Supporting Information

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SI Materials and Methods

Plant Growth Conditions and Ca Translocation Experiment. The *Brassica juncea* plants were grown on rock-wool block containing hydroponic nutrient solution [1.25 mM KNO_3, 1.50 mM Ca $(NO_3)_2$, 0.75 mM $MgSO_4$, and 0.50 mM KH_2PO_4] and the micronutrients [50 μM KCl, 50 μM H_3BO_3, 10 μM $MnSO_4$, 2.0 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.1 mM Na_2O_3Si, and 72 μM Fe-EDTA]. For the $^{45}CaCl_2$ uptake experiment, anti-BjPCR1 (anti-*B. juncea* plant cadmium resistance 1 protein) lines and WT *B. juncea* plants were grown in half-strength hydroponic medium for 3 wk. The plants were then incubated in medium supplemented with 0.4 MBq of $^{45}CaCl_2$ for 12 h, and the shoots were separated from the roots. The roots, which came into direct contact with the radioactive medium, were washed briefly with hydroponic solution. The samples were placed in 1-mL or 0.2-mL pipette tips, frozen, and subsequently thawed. The sap from each sample was collected by centrifugation. *A. thaliana* plants were grown on half-strength Murashige and Skoog (MS) agar medium supplemented with 1% sucrose with or without an additional 1.5–30 mM $CaCl_2$ for 3–4 wk for phenotype analysis. For Ca^{2+} translocation assays, plants were grown vertically on half-strength MS agar plates containing 1.5 mM $CaCl_2$ for 2 wk and then precultured in a hydroponic medium containing 1 mM $CaCl_2$ for 2 d. Subsequently, the plants were transferred to a hydroponic medium containing 1 mM $CaCl_2$ supplemented with 0.4 MBq of $^{45}CaCl_2$ for 12 h. After this incubation period, roots were washed with hydroponic medium containing 1 mM $CaCl_2$ and the roots and shoots were collected separately to detect ^{45}Ca. The radioactivity was measured using a liquid scintillation counter (Perkin–Elmer). Autoradiography of $^{45}CaCl_2$ was performed on plants incubated in the medium supplemented with 0.4 MBq of $^{45}CaCl_2$ for 12 h.

Yeast Strains. A Cd-sensitive yeast strain, DTY 167 (*MATa ura3 leu2 his3 trp3 lys2 suc2 ycf::hisG*) (1), which cannot express *YCF1*, was used for the Cd resistance test. For the Ca^{2+} transport experiment, we developed the SM17 yeast strain (*MATa pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ade2, ura3, vcx1::KanMX4*) by disrupting the VCX1 gene in the K616 (*MATa pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ade2, ura3*) (2) yeast strain. The *vcx1::KanMX4* was amplified by polymerase chain reaction assay using genomic DNA from the Y13825 strain (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ, ydl128w::kanMX4* (*VCX1*); EUROSCARF) as a template and a pair of primers (YDL128W-F: TTAATCTAATGCCTCCAACTGTCTG and YDL128W-R: ATGTGTTTCAGCTGGCATTCTCACTC). The *vcx1::KanMX4* fragment was introduced into K616 to induce homologous recombination, and the transformed cells were selected on a yeast extract, peptone, and dextrose (YPD) plate containing 100 μg/mL G418. The genotype of the surviving cells was confirmed by polymerase chain reaction assay using YDL128W-F and YDL128W-R primers.

Identification of *BjPCR* Gene Members in *B. juncea*. Genomic DNAs and cDNAs of *BjPCR* were amplified by genomic polymerase chain reaction and RT-polymerase chain reaction assays using genomic DNA and RNA isolated from *B. juncea,* respectively, as templates and *AtPCR1* primers (AP1-F: 5′GAATTCATGGAAGCTCAACTTCATGCCAAG3′ and AP1-R: 5′CTCGAGGCGGGTCATGCCGCCTTGGAAGGC3′). The polymerase chain reaction-products were cloned into the PYES2-NT/C vector after EcoRI and XhoI digestion. The plasmids from 10 positive clones were sequenced and analyzed using ClustalW (http://align.genome.jp). For the Cd sensitivity test, all vector constructs were introduced into the DTY167 yeast strain. The transformants were then spotted onto Cd-containing half-strength synthetic galactose plates lacking uracil (half-strength SG-ura) and grown for 3 to 5 days.

Mutagenesis of *BjPCR1* and *AtPCR2*. To identify the domain in AtPCR2 and BjPCR1 that is important for Cd resistance, chimeric mutant constructs of AtPCR2 and BjPCR1 were made. The 5′ fragments of *AtPCR2* and *BjPCR1* were extracted from pYES2 NTC-*BjPCR1* and NTC-*AtPCR2* by digestion with EcoRI and BalI, and the 5′ fragment of *AtPCR2* was then swapped for the 5′ fragment of *BjPCR1*. Site-directed mutagenesis of *BjPCR1* was performed by polymerase chain reaction assay using Q11H (GAATTCATGGAAGCTCAACACCTTCAT GCTAAG CCTCATGCT) and 34L (TGGCCAAATGTAATACATGGACACCATAATGT) primers, the sequences underlined indicate sequences to change glutamine (CAA) to histidine (H; CAT) and cysteine (C; TGT) to leucine (L; TTA). The vector constructs were introduced into the Cd-sensitive DTY167 (*MATa ura3 leu2 his3 trp3 lys2 suc2 ycf::hisG*) yeast strain. The transformants were spotted onto Cd-containing half-strength SG-ura plates and grown for 3–5 d.

Root Hair-Specific Expression of BjPCR1 in *Arabidopsis*. To express BjPCR1 in the epidermis of *Arabidopsis* roots, a vector containing the AtEXP7 promoter was constructed. The *AtEXP7* promoter (−1422-0) was amplified by polymerase chain reaction assay using EXP7pF: CCACCATGTTGGATCTATAACTGTAGTTAGATGATTAC and EXP7p-R: GAATTCTAGCCTCTTTTTCTTTATTCTTAGGG primers. The fragment of the *AtEXP7* promoter was inserted into the BstXI and EcoRI cloning sites of the MPC3300 binary vector (3). *BjPCR1*-containing *V5* tag or *GFP* was then inserted into the MPC3300_AtEXP7p vector using the EcoRI and XbaI restriction sites. The MPC3300_AtEXP7p-BjPCR1-V5 and MPC3300_AtEXP7p- BjPCR1-GFP vectors were introduced into *A. thaliana* using the flower-dipping method (4).

Assay of Organ- and Tissue-Specific Expression of *BjPCR1* and *BjPCR2*. To test *BjPCR1* and *BjPCR2* expression levels in *B. juncea*, an RT-polymerase chain reaction assay was performed using total RNA from the roots, leaves, stems, and flowers of 7-wk-old *B. juncea* plants grown in hydroponic medium. Because *BjPCR1*, *BjPCR2*, and *BjPCR3* have high levels of sequence similarity, an RT-polymerase chain reaction assay to analyze their individual expression patterns was performed using specific primer sets (BjPCR1RT-F: 5′-TATACACGTTGATAAGTTGTTTC-3′, BjPCR2RT-F: 5′-TATACGCGTTAATAAACGCAGTA-3′, BjPCR1RT-R: 5′-AGACTGGAGCACCCATCGCCACG-3′, and BjPCR2RT-R: 5′-AGACAGGAGCACCCATCGCGGCC-3′). As a control for the RT-polymerase chain reaction assay, actin1 (5′-CATCAGGAAGGACTTGTACGG-3′) and actin2 (5′-GATGGACCTGACTCGTCATAC-3′) primers were used to detect expression of the Actin gene. For root layer-specific expression of *BjPCR1* and *BjPCR2*, roots were collected from *B. juncea* grown on agar plates for 5 d and then frozen in liquid nitrogen. The frozen roots were ground gently with a mortar and pestle, and the powder from the roots was collected using an 80-mesh sieve. The roots that remained on the mesh were collected and ground again using the same method. This process was repeated four times to obtain sequential layers located from the epidermis to the stele of the roots. RNA was extracted from the powdered

samples, and the transcript levels of the four genes, *BjPCR1, BjPCR2, BjEXPA7,* and *BjHMA4*, were detected using specific sets of primers for the genes. The transcript levels of *EXPA7* and *HMA4* were analyzed as markers for root hair cells and vascular tissue cells using gene-specific primers (BjEXPA7-F: AAACCTTTTTAACAGCGGCTACGG, BjEXPA7-R: AAGAAGTGACCCGGAAAGAGAGAG, BjHMA4-F: ATCAGAAGGATTGTCTGGAGACTG, and BjHMA4-R: TCAAAGCTATTCAGTCACAGTCTCC). BjEXPA7 primers were developed using sequences from *Brassica oleracea EXPA7* (accession no. DQ899785), and *BjHMA4* primers were designed using *B. juncea HMA4* (accession no. EU418580). To determine the transcription levels of *BjPCR* genes under Ca-excess and Ca-deficient conditions, an RT-polymerase chain reaction assay was performed using RNA extracted from 3-wk-old plants treated with excess (10 mM $CaCl_2$) or without Ca^{2+} for the indicated amounts of time.

In Situ Hybridization. To perform the whole-mount in situ hybridization, sense- and antisense-oriented *BjPCR1* vectors were developed. The sense-oriented BjPCR1 vector was made by ligating the pBluescript vector (Stratagene) with the *BjPCR1* fragment excised from pYESNT/C-BjPCR1 using EcoRI and XbaI. The fragment of polymerase chain reaction for the antisense-oriented *BjPCR1* was amplified by polymerase chain reaction assay using a primer set (anti-BjPCR1 F: CGCGAGATGGAAGCTCAACACCTTC, anti-BjPCR1 R: GAATTCTTAGCGGGTCATGCCGCCTTG) and inserted into the XhoI and EcoRI sites of the pBluescript vector. To generate the probes, the vectors were linearized and cRNAs were synthesized using the Fluorescein-12-UTP RNA Labeling Mix (Roche) and T7 RNA Polymerase (Promega). Whole-mount in situ hybridization was performed using roots from 5-d-old *B. juncea* seedlings according to the protocol described by Bauwens et al. (4). Fluorescent optical sections of the samples were acquired using a confocal laser scanning microscope with a 488/530-nm excitation/emission filter set (Zeiss).

Ca^{2+} and Zn^{2+} Imaging Using Fluo-3 and Zinpyr-1 Fluorescent Dyes. *B. juncea* plants were grown on agar plates containing the salts present in the hydroponic medium for 5 d. Roots were harvested and incubated in PBS solution containing 50 μM Fluo-3-AM (Molecular Probes) for cytosolic Ca^{2+} detection or 50 μM zinpyr-1 (Sigma–Aldrich) for Zn^{2+} detection at room temperature in darkness for 4 h. The samples were then washed with PBS solution and observed using a confocal laser scanning microscope with a 488/530-nm excitation/emission filter set (Flowview FW1000; Olympus).

Western Blot Analysis. Yeast cells were treated with lyticase, and their crude extract was obtained by centrifugation at 1,000 × *g* in an extraction buffer [50 mM Hepes-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 0.7 μg/mL pepstatin A, 5 μg/mL aprotinin, 20 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride]. The supernatant of the crude extract was centrifuged at 100,000 × *g* for 1 h at 4 °C to obtain microsome. The protein preparation thus obtained (10 μg) was separated by SDS/PAGE and transferred to a nitrocellulose membrane by electroblotting. The membrane was cross-reacted with the anti-V5 antibody following the protocol provided by Invitrogen. Chemiluminescence was detected using the ECL reagent according to the instructions of the manufacturer (Amersham Pharmacia Biotech), and the signals were developed with X-ray film.

Generation of *B. juncea* with Reduced Levels of *BjPCR1* Expression. To reduce endogenous *BjPCR1* expression in *B. juncea*, a *BjPCR1*-antisense vector was constructed by inserting the *BjPCR1* fragment (BalI/EcoRI) from the pGEM Teasy-BjPCR1 vector into the EcoRI and SmaI sites of the MPC3300 vector (3), which is modified from pCAMBIA3300. For transformation of anti-*BjPCR1*. *Agrobacterium tumefaciens* strain GV3301 containing MPC3300–anti-BjPCR1 was grown to reach an OD_{600} of 0.5–0.6. The cells were collected by centrifugation and suspended in MS medium. The hypocotyl explants (0.5–1.0 cm) from the 6-d-old seedlings were infected by agrobacteria and cocultivated with the bacteria for 3 d on N1B1 agar medium (MS medium containing 1 mg/mL α-naphthylacetic acid and 1 mg/mL 6-benzylaminopurine). The explants were rinsed twice with MS medium containing 100 mg/L cefotaxime and were then transferred onto SIM agar medium [MS medium containing 200 mg/L cefotaxime, 20 μM $AgNO_3$, and 10 mg/L phosphinothricin (PPT)]. The shoots that had differentiated on the SIM medium were plated onto MS medium containing 2 mg/L indole-3-butyric acid, 10 mg/L PPT, and 200 mg/L cefotaxime to induce root development. The transgenic plants thus selected were then transferred to the soil and cultivated to generate homozygous lines. To select the knock-down mutants of *BjPCR1* in anti-BjPCR1 transgenic lines, RNA blotting was performed using 30 μg of total RNA extracted from the putative transgenic plants. The RNA was separated on a 0.8% agarose gel containing formaldehyde, transferred onto a Hybond-N nylon membrane (Amersham Pharmacia), hybridized at 65 °C with a ^{32}P-labeled *BjPCR1* and *Actin* probe, and developed on X-ray film.

Ca^{2+} Flux Assays Using *B. juncea* Protoplasts. Mesophyll protoplasts were purified from the leaves of 3- to 4-wk-old *B. juncea* WT and anti-BjPCR1 lines on a 33% (vol/vol) Percoll gradient (6). Protoplasts were resuspended in bathing solution [0.5 M glycine betain, 10 mM Mes-KOH, 5 mM $KHCO_3$, 0.1% BSA, and 18.5 kBq ^3H_2O per 0.1 mL (pH 5.6)] to a density of 1×10^7 protoplasts per 1 mL. For uptake assays, 0.1 mM $CaCl_2$ and 74 kBq of $^{45}CaCl_2$ were added to 4 mL of protoplast suspension and gently shaken at room temperature (25 °C). After a fixed incubation time, 100 μL of the sample was taken and loaded on silicon oil and 33% (vol/vol) Percoll (pH 6.0) gradients. Intact protoplasts were collected after centrifugation at 8,000 × *g* for 20 s. For efflux assays, protoplasts were incubated in 4 mL of the bathing solution containing 0.1 mM $CaCl_2$, 74 kBq of $^{45}CaCl_2$, and 74 kBq of ^3H_2O for 30 min, and they were then centrifuged for 1 min at 50 × *g*. The cells were washed twice with ice-cold bathing solution containing 0.1 mM $CaCl_2$ and gently shaken in 4 mL of bathing solution containing 0.1 mM $CaCl_2$ and 74 kBq of ^3H_2O. After each time interval, 100 μL of the sample was taken and loaded on silicon oil and 33% (vol/vol) Percoll (pH 6.0) gradients. Intact protoplasts were collected, and their ^{45}Ca and ^3H_2O contents were measured using a liquid scintillation counter. The relative numbers of intact protoplasts were estimated using the radioactive counts from ^3H_2O.

Ca^{2+} Transport Assay Using Yeast Vesicles. SM17 yeast cells transformed with the empty vector (pYES2 NTC) or BjPCR1 were grown on synthetic dextrose (SD) medium and transferred to synthetic galactose (SG) medium to induce the expression of *BjPCR1*. To reduce the endogenous Ca^{2+} efflux transport activity, 2 mM EGTA was added in the SG medium. For isolation of microsomes, cells were incubated in YPD medium for 30 min, collected by centrifugation, and digested with lyticase at 30 °C for 45–90 min (1,000 units/g of fresh weight of cells). The microsomal vesicles were isolated using a method described previously (7), and they were then frozen and stored at −70 °C until used. For the Ca^{2+} transport experiment, 0.1–200 μM $CaCl_2$ solution containing 1.85 kBq of $^{45}CaCl_2$ was added to transport buffer, which consisted of 4 mM ATP, 5 mM $MgCl_2$, 10 mM creatine phosphate, 16 units/mL creatine kinase, 1 mg/mL BSA, 100 mM KCl, and 25 mM Tris·Mes (pH 7.4). Ten microliters of thawed microsome preparation (50 μg of protein) was resuspended in 90 μL of transport buffer and incubated on ice for 5 min. The leukotriene transport assay was performed using

standard transport buffer containing 0.88 nM ^{3}H-leukotriene and 4 mM Mg-ATP for 15 min. The microsomes were mixed with the radioactive transport buffer described above and incubated at 25 °C for the indicated periods of time. The reactions were stopped using 1 mL of ice-cold washing buffer [100 mM KCl, 25 mM Tris·Mes (pH 7.4)], and the samples were filtered immediately under a light vacuum through a nitrocellulose filter (Millipore) with a pore size of 0.45 μm and washed with 2 mL of washing buffer. The radioactivity remaining on the filter was counted using a liquid scintillation counter. The dissipation of the pH gradient mediated by Ca^{2+} influx in BjPCR1- or empty vector-expressing yeast vesicles was monitored by measuring the quenching and recovery of the fluorescence of ACMA (Sigma), as described by Greutert and Keller (8). Yeast membrane vesicles containing 100 μg of protein were preincubated in 0.5 mL of an assay buffer [5 mM $MgCl_2$, 1 mg/mL BSA, 100 mM KCl, 20 mM Tris·Mes (pH 7.4), and 1 μM ACMA]. The pH gradient was allowed to develop by adding 4 mM ATP to the medium; after a steady state was reached, 5 mM $CaCl_2$, 5 mM $FeSO_4$, or 5 mM $MnCl_2$ was added. The fluorescence change was monitored using a microplate reader at 428/475-nm excitation/emission conditions (Infinite F200 PRO; TECAN).

Assay of Metal Content. The roots and shoots were prepared from *B. juncea* grown in hydroponic medium for 4 wk. The samples were briefly washed using cold water and dried at 60 °C for 4 d. The dried samples were digested with 65% (wt/vol) HNO_3 for 24 h at 200 °C and dissolved in 0.1 N of HNO_3. Ion contents were measured using inductively coupled plasma-MS (ELAN DRC-e; Perkin–Elmer).

Derivation of the Exchanger Equilibrium Potential Equation. A generic antiporter of two ionic species A and B in a membrane catalyzes the following reaction:

$$nA_{ex} + mB_{in} \Leftrightarrow nA_{in} + mB_{ex} \quad \textbf{[S1]}$$

At equilibrium, the electrochemical potentials are:

$$n\Delta\mu_A = m\Delta\mu_B \quad \textbf{[S2]}$$

because for ionic species x with charge z_x,

$$\Delta\mu_x = RT\ \ln\left(\frac{C_{x,ex}}{C_{x,in}}\right) - z_x F E_m \quad \textbf{[S3]}$$

where R is the universal gas constant; T is the absolute temperature; F the Faraday constant; $C_{x,ex}$ and $C_{x,in}$ are the external and internal concentrations of the ionic species, respectively; and E_m is the membrane potential.

When the reaction (Eq. **S1**) is at equilibrium, E_m is equal to the equilibrium potential of the exchanger (E_{eq}^{exch}), and solving for E_{eq}^{exch} (Eq. **S2**), we obtain:

$$E_{eq}^{exch} = \frac{1}{mz_B - nz_A}\left(mz_B E_{rev}^{B} - nz_A E_{rev}^{A}\right) \quad \textbf{[S4]}$$

if we define $r = \frac{m}{n}$

$$E_{eq}^{exch} = \frac{1}{rz_B - z_A}\left(rz_B E_{rev}^{B} - z_A E_{rev}^{A}\right) \quad \textbf{[S5]}$$

where $E_{rev}{}^{A}$ and $E_{rev}{}^{B}$ are the reversal potential of the species A and B.

If A is H^+ and if B is Ca^{2+}, Eq. **S5** becomes:

$$E_{eq}^{exch} = \frac{1}{r-2}\left(rE_{rev}^{B} - 2E_{rev}^{A}\right) \quad \textbf{[S6]}$$

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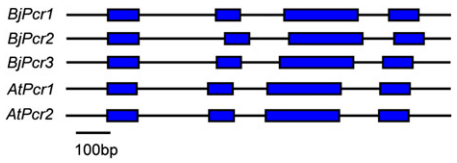

Fig. S1. Genomic structures of *BjPCR*s and *AtPCR*s. Blue boxes represent exons, and black lines represent introns.

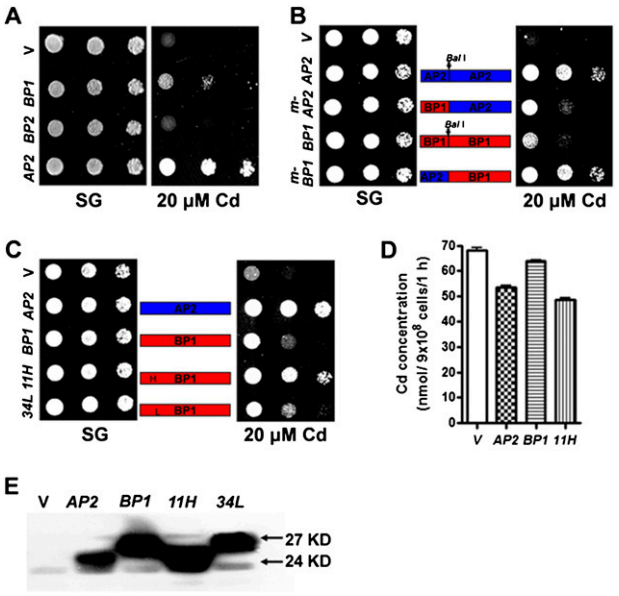

Fig. S2. Comparison of Cd resistance between *BjPCR1*-, *BjPCR2*-, and *AtPCR2*-expressing yeasts and analysis of important domains for Cd resistance. (*A*–*C*) Growth of the yeast strains expressing various constructs. The yeast strains were grown on plates without (control) or with Cd(II) at 30 °C for 4 d. Yeast strains were *ycf1* cells carrying empty vector pYES2/NTC (V), *BjPCR1* (*BP1*, red bar), *BjPCR2* (*BP2*), *AtPCR2* (*AP2*, blue bar), *mAtPCR2* (*mAP2*, red and blue bar), and *mBjPCR1* (*mBP1*, blue and red bar); *BjPCR1* replaced the 11th glutamine with histidine (11H), and BjPCR1 replaced the 34th cysteine with leucine (34L). (*D*) Cd concentration in yeast cells expressing pYES2/NTC (V), *AtPCR2* (*AP2*), *BjPCR1* (*BP1*), and BjPCR1 mutated with the 11th histidine (*11H*). The data represent averages of six samples collected in two separate experiments. (*E*) Western blot of AP2, BP1, 11H, and 34L tagged with V5 at their C termini. Proteins were separated by SDS/PAGE, blotted, and cross-reacted with V5 antibody.

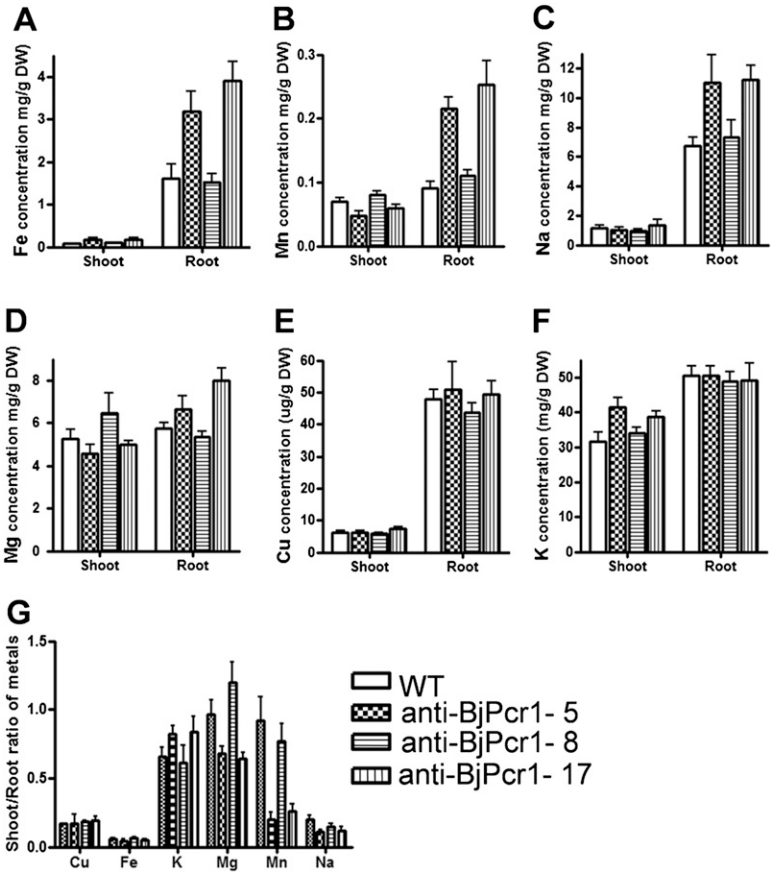

Fig. S3. Metal concentrations in the *BjPCR1* antisense lines. Fe (*A*), Mn (*B*), Na (*C*), Mg (*D*), Cu (*E*), and K (*F*) concentrations in the shoots and roots of anti-BjPCR1 plants and the corresponding WT plants. DW, dry weight. (*G*) Shoot-to-root ratios of mineral ion concentrations as shown in Fe (*A*), Mn (*B*), Na (*C*), Mg (*D*), Cu (*E*), and K (*F*). Samples were prepared from plants grown in hydroponic medium for 4 wk. Ion contents were measured using inductively coupled plasma. The values from WT and mutant plants are the means (± SE) of two independent experiments (n = 6, N = 2).

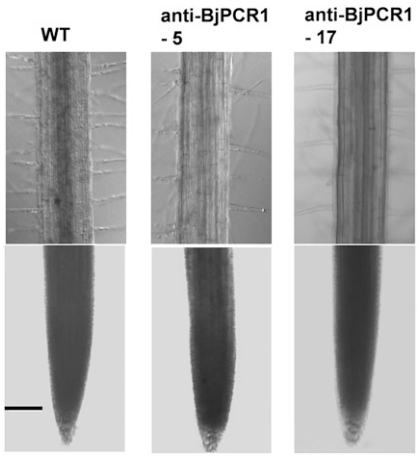

Fig. S4. Bright-field images of the roots of WT and anti-BjPCR1 plants used to visualize fluo-3 fluorescence in Fig. 2*F*. Roots of WT and anti-BjPCR1 lines were visualized by confocal microscopy. (Scale bar = 500 μm and applies to all panels.)

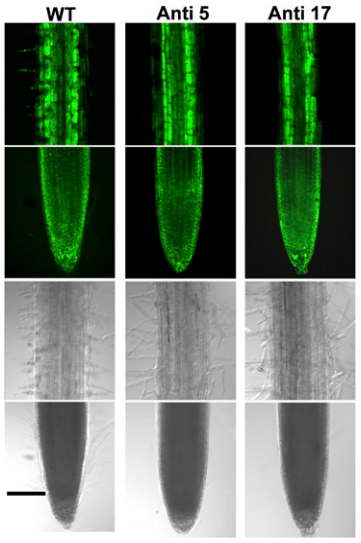

Fig. S5. Zn^{2+} distribution in the roots of WT and anti-BjPCR1 plants visualized by zinpyr-1. WT and anti-BjPCR1 lines (Anti 5, anti–BjPcr1-5; Anti 17, anti–BjPcr1-17) were grown on agar medium containing 1 μM $ZnSO_4$ for 5 d, the roots were treated with saline solution containing 50 μM zinpyr-1 for 4 h, and the Zn distribution was visualized by confocal microscopy. Green fluorescence images (*Upper*) and corresponding bright-field images (*Lower*) are shown. (Scale bar = 500 μm and applies to all panels.) Growth conditions were the same as those described for the Ca imaging experiment in Fig. 2*F*.

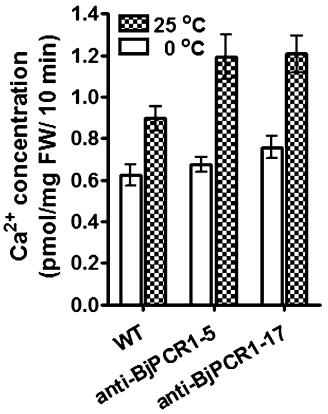

Fig. S6. Short-term ^{45}Ca uptake into *B. juncea* roots at 0 °C and 25 °C. Roots from plants grown in hydroponic medium were dissected into 5-mm segments, incubated in hydroponic medium containing 1 mM $CaCl_2$ and 0.4 MBq of $^{45}CaCl_2$ for 10 min at 25 °C or on ice, and washed with ice-cold hydroponic medium containing 1 mM $CaCl_2$. Mean ± SE is shown (n = 5). Radioactivity was counted using a liquid scintillation counter. FW, fresh weight.

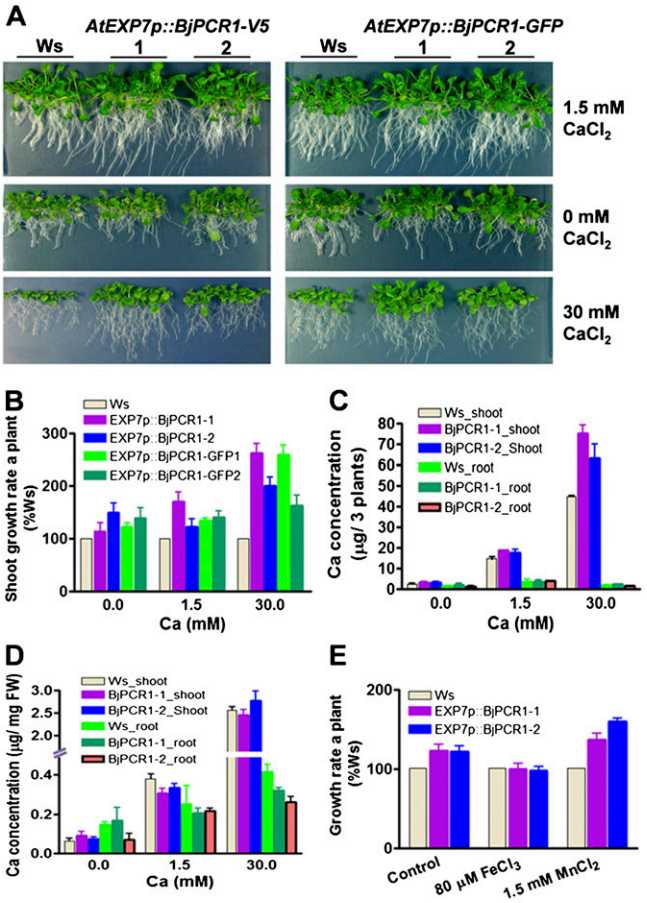

Fig. S7. Root hair-specific expression of *BjPCR1* in *Arabidopsis* results in enhanced growth and an increased Ca concentration in the shoot when grown in medium supplemented with a high level of Ca. (*A*) Phenotypes of WT plants (Ws) and AtEXP7p::BjPCR1-V5 (*Left*) and AtEXP7p::BjPCR1-GFP (*Right*) transgenic lines grown on half-strength MS plates with or without additional $CaCl_2$ for 3 wk. (*B*) Fresh weight (FW) of the shoots of plants grown as described in *A*. Note that BjPCR1-GFP as well as BjPCR1-V5 exhibited better growth than nontransgenic plants, especially in the presence of excess Ca. (*C* and *D*) Ca concentrations in the shoots and roots of the AtEXP7p::BjPCR1 transgenic *Arabidopsis* lines. Ca concentrations of three plants combined (*C*) and the same data normalized by the FW of the samples (*D*). (*E*) Growth rate (FW) of Ws and AtEXP7p::BjPCR1-V5 transgenic lines of plants grown on half-strength MS plates with or without an additional 1.5 mM $MnCl_2$ and 80 μM $FeCl_3$ for 3 wk. Mean ± SE is shown (n = 3, N = 2).

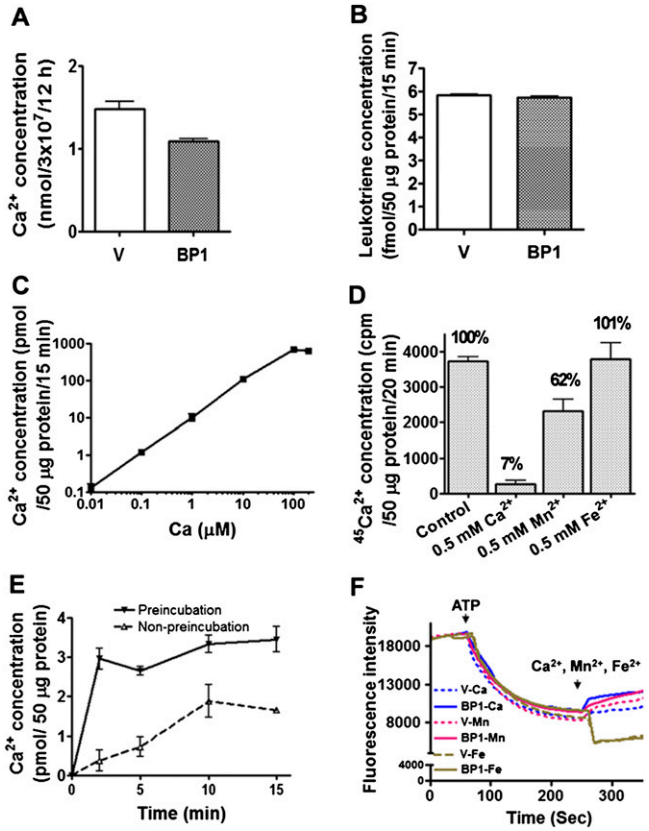

Fig. S8. Ca^{2+} transport kinetics of BjPCR1. (*A*) Ca uptake into yeast cells transformed with empty vector (V) or *BjPCR1* (BP1). Cells were cultured in SG liquid medium until OD_{600} = 1, incubated in SG liquid medium containing 0.1 mM $CaCl_2$ supplemented with 0.4 MBq of $^{45}CaCl_2$ for 12 h, washed with SG liquid medium, and collected by centrifugation. The radioactivity of samples was measured using a liquid scintillation counter. Mean ± SE is shown (n = 4, N = 2). (*B*) Leukotriene transport activity in V and BP1-expressing yeast microsome vesicles. Mean ± SE is shown (n = 4, N = 2). (*C*) Concentration-dependent Ca^{2+} uptake into BjPCR1-expressing vesicles. Ca^{2+} uptake assay was performed in the presence of 4 mM Mg-ATP in a standard transport buffer containing different concentrations of $CaCl_2$ at 25 °C for 20 min. The values represent the Ca^{2+} concentrations in vesicles expressing BjPCR1 minus those in vesicles transformed with empty vector. Mean ± SE is shown (n = 4, N = 2). (*D*) Competition assay for $^{45}Ca^{2+}$ transport activity mediated by BjPCR1 in yeast vesicles. $^{45}Ca^{2+}$ uptake was measured in the absence (Control) or presence of 500 µM of unlabeled $CaCl_2$, $MnCl_2$, or $FeCl_3$ (n = 4, N = 2). (*E*) Faster Ca^{2+} uptake into preenergized BjPCR1-expressing vesicles. The microsomes, which were preincubated in a transport buffer supplemented with 4 mM Mg-ATP for 20 min, were incubated in a standard transport buffer containing 1 µM $CaCl_2$ supplemented with 1.85 kBq of $^{45}CaCl_2$ for the indicated times. The vesicles were collected by filtration on a nitrocellulose filter. The values represent the averages of the Ca^{2+} uptake mediated by BjPCR1 (n = 4, N = 2). (*F*) Ca^{2+}-induced change in the fluorescence of ACMA in microsomal vesicles expressing BP1. The pH change was initiated by adding 4 mM ATP into the medium in which the vesicles were suspended, and when the pH was stabilized, 5 mM $CaCl_2$, 5 mM $MnCl_2$, or 5 mM $FeSO_4$ was added to the medium. The quenching and recovery of fluorescence indicate the influx and efflux of H^+ into the vesicles, respectively.

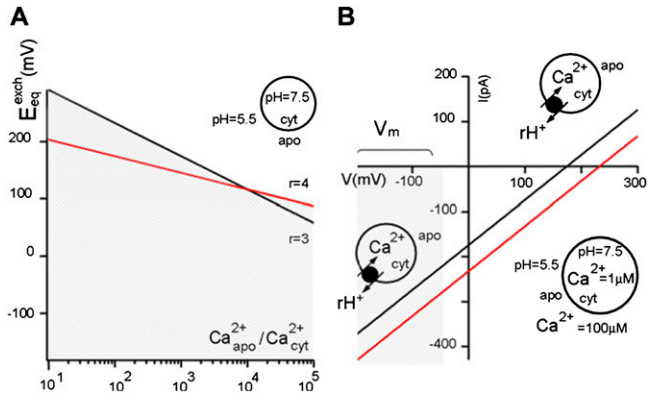

Fig. S9. Model of an H^+/Ca^{2+} exchanger. (*A*) Equilibrium potentials (E_{eq}^{exch}) of an H^+/Ca^{2+} exchanger calculated with Eq. S1 at different ratios of apoplastic-to-cytosolic Ca^{2+} concentrations ($Ca^{2+}_{apo}/Ca^{2+}_{cyt}$) in a fixed pH gradient, as indicated. When r is 3 or 4, the E_{eq}^{exch} is markedly positive, even at very high Ca^{2+} ratios of 10^5. Therefore, an H^+/Ca^{2+} exchanger with such a stoichiometry allows Ca^{2+} efflux at a membrane potential lower than E_{eq}^{exch}. (*B*) Calculated current-voltage curves of a nonrectifying exchanger in fixed Ca^{2+} and pH gradients, as indicated, and with $r = 3$ and 4. For the calculations in *A* and *B*, we used stoichiometric coefficients $r > 2$, because our transport assay shows that this stoichiometry is the most likely to occur in BjPCR1. The inhibition of BjPCR1-mediated transport by valinomycin indicates that the transport is affected by membrane potential (main text). Therefore, the transporter might be electrogenic and $r \neq 2$. If $r < 2$, the excess positive charge originates from Ca^{2+}, whereas if $r > 2$, the positive charges of the protons exceed those of Ca^{2+}. From a thermodynamic point of view, an $r < 2$ is unlikely, because Ca^{2+} charges would have to move against the membrane potential, which ranges from −80 to −140 mV across the plant plasma membrane. Moreover, if $r < 2$, a reduction in membrane potential would stimulate Ca^{2+} transport. Our transport assays show exactly the opposite; valinomycin inhibits the Ca^{2+} transport (main text). Therefore, the most likely stoichiometry for BjPCR1 is $r > 2$. (details are provided in main text). E_{eq}^{exch} is calculated using the following equation: $E_{eq}^{exch} = (rE_{H+} - 2E_{Ca}{}^{2+})/(r - 2)$, where r is the stoichiometric coefficient of the exchange mechanism rH$^+$:Ca^{2+}, and $E_{Ca}{}^{2+}$ and E_{H+} are the Nernst potentials for Ca^{2+} and H^+, respectively (details provided in *SI Materials and Methods*). The current-voltage relationships were calculated with Ohm's equation [$i = (V_m - E_{eq}^{exch})R$, where i is the current, V_m is the membrane potential, E_{eq}^{exch} is the equilibrium potential of the exchanger, and R is the membrane resistance].