Supporting Online Material for

A G Protein–Coupled Receptor Is a Plasma Membrane Receptor for the Plant Hormone Abscisic Acid

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Materials and Methods

Materials and growth conditions. All Arabidopsis thaliana ecotypes were on a Columbia background otherwise indicated. gcr2-1, gcr2-2 and gcr2-3 seeds were obtained from Arabidopsis Biological Resource Center, they were corresponding to SALK_041449, SALK_073069 and SALK_134030, respectively. All the insertions were confirmed by sequencing and only one T-DNA insertion site in each of three alleles. The primers for verifying mutants are as following (specific primer for GCR2 forward: 5' GTTTTTGCTGCTGTGCTTTG-3'; reverse: 5' GTTCACCTGGTACGCCGTTCTCT-3' and specific primer for T-DNA left border: 5' GGCGTGAGCGTCTGACACTC-3'). The primers for transcriptional analysis are as following (forward: 5'-ATGCGGAGTTTGTGACGTCCT-3'; reverse: 5' TTAGAGTTCAACCTGGAAACGAGC-3' and reverse primer for partial transcript: 5' CCCTCGGAAACGGCTAAAGTC-3'). The GPA1 mutant, gpa1-2, and overexpression line, wGα (Ws background), have been described previously (S1,S2). gcr2gpa1 was obtained by crossing gcr2-1 and gpa1-2. Full length GCR2 cDNA fused with a C-terminal FLAG (for overexpression phenotype and Co-IP) or YFP (for subcellular localization) tag was cloned in the glucocorticoid-inducible vector pTA7002, and the vector was introduced into Col-0 wild type plants via Agrobacterium tumefaciens-mediated transformation. wGaOEgcr2-1 was obtained by crossing wGa and gcr2-1. GCR2OEgpa1-2 was obtained by crossing GCR2OE and gpa1-2. Seeds were sterilized with 75% ethanol for 1 min and 15% bleach for 10 min, respectively, followed by washing three times with sterilized water, and keep in 4°C for 48 hours, then germinated on 1×MS media plates containing 1% sucrose. Plants were grown in controlled environment growth chambers under a 16-h-light (22°C)/8-h-dark (20°C) light cycle. The light intensity was 250 μmol m⁻² s⁻¹.

Vector construction and plant transformation. The full length of GCR2 cDNA was amplified by PCR using the following primers (forward: 5' cctcgagatgcggagtattttcgggaagatttag-3' and reverse: 5' gactagttttactcgtatatctgactttgttaggctatcacattcataacgag-3', which contains a FLAG tag) and cloned into pTA7002 with XhoI-SpeI site for transforming wild-type plants. A sequence of genomic DNA 1223 bp upstream of the GCR2 start codon was fused with GUS from pBIN121 followed by NOS terminus, and further cloned in pCAMBIA1300 with SalI-EcoRI site. A 1353bp fragment of GPA1 promoter was amplified by PCR using specific primers (forward: 5' ctgcagaatccaaaaccttagccttc-3' and reverse: 5' ggatccctgaagaaaaagaaatggagtcaaag-3') and cloned into GCR2::GUS plasmid but replace the GCR2 promoter by using suitable restriction endonucleases. For the ABA marker gene expression analysis, the promoter region of three typical genes, RD29A, KIN1 and ABI5, were amplified by PCR using specific primers as following (RD29A forward: 5' ctgcatcttttgaatgtttgatgtttttgatgt-3' and reverse: 5' tctgatttttcctttcagtttccatagg-3'; KIN1 forward: 5' ctgcatcttttttttttttcttttttctttttttttttttttctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
transformation was performed by *Agrobacterium*-mediated transformation (S3). The wild type (Col-0) plants harboring *RD29A::GUS*, *KIN1::GUS* or *ABI5::GUS* was crossed with *gcr2-2* plant, respectively to get the *promoter::GUS* transgenic line under *gcr2-2* background.

**Seed dormancy and seed germination.** For dormancy assay, the seeds which localized at the third and forth siliques from the complete dehydrated silique were collected and sown on the water-soaked filter paper immediately as described by Parcy *et al.* (S4). After grown for 7 d at 22°C under continuous white light (150 μmol m⁻² s⁻¹) in a growth chamber (Percival Scientific), the germination was scored and defined as an obvious protrusion of the radical through the seed coat. The image was photographed with a Leica MZ16 microscope equipped with a Canon Powershot S70 camera. In the germination assay, seeds with different genotype were sterilized as described above and sown on the filter paper on the 0.8% agar (Sigma) containing 1/2MS (Sigma, pH 5.8) and 1% (w/v) sucrose with or without indicated concentration of ABA (Sigma), chilled at 4°C in the dark for 48 h, then germinated at 22°C. After grown for 3 d under continuous white light (150 μmol m⁻² s⁻¹), the germination percentage was scored as described above.

**Seedling growth.** Wild-type, *gcr2* and GCR2 overexpressor line seeds were surface sterilized and sown on the MS plates containing 1xMS (Sigma, pH 5.8), 30 μM Dex (Sigma), 1% (w/v) sucrose and 0.3% agar (Sigma) with or without 0.3 μM ABA (Sigma), stratified at 4°C in the dark for 48 h. After 10 d grown under continuous white light (150 μmol m⁻² s⁻¹) at 22°C, the image was taken by an Olympus C-7070 digital camera.

**Stomatal aperture measurement and water loss analysis.** Stomatal response to ABA treatment assay and water loss analysis were performed according to Pei *et al.* (S5), Leung *et al.* (S6) and Pandey and Assmann (S7) with slightly modification. In briefly, for the stomata closure assay, the 5-week-old rosette leaves of *Arabidopsis* were incubated in KCl-MES buffer (50 mM KCl, 10 mM MES, pH 6.12) under continuous white light (250 μmol m⁻² s⁻¹) for 90 min to open the stomata. The strip with opened stomata from one half of a pretreated leaf was transferred to the KCl-CaCl₂ MES buffer (50 mM KCl, 1 mM CaCl₂, 10 mM MES, pH 6.12) with 25 μM ABA (Sigma) to test ABA sensitivity and the strip from the other half leaf transferred to the assay buffer without ABA as control. After incubated 2 h under light (250 μmol m⁻² s⁻¹), the stomatal apertures width was measured under microscope (magnification, ×40) for 50 randomly selected stomata. Each assay was repeated three times. The data represented means ±SE (n=150). For the stomata opening assay, rosette leaves of similar developmental stage from 5-week-old plants were clipped and dipped into the KCl-CaCl₂ MES buffer (50 mM KCl, 1 mM CaCl₂, 10 mM MES, pH 6.12) immediately. After 2 h in dark, the leaves were transferred into KCl-CaCl₂ MES buffer (50 mM KCl, 1 mM CaCl₂, 10 mM MES, pH 6.12) with or without (as control) 25 μM ABA (Sigma) and exposed to light for another 2 h under the normal growth condition as described above to open the stomata. The epidermis peels were striped and the stomatal width was measured as described above. For water loss assay, 5-6 rosette leaves were detached from indicated plants grown in the same stage and identical condition and weighed in plastic Petri immediately as fresh weight, then
placed into a laboratory bench and weighed at indicated time intervals. The water loss rate was measured as percentage of initial fresh weight.

**Patch-Clamping procedure.** The isolation of guard cell protoplasts were described by Pei *et al.* (S5) and Wang *et al.* (S8). Solutions used in patch-clamp experiments on K⁺ channels were as follows: 10 mM MES (Tris, pH 6.0), 10 mM K-glutamate, 4 mM MgCl₂, and 1 mM CaCl₂ [osmolality of 540 mmol/kg (bath solution)] and 10 mM Hepes-Tris (pH 7.8), 80 mM K-glutamate, and 20 mM KCl [osmolality of 560 mmol/kg (pipette solution)]. Fresh adenosine triphosphate (ATP) (10 mM from a 0.5 M Mg ATP stock solution in 0.5 M Tris) was added daily to the pipette solution. Final osmolalities were obtained by addition of sorbitol. Protoplasts were pretreated for at least 1.5 hours with ABA, and 25 µM ABA was added to bath solutions. Time-activated currents were calculated as the difference between average steady-state current between 3000 and 3800 ms. Whole-cell currents were measured using an amplifier (Axopatch-200A, Axon Instruments, Foster City, CA) connected to a microcomputer via an interface (TL-1 DMA Interface, Axon Instruments). pCLAMP (version 6.0.2, Axon Instruments) software was used to acquire and analyze the whole-cell currents. After the whole-cell configuration was obtained, membrane potential was clamped to -58 mV (holding potential). Standard whole-cell recording techniques were applied in this study. Glass pipettes pulled from glass capillaries (Kimax-51, Kimble, Vineland, NJ) and heat-polished before using had resistances of approximately 40 MΩ for the recordings. Whole-cell clamping was performed at room temperature (20°C ± 2°C) and the seal resistance was no less than 1 GΩ in all experiments. Cell capacitance was measured for each cell using the capacity-compensation device of the amplifier. Data were acquired 10 min after the formation of the whole-cell configuration. Whole-cell currents were filtered at 1 kHz by a four-pole Bessel filter before storage on a computer disk. Leak currents were subtracted before whole-cell current-voltage relations were generated. Leak currents for each cell were defined from the first one to three data points obtained after the membrane potential was stepped from the holding voltage to the test voltages. The mean values of time-activated whole-cell currents were determined as the average of samples obtained between 3.0 and 3.9 s after imposition of the test voltage (i.e. when the current amplitude had reached a plateau). After leak currents were subtracted, the final whole-cell currents were expressed as currents per unit capacitance (pA/pF) to account for variations in the cell surface area.

**Protein purification.** Full length cDNA of *GCR2* or *GPA1* was cloned into pET28a with *BamHI*-XhoI site. Expression and purification of GCR2 or GPA1, which carry a N-terminal His6 tag, was performed using QIAexpress Purification System by affinity chromatography to Ni-NTA agarose columns (Qiagen) according to the manufacturer’s instructions. The buffer was modified slightly as the following: lysis buffer (25 mM HEPES, 50 mM NaCl, 0.1% Triton X-100, pH8.0), washing buffer (25 mM HEPES, 50 mM NaCl, 15 mM imidazole, pH8.0) and elution buffer (25 mM HEPES, 50 mM NaCl, 250 mM imidazole, pH8.0). The elution products were further flowed through an anion exchange column (Source-Q, Pharmacia) with elution buffer A (25 mM HEPES, 3 mM DTT, pH8.0) and elution buffer B (25 mM HEPES, 3 mM DTT, 1 M NaCl, pH8.0) to get purified GCR2 or GPA1 protein. FCA (amino acids 500 to 747) truncated cDNA was cloned into pGEX-6P-1 with *BamHI*-XhoI site and protein purification was performed
using Glutathione Sepharose 4B (Amersham). The GST tag was removed by PreScission Protease (Pharmacia).

**BIACore surface plasmon resonance analysis.** Real-time protein-protein interaction was examined using a BIACore 2000 instrument (BIACore) according to the manufacturer’s manual. Purified GCR2, GPA1 (200 µg/ml) and BSA (200 µg/ml, Sigma) were individually immobilized on different flow cell of a CM5 sensor chip using an amine-coupling kit (BIACore). GCR2-GPA1 interaction was examined by flowing the indicated concentration of GPA1 through the flow cell, which was labeled BSA as control and labeled GCR2 as the test. The kinetic constants were obtained by flowing different concentrations (20, 40, 60, 80, 100, 120 nM) of GPA1 through the chip surface. Sensorgrams were subjected to global analysis using BIAevaluation software 3.2. Global fitting was used to analyze the association and dissociation data for ligand-analyte interaction. The sample 1:1 (Langmuir) binding model was employed to fit the data.

**Split-ubiquitin assay.** Yeast split ubiquitin assay and vector construction were according to Obrdlik et al. (S9) and Pandey and Assmann (S7). Briefly, each of the PCR production of GCR2, GCR2IL2 (C290-401), N terminus (N1-289), GCR1 or GPA1 was co-transformed with pNXgate (which was cleaved with EcoRI/SmaI) or pXNgate (which was cleaved with EcoRI/SmaI) into AP5 strain, respectively. And each of the PCR production of GCR2, GCR2IL2 (C290-401), N terminus (N1-289), GCR1 or GPA1 was co-transformed with pMetYCgate (which was cleaved with HindIII/PstI) into AP4 strain, respectively. Transformants were selected on the SC media (transformed AP4 on –Leu SC media and transformed AP5 on -Trp SC media), and the plasmids were extracted from yeast and amplified in *E. coli*. The DNA sequence was confirmed by sequencing. Interaction was examined by yeast diploid colonies growth or X-Gal overlay assay on the SC media containing 200 µM Met, as described by Obrdlik et al. (S9).

**Protoplast transformation and BiFC assay.** Protoplasts were isolated from 5-week-old *Arabidopsis* rosette leaves grown under the 8 h light/16 h dark light cycle as described by Asai et al. (S10). Full length (GCR2) and the N-terminal sequence (GCR2IL, amino acids 1 to 289, which is corresponding to the T-DNA insertion site of *gcr2-2*, and corresponding to the truncated GCR2 protein with deletion of the predicated third cytosol loop and free C-terminus as well) and GPA1 full length cDNA were cloned into pUC–SPYCE and pUC–SPYNE, which included a HA and cMYC tag between MCS and YFP<sup>N</sup> or YFP<sup>C</sup>, respectively (S11) in the *XbaI-StuI* site. Then GCR2:HA-YFP<sup>C</sup> and GCR2IL:HA-YFP<sup>C</sup> were amplified by PCR and cloned into pTA7002 (Dex-induced, Aoyama et al. (S12). Twenty to thirty µg plasmids of GPA1:cMYC-YFP<sup>N</sup> and GCR2:HA-YFP<sup>C</sup> or GPA1:cMYC-YFP<sup>N</sup> and GCR2IL:HA-YFP<sup>C</sup> were co-transformed into protoplast as described by Asai et al. (S10) and treated with 30 µM Dex, followed by incubation of transformed protoplasts under continuous light at 22°C for 24 h. YFP fluorescence was observed under the confocal laser scanning microscopy (LSM 510 Meta, Zeiss). The split-YFP assay has the tendency to yield false positives. In the preliminary blind assays, we found, as the control, either GPA1:cMYC-YFP<sup>N</sup> + YFP<sup>C</sup> or YFP<sup>N</sup> + GCR2:HA-YFP<sup>C</sup> has a few false positive signals, about 15% of that in GPA1:cMYC-YFP<sup>N</sup> + GCR2:HA-YFP<sup>C</sup>. To get more strict control, we selected GPA1:cMYC-YFP<sup>N</sup> +
GCR2IL:HA-YFPC as the negative control. In this case, only few protoplasts have very weak and ubiquitous fluorescence, less than 5% of that in GPA1:cMYC-YFPN + GCR2:HA-YFPC assay.

Co-immunoprecipitation assay in planta. The 14d-old seedlings of wild-type and GCR2:FLAG transgenic plants from MS plate were transferred to liquid MS media (1×MS, 1% sucrose, pH 5.8) and treated with or without Dex (Sigma, 30 µM) for 36 h. The samples were ground in liquid N2 and then on ice in 3 volumes of IP buffer (50 mM Na3PO4 pH 7.5, 200 mM NaCl, 10% glycerol, 0.1% NP40, 10 mM NaF, 2.5 mM glycerolphosphate, 1 mM Na3VO4, 0.5 mM DTT, 1 mM PMSF, 1 x plant protease inhibitor cocktail; Roche). Lysates were centrifuged at 15,000 g for 15 min at 4°C. The supernatants were incubated with 40 µl ANTI-FLAG® M2 Affinity Gel (Sigma) for 4 h at 4°C. The antigen-antibody complex was collected by centrifugation at 6,000 g for 2 min at 4°C. The beads were washed 3 times with 4.5 ml IP buffer, and then boiled in SDS sample buffer followed by running on a 8% SDS-PAGE gel. The proteins were probed with anti-GPA1 and anti-FLAG antibodies, respectively.

ABA binding assay. Purified GCR2 protein was used to determine the ABA binding activity. Protein expression, purification and ABA in vitro binding assay were conducted according to Razem et al. (S13) and Zhang et al. (S14) with slightly modification. Briefly, after purifying the GCR2 or FCA protein from E. coli. as described by Razem et al. (S13), the elution buffer in the GCR2 or FCA protein solution was exchanged to the binding buffer (25 mM Tris-HCl buffer, pH 7.3, 250 mM sucrose, 5 mM MgCl2, 1 mM CaCl2) by using the HiTrap desalting columns (Amersham). Fifty ng of purified GCR2 or FCA protein was used to test ABA binding activity in vitro as described by Zhang et al. (S14) using DCC (Dextran T70-coated charcoal, Sigma) method. The incubation medium included 25 mM Tris-HCl buffer, pH 7.3 (except for testing ABA binding at different pH), 250 mM sucrose, 5 mM MgCl2, 1 mM CaCl2, 50 nM 3H-(±)ABA (Amersham, 40 Ci/mmol; except for the determination of GCR2-ABA binding kinetics) and 50 ng GCR2, FCA or GPA1. The free ABA was removed by the addition of 100 µl of 0.5% (w/v) DCC. After removing the DCC by centrifugation, the resultant supernatant was counted in a MicroBeta TriLux scintillation counter (PerKin Elmer) and the data was analyzed using SigmaPlot 2000 and DynaFit (S15). For stereospecific binding assay, 50 ng GCR2 was added into the binding buffer (100 µl) with 50 nM 3H-(+)ABA and each of the four ABA analogs (±)ABA, (+)ABA and (–)ABA from Sigma and trans-ABA from A.G. Scientific) in the concentrations from 1 to 1000-fold molar excess of [3H]-ABA. The conditions of binding assay were the same as described above.

Liquid β-galactosidase assay and Co-IP in yeast. Yeast diploid colonies came from split-ubiquitin assay system were examined for β-galactosidase activity and GCR2-GPA1 interaction by Co-IP. The β-galactosidase activity was measured according to the Yeast Protocols Handbook (Clontech). In brief, after growing overnight at 30°C in -Leu/-Trp/-Ura SC media (OD600=1.0), yeast colonies containing GCR2-NubG+GPA1-Cub or NubG-KAT1+KAT1-Cub were transferred to fresh media to an OD of 0.2-0.3, and finally reach to an OD600 of 0.5-0.8 for the following assay. Aliquots were added different form ABA analogs (10 mM stock in ethanol, (±)ABA, (+)ABA and (–)ABA
were from Sigma and trans-ABA was from A.G. Scientific) up to 10 µM and the control was added with ethanol (0.1%), and then cells were grown for another 90 min. After equalized the OD<sub>600</sub>, cells (1 ml) were pelleted, the pellets were re-suspended by 100 µl Z buffer (Na<sub>2</sub>HPO<sub>4</sub>•7H<sub>2</sub>O 16.1 g/L, NaH<sub>2</sub>PO<sub>4</sub>•H<sub>2</sub>O 5.50 g/L, KCl 0.75 g/L, MgSO<sub>4</sub>•7H<sub>2</sub>O 0.246 g/L, pH7.0) followed by treating with liquid N<sub>2</sub>/37°C bath three times and then broken with glass beads. After centrifuged, 100 µl supernatant was added 0.7 ml of Z buffer containing 0.27% (v/v) β-mercaptoethanol and 160 µl of ONPG (4 mg/ml in Z buffer, Sigma) to both the reaction and a blank tubes (containing 100 µl Z buffer only). Incubate the tubes at 30°C until the yellow color developing, and then add 0.4 ml of 1 M Na<sub>2</sub>CO<sub>3</sub> to both the reaction and blank tubes, record the elapsed time in minutes. Measure the OD<sub>420</sub> of the supernatants of the reaction tubes against the blank. The β-galactosidase units were calculated as following: 1 unit of β-galactosidase is defined as the amount which hydrolyzes 1 µmol of ONPG to o-nitrophenol and D-galactose per min per mg protein. For Co-IP assay, yeast colonies were grown in -Leu/-Trp/-His/-Ade SC media as described above until the OD<sub>600</sub> reach 0.8. The cells were treated with ABA as described above for 20 min. Cells (3 ml) were collected by centrifugation and the pellets were re-suspended with IP buffer (50 mM Na<sub>3</sub>PO<sub>4</sub> pH7.5, 200 mM NaCl, 10% glycerol, 0.1% NP40, 10 mM NaF, 2.5 mM glicerolphosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM DTT, 1 mM PMSF, 1 x plant protease inhibitor cocktail; Roche) plus 100 µl glass beads (Sigma). After vortex at the max speed for 5 min (30 s interval in ice per 1 min), the sample was centrifuged at 14,000 rpm for 10 min. Two µg anti-HA antibody (Sigma, for IP GCR2-NubG-HA fusion protein) was added to the supernatants and mixed gently on a shaker table at 4°C for 4 h followed by adding 20 µl protein A-Sepharose (pretreated with TBS for three times, Sigma) and incubated another 2 h. The beads were collected by centrifuged at 6,000 g for 2 min followed washing 3 times with 4.5 ml IP buffer and boiled in SDS sample buffer followed running on a 12% SDS-PAGE gel. The sample was probed by anti-HA as a GCR2 input control and anti-GPA1 as the test, respectively. The relative intensity of immuno-signal of GCR2 or GPA1 was measured using MetaVue 6.2r4 (Universal Imaging Corp.)

**Protein extraction and western blotting.** Total proteins were extracted from *Arabidopsis* plants by grinding whole seedlings in liquid N<sub>2</sub> and then on ice in 3 volumes of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM sucrose, 1 mM PMSF, 0.1% Triton X-100, and 1x protease inhibitor cocktail; Roche). The debris was cleared from the lysates by centrifugation at 15,000g for 20 min at 4°C. Cleared lysate was centrifuged at 100,000g at 4°C for 1 h to separate the membrane (pellet) and soluble fractions if it is necessary. Protein concentrations in the extracts were measured by the Bradford (S16) assay (Bio-Rad). Twenty micrograms of proteins from each extract was separated on a 12% or 8% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. The blots were probed with polyclonal antibodies against GPA1, anti-GFP antibody (Santa Cruz) or anti-FLAG antibody (Sigma). The antibodies were diluted in blocking buffer (1x Tris-buffered saline [TBS] containing 5% nonfat milk and 0.5% Tween 20) to 1:8,000 for anti-GPA1 antibody, 1:500 for anti-GFP antibody, and 1:1,000 for anti-FLAG antibody. After washing in 1x TTBS (TBS containing 0.5% Tween 20), the blots were probed with appropriate secondary antibodies conjugated with horseradish
peroxidase (1:10,000 dilution). The antibody-bound proteins were detected by a chemiluminescence reaction using the SuperSignal Kit (Pierce, Rockford, IL).

**Preparation of anti-GPA1 antibody.** N-terminal His$_6$ tagged full length GPA1 was expressed and purified from *E. coli*. The purified GPA1 protein was used to immunize rabbit to generate antiserum. The specific of anti-GPA1 antibody was tested in planta using wild type, *gpa1* and GPA1 overexpression lines, and the antibody is highly specific for GPA1.

**Determination of GUS activity in transgenic lines.** Histochemical GUS staining of transgenic *Arabidopsis* plants was performed as described (S17). For quantitative GUS activity assay, 2-week-old seedlings were grinded in liquid N$_2$ and then diluted with GUS assay buffer (50 mM NaPO$_4$, pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% Triton X-100) on ice. Lysates were centrifuged at 12000g for 20 min at 4°C. The protein concentration was measured using Bradford (S16) assay. Twenty µl supernatant and 100 µl MUG (Sigma, 5 mM in GUS assay buffer) were mixed with 380 µl GUS assay buffer and warmed in 37°C. After exactly 10 or 20 min, the sample was added with 500 µl stop buffer (0.2 M Na$_2$CO$_3$, pH9.5). Keep the samples covered in foil to keep the fluorescence from fading. The MU fluorescence was measured with excitation wavelength 365 nm and emission wavelength 455 nm. The 4-MU (Sigma, 10 mM in water) standard curve was generated by diluting the 4-MU stock to 100 nM, 250 nM, 500 nM, and 1 µM in the stop buffer. The GUS activity was calculated as nmoles MU min$^{-1}$ mg$^{-1}$ protein (S18).
Supplementary Figures

**Fig. S1** GCR2 trans-membrane prediction and subcellular localization. (A) and (B) GCR2 is a 7 trans-membrane protein as analyzed by DAS and TMpred, respectively. (C) GCR2 is localized in the plasma membrane in roots of transgenic plant expressing GCR2-YFP. The scale bar represents 50 μm. (D) GCR2 is predominantly associated with the membrane fraction. Total protein (T) was isolated from transgenic plants expressing GCR2-YFP or 35S::YFP. Proteins were fractionated into soluble (S) or membrane (M) fractions. Equal amounts of protein were separated on SDS-PAGE and subjected to immunoblotting using anti-GFP antibodies. The samples were isolated from the transgenic plant under dex-inductive condition.
Fig. S2 Purification of GCR2 and GPA1 proteins from *E. coli*. M: molecular weight makers.
**Fig. S3** Physical interaction between GCR2 and GPA1 by SPR assay. (A and B) Representative surface plasmon resonance experiments show the binding of GPA1 to immobilized GCR2 (A) and nonbinding of GPA1 to immobilized BSA (B). (C) The on (ka, M$^{-1}$s$^{-1}$) and off (kd, s$^{-1}$) rates and the calculated dissociation constants ($K_d$, M) based on the surface plasmon resonance experiments (mean ± s.d; n= 3).
Fig. S4 Physical interaction between GCR2 and GPA1 by Split-ubiquitin assay. (A) Split-ubiquitin assay shows the full length of GCR2 interaction with GPA1 in yeast. Left panel: Interaction assay using GCR2-Cub as bait. (I) GPA1-NubG + GCR2-Cub, (II) NubG-GPA1 + GCR2-Cub, (III) GPA1-NubG + GCR1-Cub, (IV) NubG-GPA1 + GCR1-Cub, (V) GPA1-Nubwt + GCR2-Cub, (VI) Nubwt-GPA1 + GCR2-Cub, (VII) NubG-KAT1 + KAT1-Cub, (VIII) NubG-SUC2 + KAT1-Cub. Right panel: Interaction assay using GPA1-Cub as bait. (I) GCR2-NubG + GPA1-Cub, (II) NubG-GCR2 + GPA1-Cub, (III) GCR1-NubG + GPA1-Cub, (IV) NubG-GCR1 + GPA1-Cub, (V) GCR2-Nubwt + GPA1-Cub, (VI) Nubwt-GCR2 + GPA1-Cub, (VII) NubG-KAT1 + KAT1-Cub, (VIII) NubG-SUC2 + KAT1-Cub. (I)-(IV) contain a test construct in which GCR1 serves as a positive control, (V)-(VII) are internal positive controls and (VIII) is an internal negative control. Top panel: yeast growth assay. Bottom panel: X-gal overlay assay. The corresponding \textit{LacZ} activity for each of yeast strain was shown in the bottom of the figure. (B) Split-ubiquitin assay shows the C terminus (C\textsubscript{290-401}) of GCR2 interaction with GPA1 in yeast. Left panel: Interaction assay using GCR2\textsubscript{C}-Cub as bait. (I) GPA1-NubG + GCR2\textsubscript{C}-Cub, (II) NubG-GPA1 + GCR2\textsubscript{C}-Cub, (III) GPA1-Nubwt + GCR2\textsubscript{C}-Cub, (IV) Nubwt-GPA1 + GCR2\textsubscript{C}-Cub, (V) NubG-KAT1 + KAT1-Cub, (VI) NubG-SUC2 + KAT1-Cub. Right panel: Interaction assay using GPA1-Cub as bait. (I) GCR2\textsubscript{C}-NubG + GPA1-Cub, (II) NubG-GCR2\textsubscript{C} + GPA1-Cub, (III) GCR2\textsubscript{C}-Nubwt + GPA1-Cub, (IV) Nubwt-GCR2\textsubscript{C} + GPA1-Cub, (V) NubG-KAT1 + KAT1-Cub, (VI) NubG-SUC2 + KAT1-Cub. (I)-(II) contain the test construct, (III)-(V) are internal positive controls and (VI) is an internal negative control. Top panel: yeast growth assay. Bottom panel: X-gal overlay assay. The corresponding \textit{LacZ} activity for each of yeast strain was shown in the bottom of the figure. (C) Split-ubiquitin assay shows the N terminus (N\textsubscript{1-289}) of GCR2 can not interact with GPA1 in yeast. Left panel: interaction assay using GCR2IL-Cub as bait. (I) GPA1-NubG + GCR2IL-Cub, (II) NubG-GPA1 + GCR2IL-Cub, (III) GPA1-
Nubwt + GCR2IL-Cub, (IV) Nubwt-GPA1 + GCR2IL-Cub, (V) NubG-KAT1 + KAT1-Cub, (VI) NubG-SUC2 + KAT1-Cub. Right panel: interaction assay using GPA1-Cub as bait. (I) GCR2IL-NubG + GPA1-Cub, (II) NubG-GCR2IL + GPA1-Cub, (III) GCR2IL-Nubwt + GPA1-Cub, (IV) Nubwt-GCR2IL + GPA1-Cub, (V) NubG-KAT1 + KAT1-Cub, (VI) NubG-SUC2 + KAT1-Cub. (I)-(II) contain the test construct, (III)-(V) are internal positive controls and (VI) is an internal negative control. Top panel: yeast growth assay. Bottom panel: X-gal overlay assay. The corresponding \textit{LacZ} activity for each of yeast strain was shown in the bottom of the figure. The scale bar represents 1 cm.
Fig. S5 Characterization of gcr2 mutant. (A) T-DNA insertion sites for the three gcr2 alleles. Boxes and lines represent exons and introns, respectively. The insertion sites were verified by sequencing. (B) RT-PCR analysis of GCR2 expression in wild-type and three gcr2 homozygous lines using primers flanking the T-DNA insertion sites. Actin serves as control. (C) Schematic model indicated the positions of the three insertions of the three mutant alleles with respect to the predicted structural features of GCR2. PM: plasma membrane.
Fig. S6 The expression of ABA maker genes in gcr2. (A) Histochemical assay for GUS activity. The scale bar represents 2 mm. (B) Quantitative GUS activity. The seedlings were incubated in 0.3 µM ABA for 12 hours before GUS activity assay.
Fig. S7 Water loss response in gcr2 and GCR2 overexpressor leaves. Water loss is greater in gcr2 leaves and reduced in GCR2 overexpressor leaves than in wild-type leaves (mean ± s.d; n= 6).
**Fig. S8** Genetic interaction between GCR2 and GPA1. (A and B) The expression pattern of *GCR2* (A) and *GPA1* (B). (C) *gcr2*, *gpa1*, and *gcr2gpa1* display similar ABA defects, and overexpression of *GPA1* confers increased ABA-sensitivity in a GCR2-dependent manner (mean ± s.d; n= 3). (D) Overexpression of *GCR2* confers increased ABA-sensitivity in a GPA1-dependent manner (mean ± s.d; n= 3). (E) Representative stomata from plants of various genetic backgrounds in response to ABA. The scale bars were indicated in the corresponding panels.
Fig. S9 The binding of ABA to GCR2 is pH-dependent. Fifty ng GCR2 and 50 nM $^3$H-(+)ABA were added in the binding buffer with different pH as indicated in the figure (mean ± s.d; n=3).
ABA represses the expression of reporter gene lacZ in a GCR2-GPA1 reconstituted system in yeast. Ten µM ABA was added to the medium, and lacZ activity was determined 90 min (one yeast generation after the addition of ABA) after the addition of ABA. Only physiologically active ABA represses lacZ expression, and ABA does not affect lacZ expression in the KAT1-KAT1 interaction (mean ± s.d; n= 3). The Miller unit (µmol ONPG/min/mg protein) for 100% activity of GCR2-GPA1 or KAT1-KAT1 system was shown in the bottom of control column, respectively.
Fig. S11 Transformation of GCR2 cDNA to gcr2 mutant partial complements gcr2 defect in stomatal closure
Supplementary References