured the viability and number of cells after shifting to galactose medium from glucose medium. Fig. 7B shows that MCA1-173-expressing cells maintained 80% viability and MCA2-173-expressing cells 70% viability until 48 h after shifting to galactose medium. During this period, both MCA1-173-expressing and MCA2-173-expressing cells grew at low rates compared with control cells (Fig. 7C). We speculated that the low growth rate of MCA1-173-expressing and MCA2-173-expressing cells was related to Ca<sup>2+</sup> accumulation. The Ca<sup>2+</sup> accumulation assay, using a GAL1 mid1Δ strain defective in Ca<sup>2+</sup> uptake activity as a host, showed that both MCA1-173-expressing and MCA2-173-expressing cells had Ca<sup>2+</sup> uptake activity and that MCA2-173-expressing cells had extremely higher activity than MCA1-173-expressing cells (Fig. 7D).

To investigate which region is important for the activity of mediating Ca<sup>2+</sup> uptake, we also assayed Ca<sup>2+</sup> accumulation in cells expressing the truncated mutants described above, in addition to MCA1-173 and MCA2-173 (Fig. 8). MCA1-185 and MCA2-186 showed no and partial activity, respectively. MCA1-185 and MCA2-186 showed partial and full activity, respectively. MCA1-237-expressing cells showed increased Ca<sup>2+</sup> uptake activity, while MCA1-237-expressing cells did not. Both MCA1-342 and MCA2-342 displayed no activity. Part of each truncated protein expressed in yeast cells, including MCA1-135, MCA2-135, MCA1-173, MCA2-173, MCA1-237, MCA2-237, MCA1-342 and MCA2-342, was shown to be present as an integral membrane protein (Fig. 9). Among them, MCA2-135 was hardly present in the soluble fraction following NaCl wash (Fig. 9B) and retained Ca<sup>2+</sup> uptake activity (Fig. 8). We suppose that MCA2-135 may have a structural feature that is susceptible to stabilization of the assembled form a putative channel at high ionic strength. In addition, we noted that MCA1-185 and MCA2-186 were present almost exclusively as integral membrane proteins. Since MCA1-173 and MCA2-173 were not completely integrated into membranes, the C-terminal regions of MCA1-185 and MCA2-186 ranging from Ser174 to Pro185 and Thr174 to Pro186, respectively, may play a positive role in membrane integration. A high efficiency of membrane integration of MCA1-185 and MCA2-186 may be the basis of their significant Ca<sup>2+</sup> uptake activity (Fig. 8).

Taken together, the following are suggested: in MCA1, the ARPK domain with the EF hand-like region has the activity of mediating Ca<sup>2+</sup> uptake. The C-terminal half is necessary for full activity and the coiled-coil motif has a negative effect on activity. In MCA2, the ARPK domain only has partial activity, the coiled-coil motif has a positive effect and the C-terminal half is unnecessary for full activity.

**Secondary structure analysis of the N-terminal region**

In our previous study, we predicted that MCA1 and MCA2 have at least two TM segments in the C-terminal region containing the PLAC8 motif (Nakagawa et al. 2007). However, our present study showed experimentally that MCA2-135, MCA1-173, MCA2-173, MCA1-185, MCA2-186 and MCA2-237, all of
Fig. 6 Analysis of truncation mutants lacking the N-terminal or C-terminal regions of MCA1 and MCA2. (A) Location of a series of truncation mutants lacking the N-terminal or C-terminal region of MCA1 and MCA2. A summary of the Ca$^{2+}$ uptake activity of the mutants is shown. (B) Cells of strain H319 (cch1 mid1) constitutively expressing MCA11–173 and MCA21–173 exhibit a lethal phenotype. Cells were incubated for 3 d at 30°C on SD plates. (C) Spot testing on galactose-based SG plates of cells of strain KA31-1A expressing MCA11–173 or MCA21–173 under the control of the GAL1 promoter, which is repressed by glucose and induced by galactose. Cells were incubated for 3 d at 30°C on SD (Glucose) or SG (Galactose) plates. #1–3 represent the transformant number.
which are N-terminal regions, have the activity of mediating Ca\(^{2+}\) uptake and can be present as integral membrane proteins (Figs. 8, 9A, B, 10). This prompted us to analyze the secondary structure of the N-terminal region with online programs. Using the secondary structure prediction program SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), we found that MCA2 has a putative TM segment (Ile11–Ala33). The MCA1 sequence corresponding to the MCA2 Ile11–Ala33 segment is 82.6% identical in amino acid sequence, but was not predicted as a TM segment. In contrast, the ConPred_v2 program in the Aramemnon database (http://aramemnon.uni-koeln.de/) predicted that essentially the same region (Ala12–Ala32) is a putative TM segment for MCA1 but not for MCA2. Analysis by the HMMTOP method using the Quick2D toolkit (http://toolkit.lmb.uni-muenchen.de/quick2_d) predicted that essentially the same region (Ile11–Val30) was a putative TM segment in both MCA1 and MCA2. On the other hand, the Phobius program (http://phobius.sbc.su.se/) did not predict this region as a significant TM segment in either protein, although it could be a TM segment with low probability. A reason for inconsistency between the TM prediction programs could be attributed to biochemical features of this segment. It is pointed out that pore-forming segments of ion transporters are too difficult to predict as being TM regions because they have polar amino acid residues (Möller et al. 2001). Indeed, the segments of both MCA1 and MCA2 have four highly polar residues (Fig. 1C).

![Figure 7](https://academic.oup.com/pcp/article-abstract/52/11/1915/1832032)
Irrespective of this contradiction in prediction, part of both the truncated MCA1 and MCA2 proteins was found to be present as integral membrane proteins (Fig. 9). Taken together, we suggest that the Ile11–Ala33 or Ile11–Val30 region is a TM segment.

To investigate whether the Ile11–Ala33 region (designated TM1) plays a key role in Ca\(^{2+}\) uptake, we constructed deletion mutants of this region, named MCA1\(^{1–185}\)/C1\(^{TM1}\) and MCA2\(^{1–237}\)/C1\(^{TM1}\), and examined their activity to mediate Ca\(^{2+}\) uptake. The result showed that no activity was detected in either deletion mutant protein, although their expression levels were not significantly changed compared with those of their wild-type counterparts (Fig. 10A, B). High-speed centrifugation showed that part of both deletion mutant proteins expressed was detected in the membrane fraction (Fig. 10C), although their amount was slightly smaller than that of their wild-type counterparts, especially in MCA1\(^{1–185}\)/C1\(^{TM1}\) compared with MCA1. Subcellular fractionation experiments revealed that part of both deletion mutant proteins was present in the fractions containing Pma1. However, the majority of the proteins were found to be in other fractions containing Pho8 (Supplementary Figs. S3A, B). This distribution pattern is similar to that of wild-type MCA2 (Supplementary Fig. S3F), but not to that of wild-type MCA1 (Supplementary Fig. S3E). Thus, it is possible that deletion of TM1 from MCA1 results in mislocalization to some degree.

Helical wheel analysis demonstrated the amphipathic nature of the Ile11–Ala33 region. Fig. 1C shows that highly hydrophilic residues, such as aspartate, lysine and glutamine in MCA1 and MCA2, as well as asparagine in MCA2, are lined up on one side of the helix. It is conceivable that this side faces the lumen of putative ion channels because of its hydrophilic property. In the pore domain of voltage-gated Ca\(^{2+}\) channels, a highly conserved glutamate or aspartate residue is important for Ca\(^{2+}\) coordination (Hofmann et al. 1994, Catterall 1995). In the Ile11–Ala33 region of both MCA1 and MCA2, Asp21 is the only acidic amino acid residue and is found to be highly conserved among MCA orthologs (Supplementary Fig. S4). We therefore tested the possibility that Asp21 plays a role in Ca\(^{2+}\) uptake. To do this, Asp21 in the full-length MCA1 and MCA2 was replaced with asparagine to produce MCA1\(^{D21N}\) and MCA2\(^{D21N}\), respectively. Fig. 10A shows that no Ca\(^{2+}\) uptake activity was detected in MCA1\(^{D21N}\) and partial activity was detected in MCA2\(^{D21N}\), although the levels of the mutant proteins were not reduced (Fig. 10B). High-speed centrifugation and subcellular fractionation experiments for MCA1\(^{D21N}\) and MCA2\(^{D21N}\) gave results similar to those of MCA1 and MCA2 (Fig. 10C, Supplementary Fig. S5).

**Discussion**

**Structural features of MCA1 and MCA2 and their functional implication**

In the present study, we have shown using the yeast expression system that MCA1 and MCA2 form oligomeric structures, including a dimer and tetramer that is the largest detected. Since this was demonstrated by non-reducing SDS–PAGE...
followed by Western blotting, it is possible that these oligomers were produced mainly by disulfide bonding. In addition, co-expression experiments with SDS–PAGE under non-reducing conditions have suggested that MCA1 and MCA2 form homo-oligomers individually, but not hetero-oligomers (Supplementary Fig. S2). This finding is rather unexpected because the two proteins share 72.7% identity and 89.4% similarity in amino acid sequence. However, the exclusive homo-oligomer formation could be reasonable because our previous studies have shown that MCA1 and MCA2 have some distinct roles in planta (Yamanaka et al. 2010). For example, MCA1 is mainly involved in touch sensing at the root tip and bolting, while MCA2 is involved in Ca^{2+} uptake in the root (Nakagawa et al. 2007, Yamanaka et al. 2010). Their expression

![Figure 9](https://academic.oup.com/pcp/article-abstract/52/11/1915/1832032)
is also distinct in some tissues. MCA1 is expressed in the pro-
meristem and adjacent elongation zone of the primary root
where MCA2 is not expressed. Additionally, MCA1 is not ex-
pressed in mesophyll cells where MCA2 is expressed. This dif-
ferential expression in planta together with homo-oligomer
formation in yeast cells suggest that MCA1 and MCA2 function
individually in plant cells, even though both proteins are
co-expressed in some tissues, such as vascular tissues of roots,
inflorescences and leaves.

The presence of the cysteine-rich PLAC8 or DUF614 motif in
MCA1 and MCA2 does not necessary imply that this motif is
responsible for homo-oligomer formation through disulfide
bonding. No data supporting this implication have been re-
ported so far. Instead, the cysteine-rich region has been postu-
lated to be a TM segment. Arabidopsis PCR1 (for plant
cadmium resistance 1) and maize CNR1 (for cell number regu-
lator 1), both of which are evolutionarily related to MCA1 and
MCA2 because of the presence of the PLAC8 motif, have a
cysteine-rich region in the motif (Song et al. 2004, Guo et al.
2010). PCR1 and CNR1 have two putative TM segments in the
motif, and one of the two segments (nearest the N-terminus)
has the CCXXXXCPC domain in PCR1 or the CLXXXXCPC

Fig. 10 Ca\(^{2+}\) accumulation activity and expression of MCA1\(^{ΔTM1}\), MCA2\(^{ΔTM1}\), MCA1\(^{D21N}\) and MCA2\(^{D21N}\). Ca\(^{2+}\) accumulation assays and
Western blotting were performed as described in Materials and Methods. (A) Ca\(^{2+}\) accumulation in cells expressing MCA1\(^{ΔTM1}\), MCA2\(^{ΔTM1}\),
MCA1\(^{D21N}\) and MCA2\(^{D21N}\). Data are the mean ± SD for the three independent experiments. *P = 0.837, MCA1\(^{ΔTM1}\) vs. the vector; *P = 0.430,
MCA1\(^{D21N}\) vs. the vector; *P = 0.811, MCA2\(^{ΔTM1}\) vs. the vector; **P = 0.020, MCA2\(^{D21N}\) vs. the vector. (B) Expression levels of MCA1\(^{ΔTM1}\),
MCA2\(^{ΔTM1}\), MCA1\(^{D21N}\) and MCA2\(^{D21N}\). Each protein was expressed from a multicopy plasmid and detected with the monoclonal anti-HA
antibody 12CA5. (C) Examination of TM1 mutant proteins for their ability to integrate into membranes. Experiments were carried out as
described in the legend to Fig. 3.
The N-terminal region is necessary and sufficient for mediating Ca\(^{2+}\) uptake

The most remarkable finding in this study is that MCA1\(^{1–173}\) and MCA2\(^{1–173}\) have the activity of mediating Ca\(^{2+}\) uptake (Fig. 7D). To explain this activity, two possible mechanisms are conceivable: one possibility is that the proteins themselves form channels and the other is that they activate an intrinsic Ca\(^{2+}\)-permeable ion channel in yeast. Two lines of evidence favor the former possibility. First, our experiments using ionic reagents that solubilize peripheral membrane proteins have shown that part of the MCA1\(^{1–173}\) and MCA2\(^{1–173}\) proteins is present as an integral membrane protein in yeast cells (Fig. 9). In addition, slightly longer constructs MCA1\(^{1–186}\) and MCA2\(^{1–186}\), which also possess Ca\(^{2+}\) uptake activity (Fig. 8), have been shown to be predominantly present as integral membrane proteins. Therefore, the truncated MCA1 and MCA2 proteins would function as integral membrane proteins to incorporate Ca\(^{2+}\). It is unlikely that the truncated proteins chelate Ca\(^{2+}\) because MCA2\(^{1–135}\), which has Ca\(^{2+}\) uptake activity, has no potential Ca\(^{2+}\)-binding motif.

Secondly, TM segment prediction with some online programs has shown that these four constructs have a TM segment in the most N-terminal position. Deletion of this segment, designated TM1 herein, from the full-length MCA1 and MCA2 proteins led to a complete loss of Ca\(^{2+}\) uptake activity (Fig. 10A). Helical wheel analysis demonstrated the amphiphilic nature of TM1 (Fig. 1C). Interestingly, the highly hydrophilic residues in TM1 line the surface of the helix, and hydrophobic residues are clustered on the opposite face. Thus, when MCA1\(^{1–173}\) and MCA2\(^{1–173}\) assemble in the membrane, the TM1 and MCA2 proteins localize to the plasma membrane? There are three Ca\(^{2+}\) transporters in the vacuole that function as a Ca\(^{2+}\) store in Saccharomyces cerevisiae cells (Ton and Rao 2004, Cunningham 2011): Pmc1, Ca\(^{2+}\)-ATPase responsible for detoxification of the cytosolic Ca\(^{2+}\) by valuar sequestration; Vcx1, a H\(^{+}\)/Ca\(^{2+}\) exchanger whose function is the same as that of Pmc1; and Yvc1, a homolog of the mammalian TRP channel mediating Ca\(^{2+}\) release from the vacuole in response to hyperosmotic shock. For the stimulation of Ca\(^{2+}\) influx across the plasma membrane, the [Ca\(^{2+}\)]\(_{cyt}\) of MCA1- or MCA2-expressing cells should be lower than normal levels. In this context, it is possible to speculate that MCA1 and MCA2 activate Pmc1 and/or Vcx1 to lower [Ca\(^{2+}\)]\(_{cyt}\) while it is unlikely that MCA1 and MCA2 activate Yvc1. Also, it is unlikely that MCA1 and MCA2 function as vacuolar channels because if both proteins act as channels, Ca\(^{2+}\) should be released from the vacuole to the cytoplasm to increase [Ca\(^{2+}\)]\(_{cyt}\) due to the concentration gradient of Ca\(^{2+}\) across the vacuolar membrane.

Comparison of their primary structures clearly reveals that MCA1 and MCA2 do not resemble structurally typical ion channels, such as voltage-gated ion channels, cyclic nucleotide-gated ion channels, glutamate receptor ion channels or TRP channels. Untypical ion channels with considerably short amino acid sequences have been reported. For example, Alzheimer’s disease amyloid β-protein, AβP(1–40), composed of 40 amino acid residues, forms cation channels with variable conduction states which lead to three models of channel structure comprised of four or six protein molecules (Durell et al. 1994,
Kawahara et al. 1997). The coat protein of the filamentous bacteriophage Pf3 of *Pseudomonas aeruginosa*, composed of 44 amino acid residues, forms voltage-gated ion channels with a tetrameric α-helix bundle (Pawlak et al. 1994, Kiefer and Kuhn 1999). MCA1 and MCA2 might constitute a new family of proteins mediating Ca^{2+} uptake in plants. A BLAST search showed the presence of MCA orthologs in plants, such as tracheophytes and bryophytes, but not in bacteria, fungi and animals (data not shown).

**Differential regulation of the function of MCA1 and MCA2 by intramolecular motifs**

On the basis of the MCA1 and MCA2 truncation experiments, the coiled-coil motif seems to have a different effect on the activity of the two proteins. MCA1<sub>1–237</sub> containing both the EF hand-like region and coiled-coil motifs did not show Ca^{2+} uptake activity, whereas the same construct of MCA2, MCA2<sub>1–237</sub>, showed increased activity compared with wild-type MCA2 (Fig. 8). Deletion of the coiled-coil motif from these constructs, producing MCA1<sub>1–185</sub> and MCA2<sub>1–186</sub>, resulted in both proteins differentially.

Regulation by the EF hand-like region may also be different between the two proteins. Deletion of this region, producing MCA1<sub>1–237</sub> containing both the EF hand-like and coiled-coil motifs may regulate the activity of MCA1 and MCA2 differentially, although the primary structure brings about a large difference in their activity. Another possibility is that heterologous expression may result in differential modification of the intrinsic properties of MCA1 and MCA2 because of a slight difference in amino acid residues. To clarify these points, a future study should include similar experiments because of a slight difference in amino acid residues. To clarify modification of the intrinsic properties of MCA1 and MCA2 differentially, although the primary structure is 72.7% identical and 89.4% similar between the two proteins? It is possible that a slight difference in the amino acid sequence between the two proteins; the antibody (Apep1) raised against MCA1-peptide 1 specifically recognized the MCA1 protein; the antibody (Apep2) raised against MCA1-peptide 2 specifically recognized both the MCA1 and MCA2 proteins; the antibody (Apep3) raised against MCA1-peptide 3 specifically recognized the MCA1 protein; and the antibody (Bpep4) raised against MCA2-peptide 4 specifically recognized the MCA2 protein, as revealed by Western blot analysis of total cell extracts of yeast transformants expressing MCA1 or MCA2.

**Preparation of anti-MCA1 and MCA2 antibodies**

PCR fragments encoding the MCA1 peptide spanning from Lys31 to Arg120 (MCA1-peptide 1) (Nakagawa et al. 2007), Asp132 to His398 to His421 (MCA1-peptide 2) and Gln393 to His421 (MCA1-peptide 3) were cut with BamHI and SalI and inserted into pQE30 (QIAGEN, Inc.) to be conjugated with a 6 × His tag at the N-terminus of MCA1-peptide 1, 2 and 3. PCR fragments encoding the MCA2 peptide spanning from Gly398 to Gln416 (MCA2-peptide 4) were treated as above. The 6 × His-tagged MCA1-peptide 1, 2 and 3 and MCA2-peptide 4 were purified from cell extracts of an isopropyl-β-D-thiogalactopyranoside (IPTG)-treated *Escherichia coli* transformant (strain JM109) carrying this plasmid, using Ni-NTA agarose beads (QIAGEN) under denaturing conditions. Rabbit polyclonal antisera against these peptides were prepared by Promega Co. The antibody (Apep1) raised against MCA1-peptide 1 specifically recognized the MCA1 protein; the antibody (Apep2) raised against MCA1-peptide 2 specifically recognized both the MCA1 and MCA2 proteins; the antibody (Apep3) raised against MCA1-peptide 3 specifically recognized the MCA1 protein; and the antibody (Bpep4) raised against MCA2-peptide 4 specifically recognized the MCA2 protein, as revealed by Western blot analysis of total cell extracts of yeast transformants expressing MCA1 or MCA2.

**Materials and Methods**

**Yeast strains and media**

The parental strain used was H207 (MATα his3Δ1 leu2-3,112, trp1-289 ura3-52 sst1-2 gal–) (Iida et al. 1994). The following mutant strains were derivatives of H207: H311 (mid1Δ5–Δ5::HIS3) (Tada et al. 2003) and H319 (mid1Δ5–Δ5::HIS3 cch1Δ5–Δ5::HIS3) (Nakagawa et al. 2007). Note that the mid1 single and mid1 cch double mutants show the same phenotype regarding Ca^{2+} uptake activity. For galactose induction experiments, KA31-1A (MATα his3 leu2 trp1 ura3 GAL+) and its derivative KA31-D22 (mid1Δ4–Δ4::HIS3) (Iida et al. 1994) were used. SD and SD.Ca100 media, which contain 681 and 100 μM CaCl<sub>2</sub> as a Ca^{2+} source, respectively, were described previously (Iida et al. 1990, Iida et al. 1994). The constituents of SG and SG.Ca100 media were the same as those of SD and SD.Ca100 media, except that 5% galactose was included instead of 1% glucose as a carbon source. Strain H207 and its derivatives were transformed with multicopy plasmids: YEptDHXho-MCA1 (Nakagawa et al. 2007), YEptDHXho-MCA2 (Yamanaka et al. 2010), YEpt-MCA1-HA4, YEpt-MCA2-HA4 or the empty vector YEptDHXho. Strains KA31-1A and KA31-D22 were transformed with YEpgAL1pMCA1 (1–173), YEpgAL1pMCA2 (1–173) or the empty vector YEpgAL1pADH3::HA ‘HA4’ written in the above plasmids represents a quadruple HA epitope tag, and ‘HA’ has been attached to the gene products MCA1 and MCA2 in the text when necessary. In most experiments in this study, the multicopy plasmids generally were used. Therefore, the word ‘multicopy’ was not described, unless otherwise noted. Yeast transformation was performed by a method described previously (Mount et al. 1996).

**Preparation of anti-MCA1 and MCA2 antibodies**

PCR fragments encoding the MCA1 peptide spanning from Lys31 to Arg120 (MCA1-peptide 1) (Nakagawa et al. 2007), Asp132 to His398 to His421 (MCA1-peptide 2) and Gln393 to His421 (MCA1-peptide 3) were cut with BamHI and SalI and inserted into pQE30 (QIAGEN, Inc.) to be conjugated with a 6 × His tag at the N-terminus of MCA1-peptide 1, 2 and 3. PCR fragments encoding the MCA2 peptide spanning from Gly398 to Gln416 (MCA2-peptide 4) were treated as above. The 6 × His-tagged MCA1-peptide 1, 2 and 3 and MCA2-peptide 4 were purified from cell extracts of an isopropyl-β-D-thiogalactopyranoside (IPTG)-treated *Escherichia coli* transformant (strain JM109) carrying this plasmid, using Ni-NTA agarose beads (QIAGEN) under denaturing conditions. Rabbit polyclonal antisera against these peptides were prepared by Promega Co. The antibody (Apep1) raised against MCA1-peptide 1 specifically recognized the MCA1 protein; the antibody (Apep2) raised against MCA1-peptide 2 specifically recognized both the MCA1 and MCA2 proteins; the antibody (Apep3) raised against MCA1-peptide 3 specifically recognized the MCA1 protein; and the antibody (Bpep4) raised against MCA2-peptide 4 specifically recognized the MCA2 protein, as revealed by Western blot analysis of total cell extracts of yeast transformants expressing MCA1 or MCA2.

**Subcellular fractionation**

Yeast cell extracts, prepared from 2 × 10<sup>8</sup> cells as described previously (Iida et al. 1994), were subjected to subcellular...
fractionation by sucrose density gradient centrifugation as described previously (Egger et al. 1995). After centrifugation, proteins in each fraction were precipitated with 10% trichloroacetic acid, washed twice with cold acetone and dissolved in SDS sample buffer (Laemmli 1970). The separated samples were subjected to SDS–PAGE and Western blotting as described below.

**Determination of soluble and peripheral membrane proteins vs. integral membrane proteins**
Yeast cell extracts were prepared from 2 × 10^8 cells by the method of lida et al. (1994). Yeast cell extracts prepared as above were incubated with a solubilizing agent, 0.5 M NaCl, 0.1 M Na_2CO_3, 1.6 M urea, 1% Triton X-100 or 1% SDS, for 1 h at 4°C and centrifuged at 20,000 × g for 1 h to separate the pellet (P, membrane fraction) and the supernatant (S, soluble fraction). The plasma membrane H^+–ATPase Pma1 and the cystosolic soluble enzyme enolase were used as markers. The separated samples were subjected to SDS–PAGE and Western blotting as described below. Peripheral membrane proteins are expected to be collected in the supernatant fraction after the cell extracts are incubated with either 0.5 M NaCl, 0.1 M Na_2CO_3 or 1.6 M urea.

**SDS–PAGE and Western blotting**
Yeast cell extracts were prepared from 2 × 10^8 cells. For reducing SDS–PAGE, total cell extracts were treated with SDS sample buffer (Laemmli 1970) containing 2% β-mercaptoethanol (β-ME) or dithiothreitol (DTT; 0.001–1.0 mM). For non-reducing SDS–PAGE, total cell extracts were treated with SDS sample buffer containing neither β-ME nor DTT. The samples were separated by SDS–PAGE on appropriate concentrations of polyacrylamide gels with 0.1% SDS and subjected to Western blotting as described elsewhere (Iida et al. 1994). The proteins were detected with the antibodies specifically recognizing MCA1 and/or MCA2 described above, or a monoclonal antibody against HA antigen (12CA5) purchased from Babco using the Immobilon Western Detection Reagent (Millipore Corp.). The antibodies against Pma1 and enolase were described previously (Iida et al. 1994).

**Ca^{2+} accumulation assays**
The method of lida et al. (1990) was followed. In experiments shown in Figs. 5, 8 and 10, yeast cells were grown to 2 × 10^8 cells ml^−1 in SD.Ca100 medium at 30°C and incubated for 2 h with 185 kBq ml^−1 45CaCl_2 (1.8 kBq mmol^−1). In experiments shown in Fig. 7D, yeast cells growing in SD.Ca100 were shifted to SG.Ca100 medium, washed twice with SG.Ca100, pre-incubated for 1 h in SG.Ca100 medium and then incubated in the same medium for 0, 8, 24 and 48 h at the same temperature and 45CaCl_2 concentration as described above. Samples (100 µl each) were filtered with Millipore filters (type HA, 0.45 µm) pre-soaked with 5 mM CaCl_2 and washed five times with the same solution. The radioactivity retained on each filter was measured as described previously (Iida et al. 1990).

**Construction of truncation mutants**
To construct truncation mutants with an HA4 tag at the 3’ end, MCA1^1–135, MCA2^1–135, MCA1^1–173, MCA2^1–173, MCA1^1–185, MCA2^1–186, MCA1^1–237 and MCA2^1–237, MCA1 and MCA2 having a BamHI site at the 5’ end and a SalI site at the 3’ end were synthesized by PCR using the plasmid YEpT-MCA1-HA4 or YEpT-MCA2-HA4 as a template with the forward primer TDI-F2 and the reverse primer SalRAM1AORF405, SalRAM1BORF405, SalRAM1AORF519, SalRAM1BORF519, SalRAM1AORF555, SalRAM1BORF558, SalRAM1AORF711 or SalRAM1BORF711. The nucleotide sequences of the primers are shown in Table 1. To construct MCA1^173–421 and MCA2^173–417, MCA1 and MCA2 having a BamHI site at the 5’ end and SalI and NotI sites at the 3’ end were synthesized by PCR using YEpT-MCA1-HA4 or YEpT-MCA2-HA4 as a template, the forward primer BmFAM1AatgORF520 or BmFAM1BtagORF520, and the reverse primer ADH-R. The resulting products were cut with BamHI and SalI and NotI and inserted between the BamHI and SalI or NotI sites of YEpT-MCA1-HA4 and YEpT-MCA2-HA4.

To construct galactose-inducible MCA1^1–173 and MCA2^1–173 constructs, which are transcribed under the control of the galactose-inducible GAL1 promoter (GAL1p), the BamHI–SalI fragments of the above plasmids were inserted between the BamHI and SalI sites of a vector, YEpGAL1pADH3t. DNA sequences were confirmed with an ABI 310 or 3130 Genetic Analyzer (Applied Biosystems). The expression of these truncated fragments was checked by Western blotting.

**Construction of a substitution mutant of the TM1 segment**
To delete the TM1 (Ile11–Ala33) region, a KOD mutagenesis kit (Toyobo Co. Ltd.) was used. The primers used were MCA1–ΔTM1-F, MCA1–ΔTM1-R, MCA2–ΔTM1-F and MCA2–ΔTM1-R, and the plasmid YEpT-MCA1-HA4 or YEpT-MCA2-HA4 was used as a template. We also used the mutagenesis kit to replace the Asp21 residue with asparagine. The primers used were MCA1–D21N-F, MCA1–D21N-R, MCA2–D21N-F and MCA2–D21N-R, and the plasmid YEpT-MCA1-HA4 or YEpT-MCA2-HA4 was used as a template. DNA sequences and expression of the resulting products were confirmed as described above.

**Effect of the expression of MCA1^1–173 and MCA2^1–173 on yeast cell proliferation**
Since the constitutive expression of MCA1^1–173 and MCA2^1–173 results in the arrest of yeast cell proliferation (see the Results), their expression was designed to be under the control of the GAL1 promoter. Yeast cells bearing YEpGAL1pMCA1(1–173) or YEpGAL1pMCA2(1–173) were grown to 2 × 10^7 cells ml^−1 in SD medium at 30°C and centrifuged for 5 min at 3,000 r.p.m.
The pellet was resuspended in distilled water to give a cell density of $2 \times 10^8$ cells ml$^{-1}$. This suspension was diluted sequentially with distilled water to give $2 \times 10^7$, $2 \times 10^6$, $2 \times 10^5$, $2 \times 10^4$ and $2 \times 10^3$ cells ml$^{-1}$. Each suspension (5 ml) was spotted on SD and SG plates (final cell numbers: 10$^6$, 10$^5$, 10$^4$, 10$^3$ and 10$^2$ cells). These plates were incubated at 30°C for 2 d.

The effect of MCA1$^{1–173}$ and MCA2$^{1–173}$ was also examined using liquid medium. The yeast strains described in the above paragraph were grown to $2 \times 10^6$ cells ml$^{-1}$ in SD medium at 30°C, washed twice with SG medium, and resuspended in the same medium at a cell density of $2 \times 10^6$ cells ml$^{-1}$, after which viability and cell density were examined at 0, 8, 24 and 48 h after the start of incubation.

### Online programs

- **SOSUI**, http://bp.nuap.nagoya-u.ac.jp/sosui/
- **Quick2D**, http://toolkit.lmb.uni-muenchen.de/quick2_d
- **Phobius**, http://phobius.sbc.su.se/
- **Helical Wheel Projections**, http://rzlab.ucr.edu/scripts/wheel/wheel.cgi
- **Aramemnon database**, http://aramemnon.uni-koeln.de/

### DNA sequence data of MCA1 and MCA2

DNA sequence data can be found in the GenBank/EMBL/DDBJ data libraries under accession numbers AB196960 for MCA1 and AB196961 for MCA2.

### Statistical analysis

Statistical significance was determined using unpaired Student’s $t$-test with a $P$-value $<0.05$ required for significance.

### Supplementary data

Supplementary data are available at PCP online.

### Funding

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology in Japan [Grant-in-Aid for Scientific Research on Priority Areas (No. 21026009 and No. 23120509 to H.I.), Grant-in-Aid for Scientific Research B (No. 21370017 to H.I.) and Grant-in-Aid for Scientific Research.

### Table 1 The primers used in this study

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<th>Name</th>
<th>Orientation</th>
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<td>TDH-F2</td>
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The SalI (GTCGAC) and BamHI (GGATCC) sites are italicized. The initiation codon is underlined. Mutated nucleotides are in bold.
We thank Ms. Yumiko Higashi for secretarial assistance.

Acknowledgments

References