Transmembrane Topologies of Ca\(^{2+}\)-permeable Mechanosensitive Channels MCA1 and MCA2 in *Arabidopsis thaliana*\(^*\)

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Sensing mechanical stresses, including touch, stretch, compression, and gravity, is crucial for growth and development in plants. A good mechanosensor candidate is the Ca\(^{2+}\)-permeable mechanosensitive (MS) channel, the pore of which opens to permeate Ca\(^{2+}\) in response to mechanical stresses. However, the structure-function relationships of plant MS channels are poorly understood. *Arabidopsis* MCA1 and MCA2 form a homotetramer and exhibit Ca\(^{2+}\)-permeable MS channel activity; however, their structures have only been partially elucidated. The transmembrane topologies of these ion channels need to be determined in more detail to elucidate the underlying regulatory mechanisms. We herein determined the topologies of MCA1 and MCA2 using two independent methods, the Suc2C reporter and split-ubiquitin yeast two-hybrid methods, and found that both proteins are single-pass type I integral membrane proteins with extracellular N termini and intracellular C termini. These results imply that an EF hand-like motif, coiled-coil motif, and plac8 motif are all present in the cytoplasm. Thus, the activities of both channels can be regulated by intracellular Ca\(^{2+}\) and protein interactions.

Mechanical stimuli affect plant growth, development, and resistance to herbivores (1–6). Natural stimuli, such as wind and gravity, modify the height and shape of grasses and trees.

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Touch retards the elongation of inflorescence and increases resistance to herbivores in *Arabidopsis thaliana*. Thus, stimulus perception is fundamental to plants, and the elucidation of mechanosensors is important for understanding the molecular basis of plant mechanics and morphogenesis.

A Ca\(^{2+}\)-permeable mechanosensitive (MS) channel has been suggested as a component of mechanosensors (7–10). Using *Nicotiana plumbaginifolia* seedlings carrying the Ca\(^{2+}\)-dependent photoprotein, aequorin, as an intracellular Ca\(^{2+}\) indicator, a previous study reported that touch elicits an immediate increase in the cytosolic concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{cyt}\)), which may act as a Ca\(^{2+}\) signal (11). We recently identified novel Ca\(^{2+}\)-permeable MS channels in *Arabidopsis (A. thaliana)*, named MCA1 and MCA2 (mid1-complementing activity 1 and 2) (12–15). Both proteins share 74% identity in their amino acid sequences, form a homotetramer, have no homology to any known ion channels or transporters, and mediate Ca\(^{2+}\) influx upon mechanical stimulation, such as hypo-osmotic shock and membrane stretch. Genes coding for MCA orthologs are found exclusively in the plant kingdom (9, 12). Rice and tobacco MCA proteins are also shown to mediate an increase in [Ca\(^{2+}\)]\(_{cyt}\) upon hypo-osmotic shock (16, 17).

An *in silico* study suggested that MCA1 and MCA2 have several motifs, such as an EF hand-like motif, coiled-coil motif, and plac8 (DUF614) motif as well as a few predicted putative transmembrane segments (12, 13). To understand the molecular and physiological functions of both proteins and their motifs, their transmembrane topologies need to be elucidated in more detail. In the present study we determined these topologies using two different methods. The results provide evidence that MCA1 and MCA2 have one transmembrane segment near their N termini which is oriented to the outside of the plasma membrane, in contrast to the prediction with transmembrane topology algorithms available online. Thus, these proteins are identified as single-pass type I transmembrane proteins. This result suggests that all of the above-mentioned motifs are oriented toward the cytosol and provides an insight into the regulatory mechanisms of MCA1 and MCA2. Although other fam-

\(^{8}\)The abbreviations used are: MS, mechanosensitive; MCS, multi-cloning site; Endo H, Endoglycosidase H; ER, endoplasmic reticulum; Cub, C-terminal ubiquitin; Nub, N-terminal ubiquitin; TF, transcription factor.
The transmembrane topologies of MS channels, MCA1 and MCA2

| Table 1 | Primers to construct the short Suc2 reporter, Suc2C |
|---------|---------------------------------------------------|
| Underlines indicate NcoI, BamHI, EcoRI, SalI, and NotI restriction sites (from the top to the bottom). The initiation and stop codons are boldfaced. |

| Primer name | Nucleotide sequence |
|-------------|---------------------|
| S2C-SF-Nco | 5'-ccagcGAGCGACGAAAAATTAGGTAAATTAAG-3' |
| S2C-3R-Bam | 5'-ggatccGAGCGAGCGAAAAATTAGGTAAATTAAG-3' |
| S2C-SF-Rl  | 5'-ggatccGAGCGACGAAAAATTAGGTAAATTAAG-3' |
| S2C-3R-Sall| 5'-ggatccGAGCGACGAAAAATTAGGTAAATTAAG-3' |
| S2C-3R-Not | 5'-ggatccGAGCGACGAAAAATTAGGTAAATTAAG-3' |

The transmembrane topologies of plant MS channels have already been identified and characterized, their transmembrane topologies have not yet been determined experimentally (18, 19). Therefore, this is the first study to experimentally elucidate the transmembrane topologies of plant MS channels.

**Experimental Procedures**

Yeast Strains and Media—Strain H319 (MATa cch1Δ::HIS3 mid1Δ::HIS3 his3Δ3-1 leu2-3,112 trp1-289 ura3-52 sst1-2) was described previously (20), and the two-hybrid reporter strain NMY32 (MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)_4-HIS3 URA3::(lexAop)_4-lacZ ade2::(lexAop)_4-ADH2 GAL4) was obtained from a manufacturer (Dualsystems Biotech AG, Zürich, Switzerland). Synthetic SD medium was described previously (21), to which 20 mg/liter histidine, 30 mg/liter leucine, 20 mg/liter tryptophan, 20 mg/liter uracil, and 20 mg/liter adenine sulfate were added if necessary.

**Escherichia coli Strain and Medium—**Strain XL1-Blue (supE44 hisdR17 recA1 endA1 gyrA46 thi-1 relA1 lacI-1697 prpAB lacF7 lacZ ΔM15 Tn10 (tetR)) was used in the experiments (22).

Construction of MCA1-FLAG and MCA2-FLAG—To construct the low copy expression plasmids YCpT-MCA1-FLAG and YCpT-MCA2-FLAG, the open reading frames (ORF) of MCA1-HA and MCA2-HA in the multicopy expression plasmids YEpTDHXho-MCA1 (12), YEpTDHXho-MCA2 (13), YCpT-MCA1-FLAG, YCpT-MCA2-FLAG, and YCpS-MID1-16Q-FLAG, which had been linearized with the respective restriction enzymes. The resulting plasmids are listed in Table 2.

Construction of a Series of C-terminally Truncated MCA1-FLAG and MCA2-FLAG Tagged with Suc2C—To synthesize a series of truncated derivatives of MCA1 and MCA2, PCR was performed using the plasmid YCpT-MCA1-FLAG or YCpT-MCA2-FLAG as a template and BamHI site-containing forward primers and Sall site-containing appropriate reverse primers. The resulting products were cut with BamHI and Sall and inserted into the Suc2-tagged MCA1 or MCA2 plasmids described above in place of the full-length ORF. The resulting plasmids carrying the inserts encoding the appropriately truncated forms of MCA1 and MCA2 are listed in Table 2.

Construction of Bait Plasmids—The vectors pNCW, carrying the LexA-VP16-Cub cassette for N-terminal fusions, and pCCW, carrying the Cub-LexA-VP16 cassette for C-terminal fusions (25), were used to construct the bait plasmids. pCCW was modified to pCCWC, which contained an additional SacI restriction site in the MCS. The ORFs of MCA1, MCA2, and their truncated derivatives were inserted in-frame in the MCS of pNCW or pCCWC, respectively. The nucleotide sequences of the bait plasmids were confirmed by DNA sequencing. The resulting plasmids were shown in Table 3. pCCW, pNCW, and the prey plasmids pAl-Alg5 and pDL2-Alg5 were purchased from a manufacturer (Dualsystems Biotech AG, Zürich, Switzerland).

Endoglycosidase H (Endo H) Treatment and Western Blot Analysis—Yeast cells were grown to the late log phase at 30 °C, harvested by centrifugation, resuspended in TE buffer (10 mm Tris-HCl, pH 8.0, 1 mm EDTA) containing 1 mm PMSF, and disrupted by stirring at 4 °C for 7 min with acid-washed glass beads (425–600 μm, Sigma). Whole cell lysates were prepared by centrifuging the extracts for 5 min at 800 × g. The supernatant received a 1/10 volume of the denaturation buffer (5% SDS and 0.4 M DTT), was heat-denatured at 70 °C for 5 min, then received a 1/10 volume of the reaction buffer (0.5 M sodium acetate, pH 5.2) and divided into two equal parts (29 μl each). One

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Transmembrane Topologies of MS Channels, MCA1 and MCA2

Table 2

Plasmids carrying MCA1 and MCA2 tagged with SUC2C

| Plasmid name                      | Markers                                      |
|-----------------------------------|----------------------------------------------|
| YEpt-SUC2C-MCA1                   | LEU2 2-μm-ori TDH3p-SUC2C-MCA1-ADH1t amp*   |
| YEpt-SUC2C-MCA2                   | LEU2 2-μm-ori TDH3p-SUC2C-MCA2-ADH1t amp*   |
| YEpt-MCA1-SUC2C                   | LEU2 2-μm-ori TDH3p-MCA1-SUC2C-ADH1t amp*   |
| YEpt-MCA2-SUC2C                   | LEU2 2-μm-ori TDH3p-MCA2-SUC2C-ADH1t amp*   |
| YGpT-MCA1-FLAG                    | LEU2 CEN4 AR5 TDH3p-MCA1–5xFLAG-ADH1t amp*   |
| YGpT-MCA2-FLAG                    | LEU2 CEN4 AR5 TDH3p-MCA2–5xFLAG-ADH1t amp*   |
| YGpT-SUC2C-MCA1-FLAG              | LEU2 CEN4 AR5 TDH3p-SUC2C-MCA1–5xFLAG-ADH1t amp* |
| YGpT-SUC2C-MCA2-FLAG              | LEU2 CEN4 AR5 TDH3p-SUC2C-MCA2–5xFLAG-ADH1t amp* |
| YGpT-MCA1-SUC2C-FLAG              | LEU2 CEN4 AR5 TDH3p-MCA1–5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA2-SUC2C-FLAG              | LEU2 CEN4 AR5 TDH3p-MCA2–5xFLAG-SUC2C-ADH1t amp* |
| YGpT-SUC2C-MCA1-(1–185)-FLAG      | LEU2 CEN4 AR5 TDH3p-SUC2C-MCA1 (1–185)-5xFLAG-ADH1t amp* |
| YGpT-SUC2C-MCA2-(1–186)-FLAG      | LEU2 CEN4 AR5 TDH3p-SUC2C-MCA2 (1–186)-5xFLAG-ADH1t amp* |
| YGpT-MCA1-(1–185)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA1 (1–185)-5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA2-(1–186)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA2 (1–186)-5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA1-(1–338)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA1 (1–338)-5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA2-(1–323)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA2 (1–323)-5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA1-(1–373)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA1 (1–373)-5xFLAG-SUC2C-ADH1t amp* |
| YGpT-MCA2-(1–363)-FLAG-SUC2C     | LEU2 CEN4 AR5 TDH3p-MCA2 (1–363)-5xFLAG-SUC2C-ADH1t amp* |
| YGpS-MID1-FLAG                   | LEU2 CEN4 AR5 MID1p-MID1 (16Q)-5xFLAG-SUC2C-ADH1t amp* |
| YGpS-MID1(16Q)-FLAG-SUC2C        | LEU2 CEN4 AR5 MID1p-MID1 (16Q)-5xFLAG-SUC2C-ADH1t amp* |
| pCs4–14                          | URA3 2-μm-ori SUC2-His54C amp*               |

* A gift from Sengstag (24).

Table 3

Bait and prey plasmids used in this study

| Plasmid name                      | Markers                                      |
|-----------------------------------|----------------------------------------------|
| pNCW-MCA1                         | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA1-CYC1 kan |
| pNCW-MCA2                         | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA2-CYC1 kan |
| pCCW-MCA1                         | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA1-CYC1 kan |
| pCCW-MCA2                         | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA2-CYC1 kan |
| pCCW-MCA1-(1–185)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA1-CYC1 kan |
| pCCW-MCA2-(1–186)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA2-CYC1 kan |
| pCCW-MCA1-(1–338)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA1-CYC1 kan |
| pCCW-MCA2-(1–323)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA2-CYC1 kan |
| pCCW-MCA1-(1–373)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA1-CYC1 kan |
| pCCW-MCA2-(1–363)                 | LEU2 CEN/ARS CYC1p-LexA-VP16-Cub-MCA2-CYC1 kan |
| pAL1-Alg5                         | TRP1 2-mm-ori ADH1p-Alg5-HA-NubG-CYC1 kan |
| pDL2-Alg5*                        | TRP1 2-mm-ori ADH1p-Alg5-HA-NubG-CYC1 kan |

* The two prey plasmids were purchased from Dualsystems Biotech AG.

received 5 milliliters (1 μl) of Endo H (Roche Applied Science), and the other received an equal volume of Milli-Q water. The mixtures were incubated at 37 °C for 1 h, recovered Laemmli SDS sample buffer (26), and were then heated at 70 °C for 5 min. SDS-PAGE and Western blot analyses were performed essentially according to the method described by lida et al. (21). FLAG-tagged proteins were detected with a mouse anti-FLAG M2 monoclonal antibody (Sigma, catalog no. 3165) and an affinity-purified, peroxidase-conjugated, sheep anti-mouse IgG (GE Healthcare: NXA931). MCA1 and MCA2 without the FLAG tag were detected with the rabbit polyclonal antibody Apep2 that recognizes both the MCA1 and MCA2 proteins (14) and an affinity-purified, peroxidase-conjugated, donkey-anti rabbit IgG (GE Healthcare Bio-Science; NA934).

Growth Assay—Cells of the two-hybrid reporter strain NMY32 expressing Cub-TF-tagged MCA1 or MCA2 with Alg5Nubl or Alg5-NubG were grown to the mid log phase in SD liquid medium containing adenine and histidine but lacking leucine and tryptophan, harvested by centrifugation, and resuspended in saline at a density of 4 × 10^6 cells/ml, after which 5 μl of a 1:10 serial dilution was spotted on SD agar medium lacking leucine and tryptophan with or without adenine and histidine and then incubated at 30 °C for 2 to 4 days.

Results

Bioinformatic Predictions Presented Various Membrane Topologies of MCA1 and MCA2—We first predicted the membrane topologies of MCA1 and MCA2 with various web-based prediction servers, including PredictProtein (27), TMpred (28), SOSUI (29), and TOPCONS (30). As shown in Fig. 1, these servers gave different results for MCA1 and MCA2. In addition, although the amino acid sequence identities and similarities between MCA1 and MCA2 were 73 and 89%, respectively (12), the predicted topologies were different between the two proteins. These inconsistent results prompted us to determine membrane topologies experimentally.

Construction of a Short Suc2C Reporter to Determine Membrane Topology—The membrane topologies of many transmembrane proteins have been determined by a method using a SUC2-His4C dual topology reporter expressed in yeast cells (24, 31). In this reporter, invertase encoded by SUC2 may be N-glycosylated at 14 potential sites when localized in the endoplasmic reticulum (ER) lumen, whereas the C-terminal domain of the His4 protein harboring histidinol dehydrogenase activity can rescue the histidine auxotrophy of his4 cells spread on the histidinol plate when exposed to the cytoplasmic side of the ER membrane. This reporter is regarded as being very useful
because of this duality. However, this method sometimes gives self-contradictory results; i.e., no N-glycosylation (suggestive of cytoplasmic), but no rescue on medium containing histidinol instead of histidine (suggestive of luminal or extracellular) and vice versa (31–33). Furthermore, the molecular mass of a fully modified form of this reporter protein is \(110kDa\), which is markedly larger than the estimated molecular mass of full-length MCA1 and MCA2 proteins (\(50kDa\)). Because we analyzed even smaller, truncated forms of MCA1 and MCA2 in the present study, this large reporter may lead to discrepant results being obtained, similar to those described above.

To overcome this issue, we constructed a new reporter composed solely of a Suc2 protein fragment (amino acid residues 350–409) that only had four potential N-glycosylation sites. This reporter was named Suc2C because it was derived from a region close to the Suc2 C terminus.

**MCA1 and MCA2 Are Not Naturally N-Glycosylated—** Before using Suc2C as a probe, we examined the possible N-glycosylation of full-length MCA1 and MCA2 because the webserver GlycoEP (34) predicted that MCA1 has two potential N-glycosylation sites at Asn279 and Asn371, whereas MCA2 has one site at Asn363. If N-glycosylated, these sites may complicate the results of the experiments described below, in which Endo H is used to cleave N-linked oligosaccharides. To determine whether N-glycosylation occurred, MCA1 and MCA2 tagged C-terminally with five consecutive FLAG epitopes (MCA1-FLAG and MCA2-FLAG) were expressed in yeast cells, and whole cell extracts were treated with Endo H before SDS-PAGE and a Western blot analysis with the anti-FLAG antibody. Fig. 2A shows that the positions of MCA1-FLAG and MCA2-FLAG on the Western blot were not shifted by the Endo H treatment. In contrast, the position of the positive control, the yeast Mid1 protein, which is known to be N-glycosylated (21), was moved downward. These results suggest that MCA1 and MCA2 are not N-glycosylated in yeast cells.

**Determination of Membrane Topology with the Suc2C Reporter—** We fused the Suc2C reporter to the N terminus or C terminus of MCA1 and MCA2 and determined whether the resulting constructs, Suc2C-MCA1, Suc2C-MCA2, MCA1-Suc2C, and MCA2-Suc2C, were N-glycosylated using the procedures described above. Fig. 2B shows that the upper bands of Suc2C-MCA1 and Suc2C-MCA2 (an arrow in the figure) disappeared after the Endo H treatment. On the other hand, their lower bands were not shifted by this treatment, and neither were the bands of MCA1-Suc2C and MCA2-Suc2C. These results suggest that some of the Suc2C-MCA1 and Suc2C-MCA2 polypeptides are N-glycosylated, whereas the MCA1-Suc2C and MCA2-Suc2C polypeptides are not. The reason why a larger amount of Suc2C-MCA1 and Suc2C-MCA2 polypeptides

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**FIGURE 1.** Hydrophobicity plots and transmembrane topology predictions of MCA1 and MCA2. Hydrophobicity plots were made using the Kyte and Doolittle method with a window of 19 amino acids (47). The abscissa shows the amino acid number, and the ordinate represents hydrophobicity. Transmembrane topologies were predicted with the four membrane topology algorithms available online: PredictProtein, TMPred, SOSUI, and TOPCONS. Green bars represent putative transmembrane segments, named pTM1, pTM2, pTM3, and pTM4, among which pTM1 was identified as the real transmembrane segment in the present study.

**FIGURE 2.** Examination of N-glycosylation of MCA1 and MCA2 and Endo H sensitivity of MCA1 and MCA2 fused N-terminally with Suc2C. A, MCA1 and MCA2 were not N-glycosylated in yeast cells. Whole cell lysates prepared from yeast cells expressing MCA1-FLAG and MCA2-FLAG were treated with Endo H or mock-treated and subjected to Western blotting. As a positive control, the yeast Mid1 protein tagged with a 5×FLAG was treated in the same manner. The proteins were detected by an anti-FLAG antibody. The arrow indicates an Endo H-sensitive protein. B, Suc2C-MCA1 and Suc2C-MCA2 are sensitive to Endo H. Whole cell extracts prepared from yeast cells (strain H319) expressing MCA1 and MCA2 fused N-terminally or C-terminally with Suc2C were treated with Endo H or mock-treated and processed as described above. The arrow indicates an Endo H-sensitive protein. Because these constructs have no FLAG tag, they were detected with the anti-Apep2 antibody that recognizes both MCA1 and MCA2 (14).
are not N-glycosylated currently remains unknown. The amounts of both proteins may have been too much to be N-glycosylated in the ER because they are expressed under the control of the strong promoter, TDIH3p, on multicopy plasmids in yeast cells (35). We used this promoter because it was necessary to make MCA1, MCA2, and their derivatives detectable on Western blots.

To determine the membrane topologies of MCA1 and MCA2 with respect to putative transmembrane segments, the Suc2C reporter was fused to downstream positions of each of the four putative transmembrane segments (named pTM1 to pTM4) common to MCA1 and MCA2 (Fig. 1). The fused positions are shown in Fig. 3A. The resulting constructs were expressed in yeast cells, and whole cell extracts were treated with Endo H and then analyzed as described above. Fig. 3B shows that only the MCA1 (1–185)-FLAG protein N-terminally fused with Suc2C [i.e. Suc2C-MCA1 (1–185)-FLAG] was sensitive to Endo H. The same result was obtained with Suc2C-intact MCA1 (Fig. 2B). In contrast, MCA1-FLAG proteins (including truncated forms) that fused C-terminally with Suc2C, such as MCA1 (1–185)-FLAG-Suc2C, MCA1 (1–338)-FLAG-Suc2C, MCA1 (1–373)-FLAG-Suc2C, and intact MCA1 (1–421)-FLAG-Suc2C, were all insensitive to Endo H.

The intact and truncated forms of MCA2-FLAG gave similar results (Fig. 3C). Namely, only Suc2C-MCA2 (1–186)-FLAG was sensitive to Endo H, whereas MCA2 (1–186)-FLAG-Suc2C, MCA2 (1–323)-FLAG-Suc2C, MCA2 (1–363)-FLAG-Suc2C, and intact MCA2 (1–416)-FLAG-Suc2C were insensitive to this enzyme. These results suggest that the N termini of MCA1 and MCA2 are extracellular and that only pTM1 is an actual transmembrane segment.

In addition to the suggestion about membrane topology, the presence of the N-glycosylated forms of Suc2C-MCA1 (1–185)-FLAG and Suc2C-MCA2 (1–186)-FLAG suggest that neither MCA1 nor MCA2 has a signal peptidase-cleavable signal sequence at their N-terminal region (Fig. 3, B and C).

**Determination of Membrane Topology by the Yeast Two-hybrid Method**—To confirm the above suggestion, we employed the split-ubiquitin membrane-based yeast two-hybrid system as the second method that functions with a different operation principle to that of the Suc2C method (25, 36). In this system the MCA proteins and their derivatives were fused with the LexA-VP16-Cub cassette at the N terminus or Cub-LexA-VP16 cassette at the C terminus, in which Cub is C-terminal ubiquitin, and LexA-VP16 is an artificial transcription factor that can be released by ubiquitin. These fusions were used as bait. On the other hand, as prey, the yeast ER membrane protein Alg5 was fused with two kinds of Nub (N-terminal ubiquitin), NubI and NubG. The former possessed wild-type Ile13, whereas the latter carries the Ile13 to Gly mutation. If the LexA-VP16-Cub or Cub-LexA-VP16 moiety of the bait protein is localized on the cytoplasmic side of the ER and plasma membranes, the co-expressed Alg5-NubG forms split-ubiquitin (the native fold of ubiquitin), which cleaves off LexA-VP16 to activate the reporter genes ADE2 and HIS3, the expression of which renders the yeast ade2 his3 auxotroph to grow on agar medium lacking adenine and histidine. On the other hand, if the moiety is located on the extracellular side of the plasma membrane or luminal side of the ER membrane, the co-expressed Alg5-NubI cannot form split-ubiquitin. The co-expressed Alg5-NubG is used as a negative control, in which NubG cannot form split-ubiquitin even when the Cub-LexA-VP16 moiety is localized on the cytoplasmic side of the membranes.

FIGURE 3. Endo H sensitivity of C-terminally truncated forms of MCA1 and MCA2 fused with Suc2C. A, schematic diagram of the truncated derivatives of MCA1 and MCA2 fused with Suc2C. The Suc2C reporter was fused at the N terminus or downstream of the four putative transmembrane segments (pTM1–4). MCA1, MCA2, and their truncated derivatives were represented by light brown-colored boxes. The size of each protein was shown in amino acid numbers. pTM1–4 were denoted by brown-colored bars. Suc2C was indicated by gray-colored boxes. N and C represent the intrinsic N and C termini, respectively. The sizes of the proteins and the reporter are arbitrary. The FLAG tag fused to the C terminus of each construct of MCA1 and MCA2 was not drawn for the sake of simplicity. B and C, schematic diagram of the truncated derivatives fused C-terminally with Suc2C are all insensitive to Endo H. The whole cell lysate preparation, Endo H treatment, and Western blotting were performed as described under “Experimental Procedures.” Mid1(16Q)-FLAG-Suc2C, a positive control, contains the Asn to Gln mutation at all of the 16 N-glycosylation sites in Mid1 so that only the Suc2C reporter can be N-glycosylated. Arrows indicate Endo H-sensitive proteins.
described above (Fig. 3A). The bait and prey plasmids were co-introduced into the two-hybrid reporter strain NMY32 and the sequentially diluted cultures of the resulting three independent transformants were spotted onto synthetic agar medium with or without adenine and histidine. As shown in Fig. 4B, upper panels, transformants expressing the MCA1 protein fused N-terminally with the LexA-VP16-Cub cassette (i.e. LexA-VP16-Cub-MCA1), and NubI was unable to grow on selection medium lacking adenine and histidine. This was also the case with those expressing NubG as negative control prey. In contrast, transformants expressing MCA1 proteins (including truncated derivatives) fused C-terminally with the Cub-LexA-VP16 cassette (such as MCA1 (1–185)-Cub-LexA-VP16, MCA1 (1–338)-Cub-LexA-VP16, and intact MCA1 (1–421)-Cub-LexA-VP16) were able to grow on the selection medium when co-expressed with NubI but not with NubG (Fig. 4, C–F, upper panels). The same results were obtained with the MCA2 proteins (Fig. 4B–F, lower panels). These results suggest that the N termini of MCA1 and MCA2 are located extracellularly or in the ER lumen, and the C
termini of the intact and truncated forms of both proteins are located intracellularly, again suggestive of only pTM1 being the actual transmembrane segment.

**Discussion**

In the present study we demonstrated that the N termini of MCA1 and MCA2 are oriented extracellularly, whereas their C termini are located in the cytosol, using two different methods, the Suc2C reporter and split-ubiquitin methods. Our results also suggest that only pTM1 near the N terminus is the actual transmembrane segment among the four putative transmembrane segments (pTM1–4) commonly predicted for both proteins. Moreover, the presence of N-glycosylation on the Suc2C reporter fused N-terminally to MCA1 and MCA2 suggest that neither protein has a signal peptidase-cleavable signal sequence near their N termini. Therefore, we concluded that both proteins are single-pass type I transmembrane proteins without a cleavable signal sequence (Fig. 5).

This conclusion is consistent with a finding obtained in our previous study on the structure-function relationships of MCA1 and MCA2. In that study we showed that the C-terminally truncated forms of MCA1 and MCA2, namely MCA1<sup>1–173</sup>, MCA2<sup>1–173</sup>, MCA1<sup>1–186</sup>, and MCA2<sup>1–186</sup>, all of which have the N-terminal pTM1 as the only putative transmembrane segment, exhibits Ca<sup>2+</sup> uptake activity (14). Hence, based on the present results and previous findings, we hypothesize that pTM1 is necessary and sufficient for Ca<sup>2+</sup> permeation. This hypothesis is supported by the finding that the sole negatively charged amino acid residue Asp<sup>21</sup> located in the pTM1 of MCA1 and MCA2 is highly conserved during plant evolution and required for Ca<sup>2+</sup> permeation (14); deletion of the pTM1 segment results in the complete loss of Ca<sup>2+</sup> permeation activity in MCA1 and MCA2, whereas the Asp<sup>21</sup> to Asn substitution leads to the complete loss of activity in MCA1 and a partial loss in MCA2, suggesting that Asp<sup>21</sup> is involved in Ca<sup>2+</sup> coordination.

Our conclusion that MCA1 and MCA2 are single-pass type I transmembrane proteins with a transmembrane segment located near the extracellular N terminus is helpful for speculating about the regulatory mechanisms underlying their activities. According to this conclusion, it has become clear that an EF hand-like motif, coiled-coil motif, and plac8 motif are all present in the cytosol. The EF hand-like motif can sense [Ca<sup>2+</sup>]<sub>c</sub> needed to regulate the Ca<sup>2+</sup> permeation activities of MCA1 and MCA2. We previously reported that MCA1<sup>1–135</sup> lacking this motif loses this activity, whereas MCA2<sup>1–135</sup> does not, suggesting that the motif regulates the two proteins differ-

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**FIGURE 5. A model for the transmembrane topology of MCA1 and MCA2 based on the present study.** This figure was drawn using the TOPO2 program (48). MCA1 and MCA2 both have one transmembrane segment, with the N termini being located extracellularly, and C termini located intracellularly. In the transmembrane segment Asp was highlighted by red, Gln and Asn were in magenta, and Lys was in purple. Note that these hydrophilic amino acid residues are vertically disposed in an array in the transmembrane helix. This feature may enable the hydrophilic residues to face each other and the hydrophobic residues to face out toward the lipid bilayer in order to form the pore of a channel composed of four subunits (14). In the cytoplasm amino acid residues in the EF hand-like motif are blue, those in the coiled-coil motif are green, and those in the plac8 motif are brown.
entially (14). Coiled-coil motifs have been shown to mediate protein-protein interactions. Our previous findings suggested that this motif is not required for making a Ca\(^{2+}\)--permeable channel because MCA1 and MCA2 lacking the coiled-coil motif (i.e. MCA1\(^{1–173}\), MCA2\(^{1–173}\), MCA1\(^{1–185}\), and MCA2\(^{1–186}\)) maintained Ca\(^{2+}\) permeation activity. The coiled-coil motif appears to regulate the two proteins differentially because MCA1\(^{1–237}\) carrying the motif does not have this activity, whereas MCA2\(^{1–237}\) does (14).

In some proteins, the plac8 motif has been suggested to form a pore-forming channel (37, 38). However, as discussed in our previous study, the plac8 motif of MCA1 and MCA2 is unlikely to participate in forming a channel (14). We herein only show that the C-terminally truncated MCA1 and MCA2 lacking the motif, such as MCA1\(^{1–173}\) and MCA2\(^{1–173}\), exhibit Ca\(^{2+}\) permeation activities. Therefore, this motif may be unnecessary for forming a channel.

The results of the present study suggest that MCA1 and MCA2 are structurally unique MS channels in terms of the number of transmembrane segments. To date, the polypeptides of all MS channels studied structurally have multiple transmembrane segments. For example, the polypeptides of bacterial MscS and MscL have three and two transmembrane segments, respectively (39, 40). Moreover, seven MscS polypeptides and five MscL polypeptides assemble as subunits to form a pore-forming channel. Therefore, the MS channels MscS and MscL are composed of a large number of transmembrane segments (i.e. 21 and 10, respectively). In addition, the polypeptides of plant MS channels MSL (for MscS-like) and OSCA1 have been predicted to have five to six and nine transmembrane segments, respectively; however, their transmembrane topologies have not yet been determined (19, 41). In contrast, the polypeptides of MCA1 and MCA2 have a single transmembrane segment, as shown in this study, and assemble as a homotrimer to form a Ca\(^{2+}\)--permeable channel (14, 42). Chloride intracellular channels (CLICs) in mammals may have a single N-terminal transmembrane segment, but the number of subunits has not yet been established (43–46). In this context, MCA1 and MCA2 are structurally unique among MS channels, and thus, elucidation of the molecular mechanisms of MCA1 and MCA2 involved in sensing mechanical stresses, including hypo-osmotic stress and membrane stretch, and in permeating Ca\(^{2+}\) is important.

Author Contributions—S. Kamano performed the experiments by the Suc2C method and revised the paper. S. Kume performed the experiments using the yeast two-hybrid system and revised the paper. K. I. designed the Suc2C method, constructed most plasmids for Suc2C fusions and the yeast two-hybrid system, and revised the paper. K.-J. L., M. N., and Y. N. helped with the experiments and revised the paper. H. I. designed and coordinated the study and wrote and revised the paper. All authors reviewed the results and approved the final version of the manuscript.

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