Supplementary information, Data S1 Experimental material and procedures

Constructs published previously

PM-OFP, in Batistič et al., 2010.

Plasmid construction

To obtain either GFP or HA fusion proteins all constructs used in this work were amplified by PCR using a Pwo-Polymerase (peqlab) and inserted into the pGPTVII.GFP or pGPTVII.HA plasmid (Walter et al., 2004). CBL2 (GFP and HA fusion protein) was obtained using the CBL2GFPfor-CBL2revGFP primer pair and using the reading frame of At5g55990 (AtCBL2) as a template (Batistič et al., 2008). Mutants of CBL2 were obtained by using the different forward primers given in the Supplemental Table 2. To obtain CBL2 (full-length protein) mutants with multiple cysteine to serine mutations, CBL2C18S was used as a template and PCR was performed using CBL2forC12S forward primer and CBL2revGFP reverse primer which resulted in the double CBL2C12,18S mutant product. Subsequently, using the double exchange mutant as a template and the CBL2forC4S forward primer resulted in the triple exchange mutant CBL2C4,12,18S. The modified GFP (CBL2n-GFP and versions thereof) were obtained by using the respective forward primer (either GFPCBL2nterm, GFPCBL2nterm22aa, GFPCBL2nterm22CxxxS) and the GFPrevSac reverse primer. OFP-TM23 and TPK1-GFP were obtained using the respective primer pairs (Supplemental Table 2) and either an OFP reading frame (Batistič et al., 2010) or the reading frame of At5g55630 (TPK1) as templates. The identity of all plasmid constructs generated in this study was verified by sequencing.

For complementation of the *cbl2* mutant line the *CBL2* promoter region, encompassing 1546 nucleotides representing the complete intergenic region between the CBL2 gene locus (including the 5' UTR) and the upstream located gene At5g55980 was amplified by PCR using the primers CBL2-Prom For-Hind and CBL2-Prom Rev-Spe (Supplemental Table 2) and inserted into a HindIII-SpeI digested pGPTVII.bar plasmid (Walter et al., 2004) in front of the *uidA* gene thereby achieving a promoter GUS fusion. Subsequently, cDNA fragments containing either the open reading frame of CBL2 (wild type version) or mutated versions of CBL2 were inserted behind the promoter fragment by replacing the existing *uidA* reading frame using the BamHI and SacI sites. Plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 and stable transformations of *cbl2* mutant plants was achieved

using the floral-dip method (Clough and Bent, 1998). Transformed plants were selected by spraying with 0.1% glufosinate-ammonium (200 g/L) (Basta; Bayer CropScience) containing 0.1% Tween and resistant plants were repeatedly selfed to receive homozygous lines and analyzed for segregation of the resistance locus. To verify the recombinant status of plants, DNA from the homozygous lines was isolated and tested for the presence of the recombinant genes by PCR that were confirmed by sequencing.

Biochemical fractionation analyses

For biochemical fractionation analysis, the materials were resuspended in extraction buffer (50 mM HEPES, pH 7.5, 10 mM KCl, supplemented with protease inhibitor cocktail from Sigma-Aldrich) at 4°C for 45 min by slow rotation. To remove cell debris, the suspension was centrifuged for 10 min at 3,000g, and the supernatant was additionally centrifuged for 15 min at 10,000g and for 5 min at 20,000g. Finally, to separate soluble and insoluble proteins, the supernatant was centrifuged for 1 h at 100,000g. The resulting pellet was resuspended in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1.5% SDS, and 1% Triton. All steps were performed at 4°C or on ice.

For detection of GFP and GFP fusion constructs, proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane after SDS-PAGE and incubated with 1:4000 rabbit anti-GFP antibody (Invitrogen) and 1:10,000 goat anti-rabbit antibody (Bio-Rad) conjugated with horseradish peroxidase. HA tagged proteins were detected after SDS-PAGE by blotting proteins to PVDF membrane and incubating with 1:3000 anti-HA monoclonal antibody (BabCo) and 1:10,000 goat anti-mouse antibody (Bio-Rad). Detection was performed using enhanced chemiluminescence reaction.

Protein expression and determination of lipid modification

Protein purification was performed from total protein extracts by differential ammonium sulfate precipitations (20-40 % ammonium sulfate). Proteins that were precipitated at 20-40% ammonium sulfate step were further purified by ion exchange chromatography on a HiTrap^{TM} Q FF Column (GE Healthcare; catalog No.17-5053-01) using an AKTAprime plus protein purification system. The column was equilibrated with loading buffer containing 50 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1mM $CaCl_2$, 5% glycerol, 2 mM β-mercaptoethanol with or without 0.5% Triton X-100

for CBL2 or CBL2C4,12,18S, respectively. Elution was preformed with a continuous 25-800 mM NaCl gradient in the same buffers. Both CBL2 and CBL2C4,12,18S where eluted at about 400 mM NaCl. Desalting of purified protein fractions was preformed with Hi Trap^{TM} Desalting column (GE Healthcare; catalog No.17-1408-01) with a buffer containing 20 mM Tris-HCl pH 7.5, 25 mM NaCl, 1 mM $CaCl_2$, 5% glycerol, 2 mM β-mercaptoethanol with or without 0.5% Triton X-100 for CBL2 or CBL2C4,12,18S, respectively. Eluted proteins were pooled for further analysis.

Cleavage of acyl groups was performed as following: 25 µg of purified protein was dried in a vacuum concentrator inside sealable glass vials (2-mL reactive vial; Whatman catalog No. 986276) and resuspended in formic acid:ethanol (1:4, v/v). The samples were washed three times with 0.5 mL of pentane:formic acid:ethanol (10:1:4, v/v) to wash away noncovalently bound lipids. A total of 5 mg of platinum (IV) oxide was added per 400-µL sample, and proteins were hydrogenated for 120 min. Following hydrogenation, extraction of released acyl groups was done by adding 0.5 mL of pentane. Extraction procedure was repeated three times, and each time the pentane was transferred into a new tube. The pentane washes were pooled and concentrated under nitrogen to a final volume of 15 µL. Concentrated samples were analyzed by GC-MS.

Phenotypic bioassays

Guard cell measurements after ABA treatment

Detached *A. thaliana* leaves from 4–5 week-old soil-grown plants, grown under short-day conditions, were floated on incubation buffer (10 mM MES-KOH, pH 6.15, 10 mM KCl and 50 µM $CaCl_2$) at 23–24°C. After 2 h of incubation the solution was replaced with incubation buffer supplemented with ABA (0.5 and 5 µM, respectively) and incubated for another 2 h. Subsequently, epidermal peels of each line were prepared, and the length and opening width of 50 stomata were determined using an inverted microscope. Each assay was performed in triplicate and as a blind experiment.

Seedling survival assays

Seeds were sown on 0.5 Murashige & Skoog + Gamborg vitamins, 0.8 % Agar medium. After one week of horizontal growth under long day conditions five plants per line were transferred to vertical plates with 0.5 MS media supplemented with 0, 5, 10, 20 or 40 µM ABA. This was done in triplicate. After another 7 days of growth shoot and root size were visually analyzed.

Supplemental References

Clough, S.J. and A.F. Bent. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. *16*, 735-743.

Walter, M., Chaban, C., Schutze, K., Batistič, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K. and J. Kudla. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J. *40*, 428-438.