Lipid Profiling Leymus chinensis Root Insensitive to Ca Deprivation

Yang Nan
Inner Mongolia University
Yanbin Dong
Inner Mongolia University
Lili Zhang
Inner Mongolia University
Lijuan Zhang
Inner Mongolia University
Zhi Qi
Inner Mongolia University
Haiye Luan
Yancheng Teachers University
Ju Yang (yangj01@yctu.edu.cn)
Yancheng Teachers University

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Abstract

Background

Leymus chinensis (*L. chinensis*) is a perennial native forage grass widely distributed in the steppe of Inner Monoglia as the dominant species. Calcium (Ca) as an essential mineral element important for plant's adaptation to its growth environment.

Results

In this study, significance of Ca for *L. chinensis* growth and membrane stability was investigated and compared with that for *Arabidopsis*. Previously it was found that Ca deprivation strongly inhibited *Arabidopsis* seedling growth, disrupted the plasma membrane stability and selectivity, accompanied with increasing fluid-phase based endocytosis and contents of all major membrane lipids. In contrast, *L. chinensis* seedlings growth and root endocytosis activity were not affected by Ca deprivation. The plasma membrane maintained high selectivity. The PC:PE ratio, an indicator of the membrane stability, is five times higher in the *L. chinensis* than the *Arabidopsis*. In addition, Ca deprivation did not affect contents of all major lipid types in *L. chinensis*. Ca deprivation increased the MDA contents and decreased the SOD activity in *L. chinensis*, which were opposite with *Arabidopsis*. *L. chinensis* roots have higher contents of PC, PI, MDGD, PG, CL, DGDG, LPC, but less of PE, DAG, TAG, PS, LPA, LPE, LPS than those in *Arabidopsis* roots. Moreover, 31 unique lipid species were found in *L. chinensis*, and 66 unique in *Arabidopsis*.

Conclusions

This study revealed that *L. chinensis* root has unique membrane lipid composition without much Ca dependence, which might be accounted for the plant's ability to distribute wide-range of natural environment.

Background

Leymus chinensis (Trin.) Tzvel is a perennial gramineous Triticeae grass belong to the genus Leymus and serves as an high-nutrition forage for grazing livestock [1, 2]. It widely distributed in the eastern Eurasia steppe as the dominant species for the ecological conservation of the arid and semi-arid region [3, 4]. It is largely unknown how the grass achieves its adaptation to such diverse growth environments.

In plant cells, calcium (Ca) as an essential macronutrient plays an vital role in helping plant cells decipher interior and exterior cues so be able to make optimal responses [5]. In both plant and animal cells, cytosolic and organelle-level Ca^{2+} concentration are regulated by sophisticated mechanism linked with almost every aspects of signaling processes [6]. Ca in all living organisms ultimately is derived from the

soil and water bodies on the earth surface. Unfortunately, a series of long-term ecological studies indicate an significant declining trend of Ca contents in forest soils and lakes [7, 8]. Ca deprivation not only has adverse effects on plant growth and development, but plant responses to biotic and abiotic stresses [9, 10]. How plant cells cope with the stress is not fully understood yet.

In animal cells, extracellular Ca^{2+} concentration decreasing is monitored by its sensor CaSR. It regulates endocytosis and exocytosis to maintain cell membrane integrity and intercellular communication [11, 12]. In plant cells, Ca deficit could decrease the membrane stability and selectivity [13].

Ca^{2+} interacts with lipids in the cell bilayer membrane to carry out its regulatory functions on the membrane homoestasis [14]. The amphipathic glycerolipids are the dominant types of lipid in most cells. They have the glycerol backbone with nonpolar fatty acids on its *sn*-1, *sn*-2 position and a polar headgroup on the *sn*-3. The major fatty acids of plants are synthesized in the plastid and have a chain length of 16 or 18 carbons with one to three cis double bonds [15].

Glycerolipid biosynthesis occurs at the plasid inner envelope and endoplasmic reticulum (ER), in both of which the first step is to form phosphatidic acid (PA). In the plasid, the PA can directly converts to phosphatidylglycerol (PG) or gives rise to monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) through diacylglycerol (DAG) [15]. In the ER, PA can be used to synthesize phospholipids phosphatidylinositol (PI), phosphatidylserine (PS), PG, phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The PG can convert to cardiolipin (CL) in the mitochondrial inner envelope. When PA, PC, PE or PS lack one fatty acid chain at their *sn*-1 or *sn*-2 position, they would convert to lysobisphosphatidic acids (LPA), lysophosphatidylcholine (LPC), lysophosphatidylethanolamin (LPE) and lysophosphatidylserine (LPS) [16]. PC and PE exist in the outer and inner leaflet of the bilayer membrane. Higher PC to PE ratio tends to form more stable membrane [17].

Polar glycerolipids are primarily classified according to the structure of the polar head group. Each polar glycerolipid class is subclassified according to the fatty acid species in the hydrophobic tails, which vary in number of carbon chains and number and position of unsaturated bonds [18]. With advance of lipid profiling techniques, namely “lipidomics”, hundreds of lipids have been identified in plants [19]. Modification of membrane lipid composition is recognized as an efficient and universal strategy plants take to cope with various growth environment.

In this study, we found an unusual phenomenon that Ca deprivation had no obvious influences on the vegetable growth of *L. chinensis*. Moreover, by compared with model plant *Arabidopsis*, we hypothesized that the unique membrane lipid composition is an key factor for *L. chinensis* to achieve wide distribution in the eastern Eurasia steppe.

Results

Effects of the Ca deprivation on L. chinensis growth and Ca contents

L. chinensis seedlings vertically grew on solid medium supplied with different concentration of Ca^{2+} ($[Ca^{2+}]_{ext}$) for 7-day. Both of the leave and roots had reached the Petri dish's edge (Fig. 1A). The Ca deprivation (0 mM) had no inhibitory effects on the seedlings' fresh weight (Fig. 1B) and root elongation (Fig. 1C). The seedling growth showed no dependence on the medium's $[Ca^{2+}]_{ext}$ from 0 to 20 mM range (Fig. 1B, 1C).

The seedlings was significantly inhibited by nitrogen (N) deprivation (Fig. S1), which demonstrated that they were mature enough to have mineral nutrient uptake capacity from their growth environment. Consistently, the Ca contents in both leave and roots showed clear declining trend while the growth medium $[Ca^{2+}]_{ext}$ decreased. Significantly, the Ca contents in the seedlings' leaf (Fig. 1D) and roots (Fig. 1E) growth on the 0 mM $[Ca^{2+}]_{ext}$ condition were about 10% of those on the 20 mM $[Ca^{2+}]_{ext}$. It indicates that *L. chinensis* seedlings are tolerant to Ca deprivation.

L. chinensis **oxidative responses to the Ca deprivation**

H_2O_2 accumulation is an common oxidative response of plants to environmental stresses. The Ca deprivation induced strong H_2O_2 production in the root but not the leaf of the *L. chinensis* seedlings, as indicated by the diaminobenzine (DAB) reaction-dependent brown participants (Fig. 2A).

Furthermore, the Ca deprivation boasted the SOD activity (Fig. 2B), but suppressed the MDA contents in the leaves and roots of *L. chinensis* (Fig. 2C). As reported previously, the model plant *Arabidopsis* seedlings were strong inhibited by the Ca deprivation (Zhang et al., 2018), which is completely opposite to the Ca-deprivation insensitive phenotype of *L. chinensis* (Fig. 1). Under the same growth condition as the *L. chinensis*, the Ca deprivation suppressed the SOD activity (Fig. 2D), but boasted the MDA contents in the leaves and roots of *Arabidopsis* (Fig. 2E), which was opposite to the *L. chinensis* (Fig. 2B, 2C).

Endocytosis in L. chinensis **roots and its response to the Ca deprivation**

L. chinensis seedlings grown on the medium with either 1 (CK) or 0 mM $[Ca^{2+}]_{ext}$ were examined for endocytosis activity in their root cells with fluorescence membrane probe FM4-64. Rich endocytosis activities were observed in the cells of the root tip, elongation zones, maturation zones and root hairs and there were no obvious difference between the 1 and 0 mM $[Ca^{2+}]_{ext}$ (Fig. 3A).

The cells in the maturation zone were chosen for the characterization of the endocytosis process. All conserved steps of endocytosis were observed in the cells, including the initial plasma membrane invagination, subsequent early and late endosome formation, which were no obvious difference between the 1 and 0 mM $[Ca^{2+}]_{ext}$ (Fig. 3B).

Effect of the Ca deprivation on L. chinensis **root's membrane selectivity**

Alexa Fluor488 is a membrane-impermeable green fluorescent dye without biological activity and an convenient probe to track non-specific fluid phase endocytosis activity or indicator of membrane selectivity [20]. In the roots of the *L. chinensis* seedlings grown on the CK condition (1 mM $[Ca^{2+}]_{ext}$), the green fluorescent exclusively distributed around the cell boundary and no any signals were found inside the cells, which indicated that the cell plasma membrane maintained rigorous selectivity and the Alexa Fluor488 could not enter the cells through non-selective fluid phase-based endocytosis (Fig. 4A). The Ca deprivation (0 mM) had no obvious effects on the fluorescence distribution pattern, which indicated the membrane selectivity insensitive to the $[Ca^{2+}]_{ext}$ (Fig. 4A).

The endocytosis activity in the roots grown on the 1 or 0 mM $[Ca^{2+}]_{ext}$ was insensitive to Tyr A23, an inhibitor of receptor-mediated endocytosis or Tyr A51, a structural analog of Tyr A23 without inhibitory activity, served as the control (Fig. 4B)

Effect of Ca deprivation on theL. chinensis**root lipid profiles**

Totally, 16 types of membrane and storage lipid were detected in the roots of *L. chinensis*. Free fatty acid (FFA), PC, PA, PI and PE are the dominant types of lipids with contents higher than 10 nmol/g fresh weight (FW). The Ca deprivation induced significant increase of FAA contents in the roots (Fig. 5A), but had no significant effects on other 15 types of lipids (Fig. 5A, 5B, 5C).

The 16 types of lipids have 352 individual lipid molecular species. The FFA lipid type includes 5 molecular species, FFA 16:0, 16:1; 18:0, 18:1 and 18:2. It was the contents of the two dominant species FFA 16:0 and 18:0 that significantly increased as responses to the Ca deprivation (Fig. 5D).

PC is the most abundant membrane lipid in the *L. chinensis* roots and includes a group of 49 molecular species with various carbon chain lengths as well as numbers and positions of double bonds (Fig. 5A). The contents of two molecular species PC 43:2, 44:3 significantly increase and another two PC 32:2, 32:1 decreases responses to the Ca deprivation (Table S1). The content of the four PC species in total is 0.77 nmole/g FW and about 1.5% of the total PC (50 nmol/g FW), so that it didn't change the total PC content significantly under the Ca deprivation. Similar to the case of PC, there were total another 37 lipid molecular species in the 14 types of lipids showing significant changes of their contents as responses to the Ca deprivation (Table S1).

Lipid composition comparison between the roots ofL. chinensis**and**Arabidopsis

The relative abundances of PC, PI, MGDG, PG, CL, DGDG and LPC to the total lipid in the *L. chinensis* roots were significantly higher than those in the *Arabidopsis*, respectively (Fig. 6). The PE, DAG, TAG, PS, LPA, LPE and LPS in the *L. chinensis* were significantly lower than those in the *Arabidopsis*, respectively (Fig. 6A, 6B, 6C). The lipid type with the greatest relative abundance difference between the two roots was LPS, which was 0.04% in the *L. chinensis* and 0.26% in the *Arabidopsis*, with a 6.5-fold difference (Fig. 6C).

The mole ratio of PC to PE in *Arabidopsis* roots dramatically increased as a result of Ca^{2+} deprivation [13], but had no significant effect on that in the *L. chinensis* roots (Fig. 6D). In addition, the mole ratio in the *L. chinensis* roots is about five times higher than that in the *Arabidopsis* (Fig. 6D). By comparing the lipid profiles in the roots of *L. chinensis* and *Arabidopsis*, we found 31 lipid molecular species existing in the *L. chinensis* but not the *Arabidopsis* (Table S2), and 66 in the *Arabidopsis* but not the *L. chinensis* (Table S3).

Discussion

Ca is an essential mineral element for plant growth and development. Ca deficit symptoms have been described in various plants [21, 22]. In this study, the seedlings of *L. chinensis*, the dominant forage grass in the eastern steppe of Eurasian continent, showed the Ca deprivation tolerant phenotype (Fig. 1A, 1B, 1C). Two evidences excluded the possibility that the seedlings were not mature enough to have nutrient uptake capacity from its growth environment: firstly, with decreasing the $[Ca^{2+}]_{ext}$ in the medium, the Ca contents in the leaves and roots decreased significantly (Fig. 1D, 1E); secondly, the seedling at same age showed sensitive to the nitrogen deprivation (Fig. S1). *L. chinensis* is an typical monocotyledonous perennial plant which can produce seeds and propagate itself through rhizome extension. In the grassland, the plant usually takes three years to reach its full mature stage from the seed germination. To fully understand the requirement of Ca for its growth and development, a long-period experiment should be conducted.

Unique membrane properties could confer the *L. chinensis* seedlings' Ca deprivation-tolerant growth phenotype, which is supported by multiple lines of evidences in the study. Malondialdehyde (MDA) is a biomarker for oxidative damage of polyunsaturated fatty acids attached to the *sn*−2 position in glycerophospholipids within cell membranes [23]. The MDA content decreased in the *L. chinensis* roots (Fig. 2C) but increased in the *Arabidopsis* upto the Ca deprivation (Fig. 2E).

Ca deprivation significantly induced non-selective fluid-phase based endocytosis in the *Arabidopsis* roots, which led to the dramatic decrease of the membrane selectivity [13]. In contrast, the *L. chinensis* root membrane maintained high selectivity even under the Ca deprivation condition (Fig. 4). In addition, in both *Arabidopsis* and *L. chinensis*, PC, PE, PI and PA are the four dominant glyercolipids. The Ca deprivation induced significant increase of the PC, PE, PI contents, but decrease of the PA [13], but no effects on the four in the *L. chinensis* (Fig. 5A).

The relative abundance of each lipid types is significant different between the *Arabidopsis* and *L. chinensis* roots (Fig. 6), especially the PC to PE mole ratio (Fig. 6D). PC is a amphotropic lipid molecules with a hydrophilic head and a hydrophobic tail and plays an dominant role in stabilizing bilayer membrane. PC to PE ratio is well maintained in animal cells and abnormal high or low is recognized as an biomarker for disease progression [24]. In plants, the increased PC to PE ratio is an self-protective action to maintain the cell membrane stability under stress conditions [17]. The ratio in the *L. chinensis*

was five times higher than the *Arabidopsis* (Fig. 6D), which implies that the membrane stability of *L. chinensis* is much higher than the *Arabidopsis*.

The free fatty acids (FAA) is the only type of lipid that showed significant increasing up to the Ca deprivation in the *L. chinensis* roots (Fig. 5A). The 16- and 18-carbon FFAs participate in immune system regulation and response in plants, such as saturated FFA16:0 and FFA18:0 which help soybean resist high temperature stress [25, 26]. The double bonds on the fatty acyl chain of cell membrane can reduce the packing density of adjacent lipids and reducing the number of double bonds in plant cells is helpful to maintain cell membrane integrity and stability [27]. The significant increasing contents of FFA16:0 and FFA18:0 could be beneficial for the *L. chinensis* to maintain its root cell membrane stability.

There were 31 unique lipid species in *L. chinensis* roots (Table S2). There were 12 unique PC species in *L. chinensis*, 11 of which contain odd fatty acid chains (Table S2). Pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) are odd fatty acid chains with high content in animals, plants, and microorganisms, which are related to some diseases [28, 29, 30]. The specific functions of these PC molecules in *L. chinensis* roots need further investigation. Comparing lipidomic data with other plants, there are some similarities but also differences. For example, the unique PC36:0 in *Arabidopsis* roots and the unique PC35:2 and PC35:1 in *L. chinensis* are present in the epithelial cells of the dicotyledons *Mesembryanthemum crystallinum* [31]. Unique LPC16:1 and LPE18:0 in *Arabidopsis* roots were found in the monocot plant barley roots, but not unique LPC14:0 in *L. chinensis* roots [32]. The unique PC35:2, PC35:1, PC43:1, PC43:2, PC43:3, LPC14:0, LPC15:0, TAG48.0(16:0), and TAG38:3(18:3) in *L. chinensis* roots were also found in rice roots, but the unique PC39:3, PC39:4, PE39:3, PE39:4, TAG40:4(18:3), and TAG59:5(18:3) in *L. chinensis* roots do not exist in rice roots [33].

Conclusions

Arabidopsis and *L. chinensis* are two plants belong to different families. In addition, *Arabidopsis* is annual, but *L. chinensis* perennials. They have significant difference in the growth sensitive to the Ca deprivation and lipid composition, as reported previously [13] and in the current study. Further studies need to uncover if the difference exists in other plants. The data presented in this study implies that the super stable membrane conferred by its unique membrane lipid composition confers the strong adaptation ability of *L. chinensis* to wide range of environmental conditions in the eastern Eurasia steppe.

Methods

Plant material and growth conditions

L. chinensis seeds were collected from wild. *L. chinensis* plants with mature spikes in the Xilinguol grassland. These seeds were kept in 37 ℃ incubator to maintain their dryness. They were soaked in tap-water at 4 ℃ for 5 days. The sunken seeds with higher germination rate than those floating seeds were

collected and sterilized with solution containing 20% NaClO and 0.1% Triton X-100 for 2 h, which subsequently was washed with sterile water for several times. The sterilized seeds were placed on the culture medium and kept in the dark at 30 ℃ for 2 days before being placed in the growth chamber for 5–7 days.

Arabidopsis wild type seeds Columbia-0 (Col-0) were sterilized with 75% alcohol for 15 min and then washed with 100% alcohol. The sterilized seeds were placed on the culture medium, vernalized at 4 ℃ for 3 days, and then incubated in the growth chamber for 7 days. The culture medium contains 5 mM KNO_3, 1 mM H_3PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, micronutrient and Fe concentration equal to those in half-strength Murashige and Skoog medium, 5 mM MES, 1% sucrose, 1% agarose, pH 5.7 adjusted with Bis Tris Propane (Sigma).

The growth chamber was maintained at a light intensity of 75–100 μmoles $e^- m^{-2} s^{-1}$; the light cycle was 12 h light and 12 h dark, and the temperature range was 22 ± 2 ℃, under which both *L. chinensis* and *Arabidopsis* plants can achieve well growth.

Ca content analysis of L. chinensis

- After grown on medium with different concentration of Ca^{2+} for 7-day, the *L. chinensis* whole seedlings were washed with 1 mM $CuSO_4$ for 1 min and sterilized water for three times to get rid of any residual mineral elements stick to the root surface. The leaves and roots were separately collected and dried for 48 hours at 80°C. The weighted samples were grounded into fine powder with an grinder and digested with 5% HNO_3 for 48 h at 37 ℃. It was further centrifuged at 12000 rpm for 10 min and the supernatant was used for Ca content measurement with inductively coupled plasma optical emission spectrometry (ICP-OES; PQ9000).

Endocytosis Examination With Confocal

- *L. chinensis* seedlings were vertically grown on medium supplied either with 1 or 0 mM Ca^{2+} for 7-day. The seedlings with roughly 2 cm-length roots were selected for confocal examination (Zeiss LSM710) of the endocytosis activity with procedure detailed previously (Zhang et al., 2018).

Extraction and measurement of L. chinensis **root lipids**

The *L. chinensis* root sampling method was as described previously [13], which was kept in dry ice and sent to the Lipidology Platform of the Institute of Genetics and Developmental Biology at the Chinese Academy of Sciences in Beijing for lipid profiling analysis using tandard and reverse phase liquid chromatography-mass spectrometry (LC/MS) [34]. There were 5 biological *L. chinensis* root replicates for each treatment.

Determination Of Mda Content And Sod Activity

The leaves and roots of *L. chinensis* and *Arabidopsis* seedlings were frozen and ground into powder for malondialdehyde (MDA) content assay with thiobarbituric acid (TBA) and superoxide dismutase (SOD) activity measurement with nitro-blue tetrazolium (NBT) method according to the kit instructions (Comin, Suzhou).

Localization Of Ho Production In Plant Tissues

For localizing H_2O_2 production, the leaves and roots of *L.chinenssis* seedlings were stained with freshly prepared 1 mg/mL diaminobenzidine (DAB) solution and vacuumed for 1 h in dark. Afterward, the tissues were clarified by alcohol and stored in 20% glycerin for photograph with a dissection microscope.

Statistics Analysis

The number of independent experiments and replicates per experiment are indicated in table headings and figure legends. Data were compared on the basis of standard error of the mean values. Student's t-test were used to determine significance among the data.

Abbreviations

FFA, free fatty acid; PC, phosphatidylcholine; LPC, lyso-PC; PE, phosphatidylethanolamine; LPE, Lyso-PE; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerols; PS, phosphatidylserines; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyl-diacylglycerol; DAG, diacylglycerols; TAG, triacylglycerols; CL, cardiolipins; FW, fresh weight; DW, dry weight; Tyr A23, tyrphostin 23; Tyr A51, tyrphostin 51; FM4-64, N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide); LC/MS, liquid chromatography–mass spectrometry; MDA, malondialdehyde; SOD, superoxide dismutase; DAB, diaminobezidin; TBA, thiobarbituric acid, NBT, nitro-blue tetrazolium.

Declarations

Ethics approval and consent to participate

This experiment does not involve human experiments and animal experiments. The plant samples of *L.chinensis* seeds were collected from wild plants with mature spike in the Inner Mongolia grassland around middle of August under the guidance of professionals, and *Arabidopsis* wild type seeds Columbia-0 (Col-0) were ordered from The Arabidopsis Information Resource (www.arabidopsis.org). The laboratory experiments were conducted under logical legislation and permissions. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

All data generated during this study are included in this published article and its supplementary information files, and the raw data used or analysed during the current study available from the corresponding author on reasonable request.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

Ju Yang and Zhi Qi conceived and designed the experiments. Ju Yang, Yang Nan and Yanbin Dong performed the most of experiments. Ju Yang and Yang Nan did the data analysis. Nan Yang, Yanbin Dong and Ju Yang wrote the manuscript. All authors read and approved the final manuscript.

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Figures

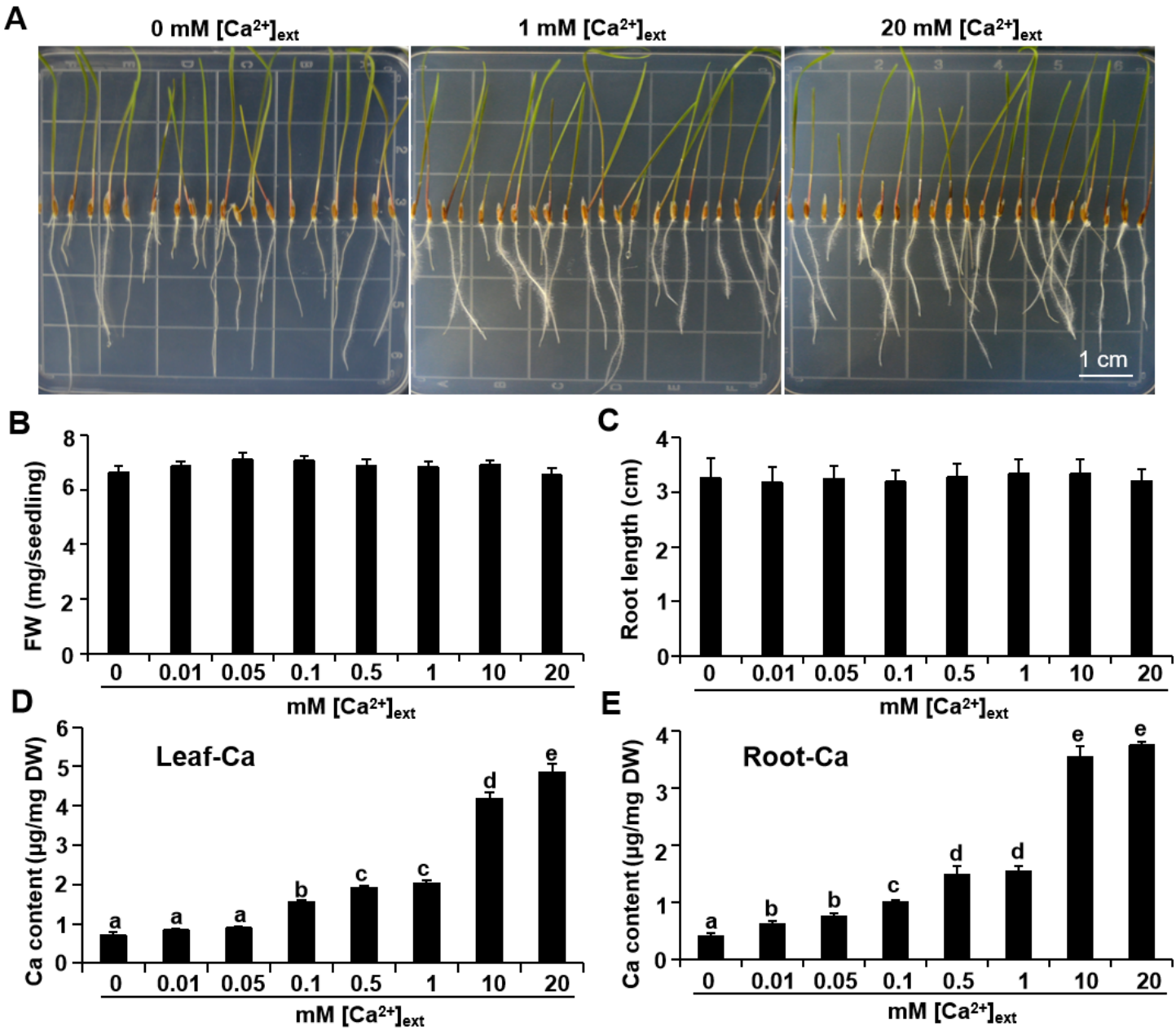

Figure 1

Effects of different $[Ca^{2+}]_{ext}$ on the growth and Ca content of *L. chinensis*. *L. chinensis* seeds were sown on medium with different Ca^{2+} concentrations, in the dark at 30 °C for 2 days; plates containing these seeds were placed vertically in a growth chamber for 7 days. **(A)** Typical growth phenotype of *L. chinensis* on the medium with different $[Ca^{2+}]_{ext}$ gradient, Bar = 1 cm. **(B)** Fresh weight of *L. chinensis* seedlings, n = 20; FW represents fresh weight. **(C)** Root length of *L. chinensis* seedlings, n = 20. **(D)** Ca content of *L. chinensis* leaves under different $[Ca^{2+}]_{ext}$. DW represents dry weight, n = 6. **(E)** Ca content of *L. chinensis*

roots under different $[Ca^{2+}]_{ext}$. n = 6. Column with different letters indicate that there are significant difference among the treatments, student t-test, $p < 0.05$.

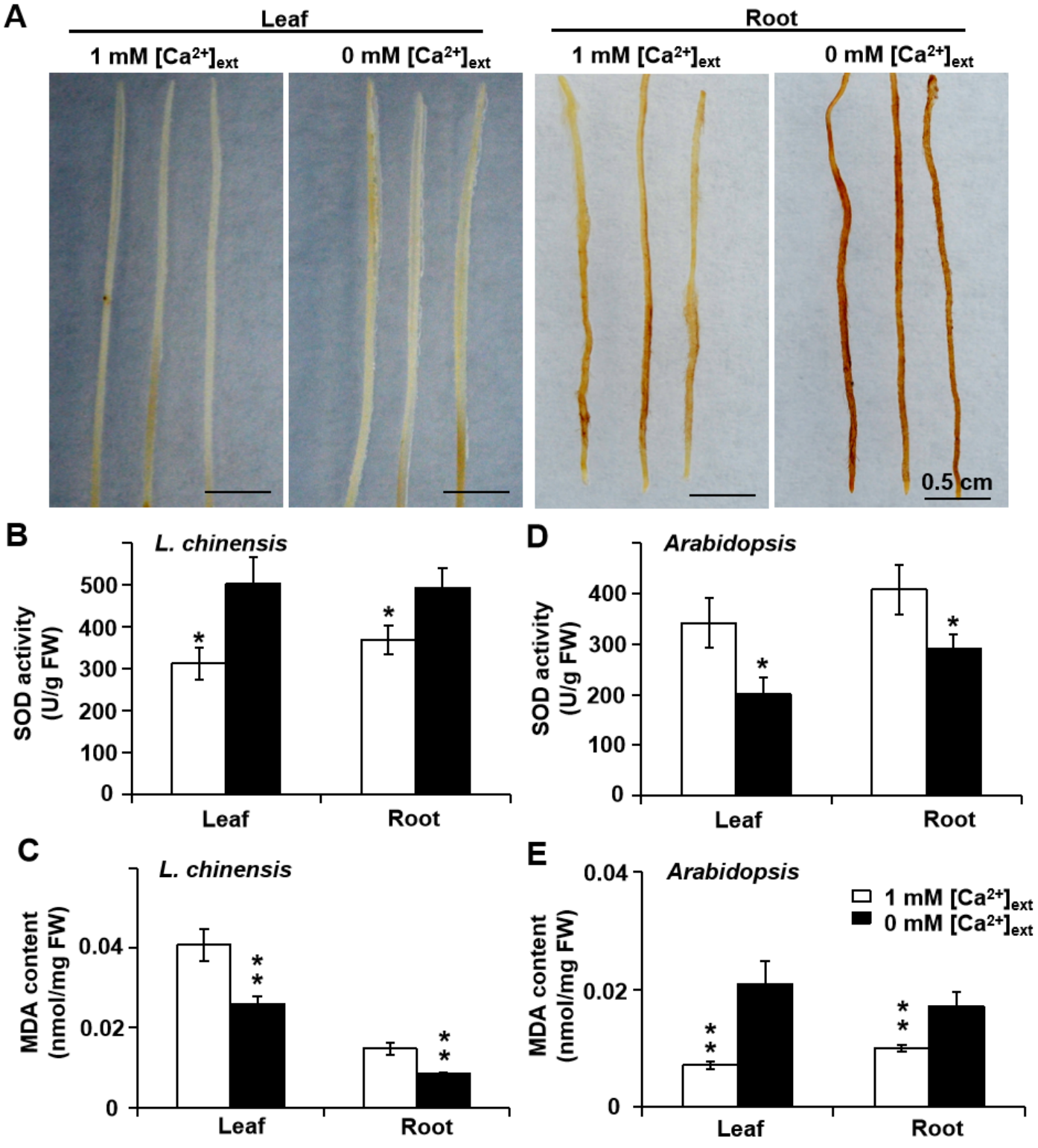

Figure 2

***L. chinensis* oxidative responses to the Ca deprivation.** Plant materials were the same as in Fig. 1. **(A)** Typical *L. chinensis* leaves and roots stained with DAB for H_2O_2 localization under CK or 0 mM $[Ca^{2+}]_{ext}$.

(B) SOD activity in the *L. chinensis.* **(C)** The MDA content in the *L. chinensis*. **(D)** SOD activity in the *Arabidopsis*. **(E)** The MDA content in the *Arabidopsis*. n = 5. *, p <0.05, **, p < 0.01 with student t-test.

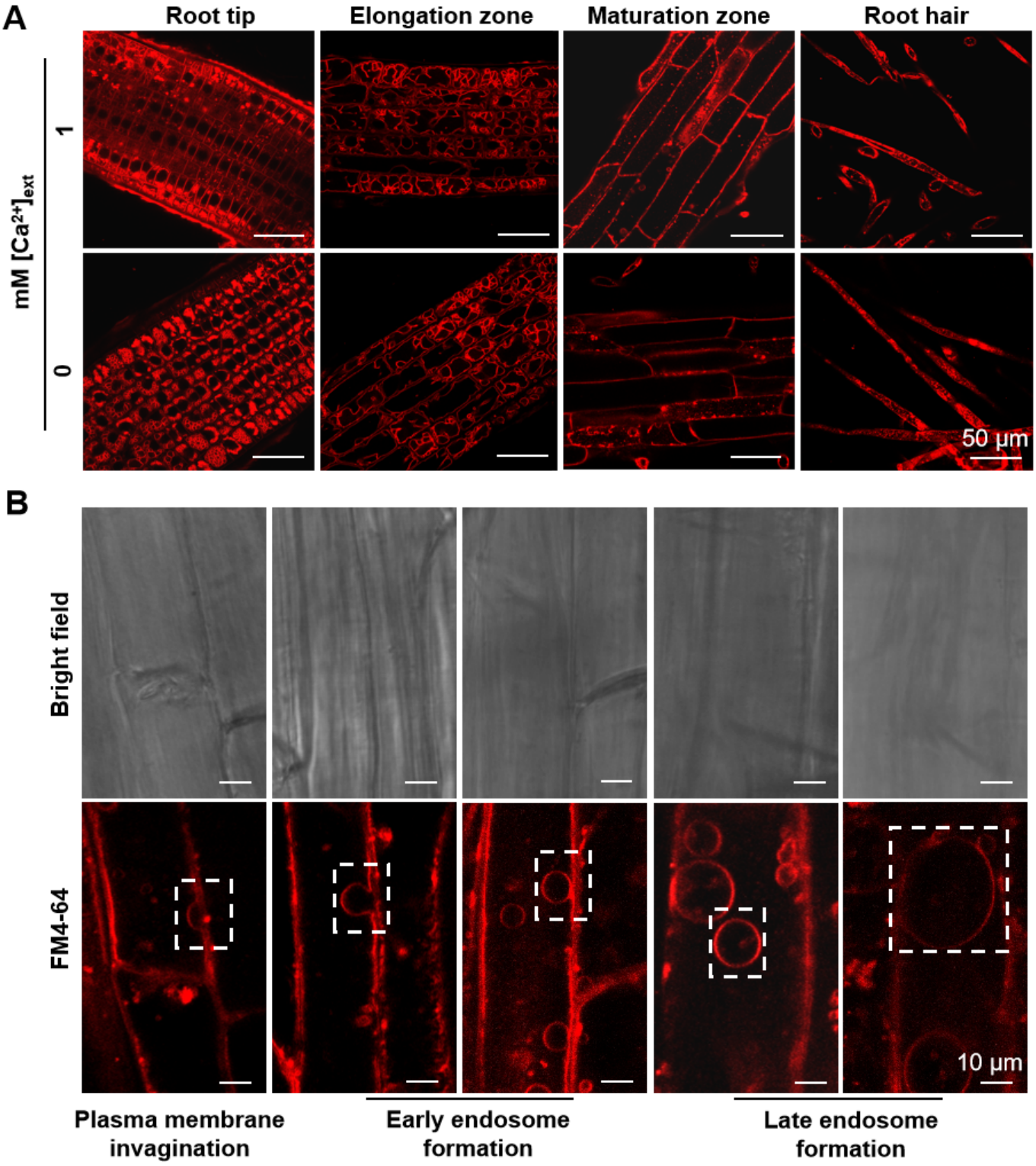

Figure 3

Endocytosis in different regions of *L. chinensis* roots and intracellular stages of endocytosis. Plant materials were the same as in Fig. 1. The endomembrane system was monitored by staining the roots

with the fluorescent membrane probe FM4-64 for 30 min. Typical bright field and confocal scanning laser microscopy images are shown. **(A)** Endocytosis in different regions of *L. chinensis* roots under CK or 0 mM $[Ca^{2+}]_{ext}$. treatment. **(B)** Intracellular stages of endocytosis in *L. chinensis* roots. The dash line regions highlight the typical endocytosis steps.

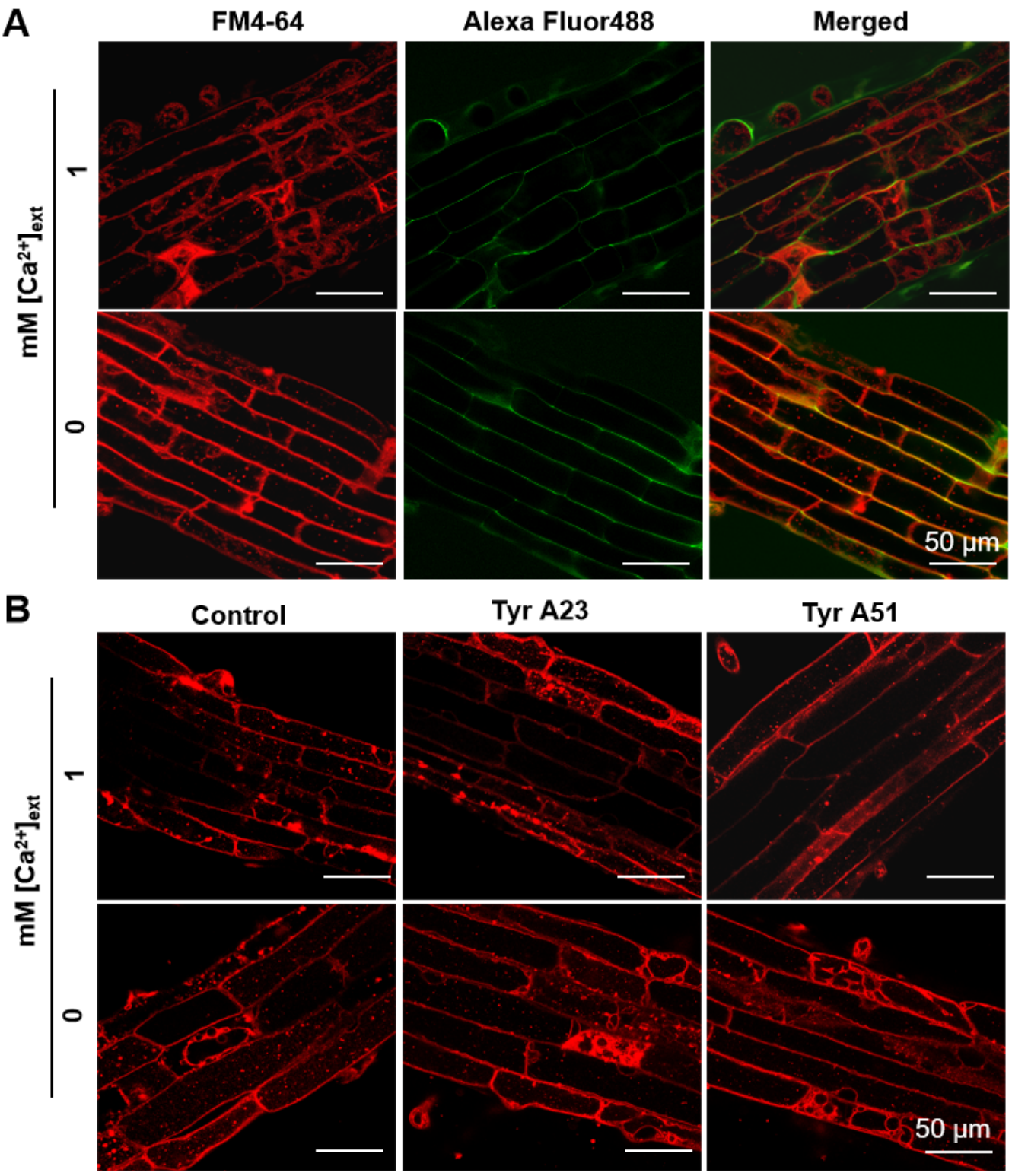

Figure 4

Effect of the Ca deprivation on *L. chinensis* root's membrane selectivity. Plant materials were the same as the Fig. 1; imaging procedure same as the Fig. 3. **(A)** Alexa Fluor 488, a membrane-impermeable green fluorescent dye without biological activity was used to track specific fluid phase-based endocytosis. *L. chinensis* seedlings were co-stained with FM4-64 and Alexa Fluor 488 for 30 min. **(B)** Tyr A23, an inhibitor of receptor-mediated endocytosis; Tyr A51, a structural analog of Tyr A23 without inhibitory activity, served as the control. *L. chinensis* seedlings were treated with 30 μM Tyr A23 or Tyr A51 in corresponding solution for 1 h, and then dyed for 30 min.

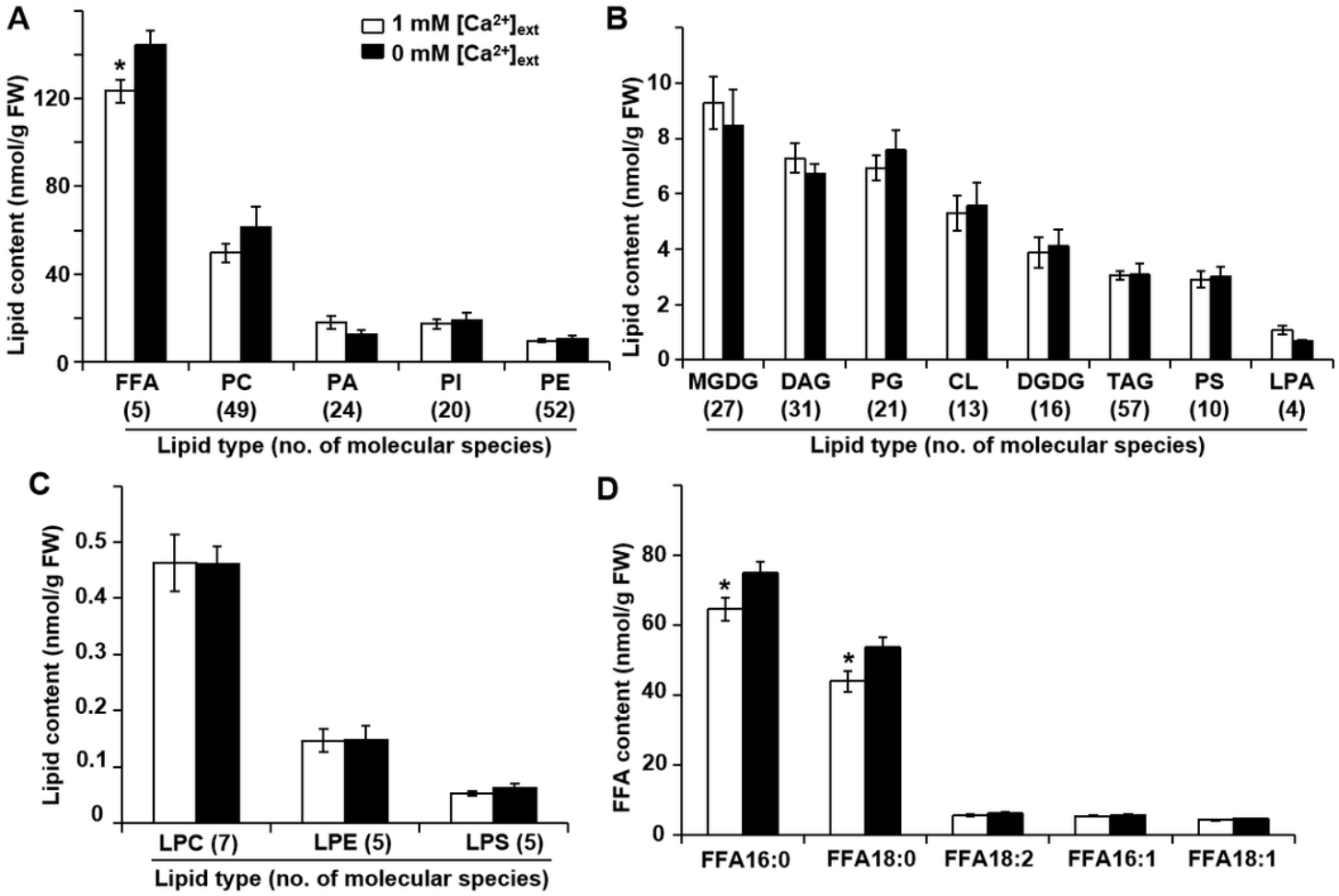

Figure 5

Effect of Ca deprivation on the *L. chinensis* root lipid profiles. Plant materials were the same as in Fig. 1. Those lipids form three distinct categories in terms of abundance, including those greater than 10 nmol/g FW **(A)**; greater than 10 but less than 10 nmol/g FW **(B)** and less than 1 nmol/g FW **(C)**. Number of the lipid molecular species in each type is in brackets. **(D)** Contents of the five molecular species of free fatty acids (FAA). FW, fresh weight. Error bars indicate average ± standard errors of five biological replicates of the root materials. *, $p < 0.05$ with student t-test.

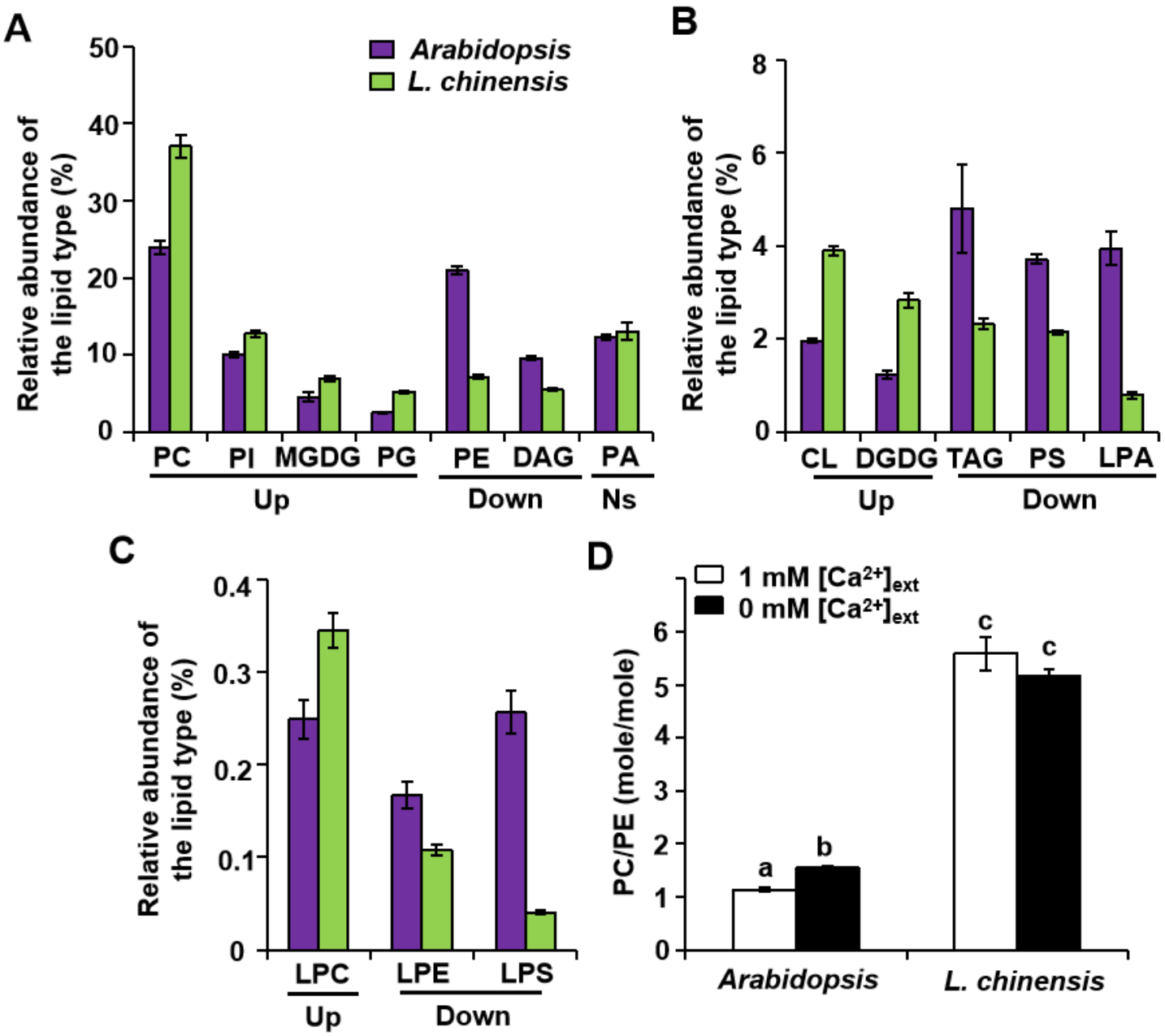

Figure 6

Lipid composition comparison between the roots of *L. chinensis* and *Arabidopsis*. Relative abundance of each lipid types to the total lipids were compared between the roots of *L. chinensis* and *Arabidopsis* (**A, B, C**). "Up" and "Down" mean that the values are higher or lower in the *L. chinensis* than *Arabidopsis*, respectively, with student t-test, $p < 0.05$, n = 5; "Ns" represents no significant difference between the two. **(D)** The molar ration of PC to PE in the roots of *L. chinensis* and *Arabidopsis* under the 1 and 0 mM $[Ca^{2+}]_{ext}$ growth conditions. Column with different letters indicate that there are significant difference among the treatments, student t-test, $p < 0.05$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- LcCaSupplementary.doc