METHODS S1

Cloning and site-directed mutagenesis

All cloning steps and primers are detailed in Table S1.

To generate point mutations, PCR was performed using primers carrying the intended mutation and annealing to the same sequence on opposite strands of the original plasmid template. A 25µl reaction mix [primers (25ng each), template plasmid (50ng), 250µM of dNTPs, Pfu polymerase (1.25U, Promega M7745) and 1X Pfu buffer] was split in two tubes (12.5µl each); one was incubated in a thermocycler [95°C – 3min; (95°C-30s, 55°C-40s, 68°C-2min/kb) x 14] while the other was kept at 4°C. Ten units of DpnI (NEB) were thereafter added to both and incubated at 37°C for 12h prior to bacteria transformation (4µL typically used). Positive clones were always confirmed by sequencing.

Y2H Assays

Y2H assays were performed as described (Saez *et al.* 2008). The full-length coding sequence of SnRK1α1 and the various deletions were cloned into pGADT7 in fusion with the GAL4 activation domain. pGADT7 constructs were faced with pGBKT7 harboring full-length SCE1 fused to the DNA binding domain of GAL4. The empty vectors were used as negative controls.

E. coli heterologous SUMOylation assay

Analyses of SnRK1 SUMOylation in *E. coli* were performed as described with slight modifications (Okada *et al.* 2009). pET28a was used to express potential SUMO targets (SnRK1α1 and its truncated and mutant variants, SnRK1β1, SnRK1β2, SnRK1γ and SnRK1βγ). BL21(DE3) cells were transformed with pACYCDuet-AtSAE1a-AtSAE2 and selected on 34µg/ml chloramphenicol (Cm) LB Agar plates. 100µl of pACYCDuet-AtSAE1a-AtSAE2 transformed BL21(DE3) competent cells were co-transformed with 30-50ng of pCDFDuet-AtSUMO1/3(AA or GG)-AtSCE1a and pET28a-SnRK1α1 (and its truncation and mutation variants)/SnRK1β1/SnRK1β2/SnRK1γ/SnRK1βγ and then selected on 17µg/mL Cm, 15µg/mL Kanamycin (Kan) and 25µg/mL Spectinomycin (Spec) LB Agar plates. Transformed cells were incubated in 34µg/mL Cm, 30µg/mL Kan and 50µg/mL Spec LB liquid media at 25°C, 200rpm, until Abs^{600nm} reached 0.5-0.8. The

expression of recombinant proteins was induced with 0.15mM IPTG (PROMV3955, Promega) at 25 °C for 12h.

For analyzing total soluble proteins, cells were harvested from 3mL cultures and lysed with 100µL of BugBuster Protein Extraction Reagent (Novagen) supplemented with 2µL of ProteoBlock (#R1321, Fermentas), 2µL of Lysozyme (L1667, Sigma) and 2µL of DNAse (PROMM6101, Promega). Samples were incubated 20min at RT and cleared by centrifugation (20,000g, 4°C, 25 min). About 100µg of soluble protein (estimated by Abs^{280nm}) were subjected to SDS-PAGE and immunoblotting with an anti-T7 antibody.

For analyses from purified proteins, bacteria were pelleted at 4000g for 30min at 4°C, resuspended in Lysis buffer [50mM Hepes-NaOH pH7.25, 0.1M NaCl, antiprotease cOmplete without EDTA (1 tablet/50mL)] and sonicated. After centrifugation (20,000g for 30min at 4°C) the soluble fraction was subjected to IMAC purification (Ni-NTA agarose, Qiagen, 30210). Beads were pre-equilibrated in Lysis buffer and incubated with the soluble protein extract under gentle shaking for 1h at RT. Beads were thereafter washed under gravity flow with Lysis buffer (5 BV, bed volume) and then with 50mM Hepes-NaOH pH7.25 until Abs^{230nm} reached 0 or stabilized at less than 0.1. A final wash was applied with 50mM Hepes-NaOH pH7.25, 20mM imidazole. Three consecutive elutions with 50mM Hepes-NaOH pH7.25 supplemented with 100, 200 or 500mM of imidazole were performed during at least 20min at RT under gentle shaking. Eluates were loaded on 30kDa Amicon columns (Millipore) for buffer exchange (to reach a 20mM imidazole concentration) and protein concentration, and were thereafter analyzed (20µg) by SDS-PAGE and immunoblotting with anti-T7.

Mass spectrometry analyses

For Mass spectrometry analyses of SUMOylated SnRK1α1-KD and SnRK1α1-RD (using SUMO3-GG or a $SUMO3^{S91R}$-GG variant), 15-30µg of concentrated eluates (see previous section) were resolved on SDS-PAGE (8%), stained with Coomassie Brilliant Blue R250 (VWR: 443283M, 0.2% w/V in 14% Acetic Acid, 14% ethanol), and destained (10% Acetic Acid, 25% ethanol) until the bands were clearly visible. Bands were excised, alkylated with iodoacetamide (carbamidomethylation of cysteines), and digested with trypsin. Eluted peptides were separated by liquid chromatography and detected with an Orbitrap Velos Pro Hybrid Ion Trap Mass Spectrometer (Thermo Scientific). An initial hit

search was done in the NCBI non-redundant database with "Arabidopsis thaliana" as a query organism. Approximately 3400 queries were made for each sample, with peptide mass tolerance ± 3 ppm and fragment mass tolerance ± 0.8 Da. For a more refined validation, a micro-database was created containing *in silico* predicted masses of the branched peptides resulting from SUMOylation was compared manually to the data set.

Generation of *SnRK1α1-GFP*, *SnRK1α1ΔKA1-GFP and SnRK1α1-GFP*$_{siz1-2}$ and *35S::GFP* lines

A homozygous insertion line for the *SnRK1α1* gene (At3g01090), was identified in the GABI-KAT collection (GABI_579E09; Figure S3) and was designated as *snrk1α1-3* [previously described *snrk1α1-1* and *snrk1α1-2* mutants are not null (Tsai and Gazzarrini 2012)]. Genotyping was performed using primers *snrk1α1*-GABIa and *snrk1α1*-GABIb in combination with a left border T-DNA primer (GABI-08409-LB). To determine the T-DNA exact insertion site, a genomic DNA fragment was amplified by PCR with a forward primer binding to the *SnRK1α1* locus (SnRK1α1-seqF Fw) and a reverse primer binding to the T-DNA right border (GABI1-RB-seq Rv). Sequencing reactions were subsequently performed on the gel-purified PCR product using the same primers. The T-DNA insertion was mapped to position 2583-2593, immediately before the last exon. The last 11 bases of the intron at the insertion site are missing. The potential presence of a second insertion in the *IMS2* gene (AT5G23020), as annotated in the GABI-Kat site, was ruled out by genotyping with primers IMS2-Fw and IMS2-Rv in combination with the GABI-08409-LB primer. The absence of SnRK1α1 protein in *snrk1α1-3* plants was confirmed by immunoblotting with antibodies recognizing epitopes well before the T-DNA insertion, a SnRK1α1-specific antibody and an AMPKα-pT172 antibody recognizing the phosphorylated T-loop of SnRK1α1 and SnRK1α2 [T175 and T176, respectively; (Baena-Gonzalez *et al.* 2007)].

The *pSnRK1α1::SnRK1α1-GFP::tSnRK1α1* and *pSnRK1α1::SnRK1α1ΔKA1-GFP::tSnRK1α1* constructs are in the pBm43GW,0 MultiSite Gateway Binary vector (Karimi *et al.* 2005) and the corresponding areas of the gene are indicated in Figure S3A. Both constructs were generated using a pDONR-P4P1R harboring the *SnRK1α1* upstream regulatory region (*pSnRK1α1*, 2000 bp upstream of the *SnRK1α1* start codon in the At3g01090.2 gene model; amplified using primers PROM-5'UTR_gSnRK1α1 attB4 Fw and PROM-5'UTR_gSnRK1α1 attB1r Rv) and a pDONR-P2RP3 harboring the

SnRK1α1 downstream regulatory region (*tSnRK1α1*, 1000 bp downstream of the *SnRK1α1* stop codon in the At3g01090.2 gene model; amplified using primers TERM-3'UTR_gSnRK1α1 attB2r Fw and TERM-3'UTR_gSnRK1α1 attB3 Rv). In *pSnRK1α1::SnRK1α1-GFP::tSnRK1α1* the middle pDONR221-P1P2 contained the full genomic sequence of *SnRK1α1* fused to GFP (primers gSnRK1α1-GFP attB1 Fw and gSnRK1α1-GFP attB2 Rv), whereas in *pSnRK1α1::SnRK1α1ΔKA1-GFP::tSnRK1α1,* it contained the coding sequence of *SnRK1α1* truncated at the KA1 domain and fused to GFP (primers gSnRK1α1-GFP attB1 Fw and gSnRK1α1-GFP attB2 Rv). The *pSnRK1α1::SnRK1α1-GFP::tSnRK1α1* and *pSnRK1α1::SnRK1α1ΔKA1-GFP:tSnRK1α1* constructs were introduced into *Agrobacterium tumefaciens* (GV3101) and *snrk1α1-3 or siz1-2* plants were transformed by the floral dip method (Clough and Bent 1998) to generate *pSnRK1α1::SnRK1α1-GFP::tSnRK1α1/snrk1α1-3* (referred as *SnRK1α1-GFP*), *pSnRK1α1::SnRK1α1ΔKA1-GFP::tSnRK1α1/snrk1α1-3* (referred as *SnRK1α1ΔKA1-GFP*), and *pSnRK1α1::SnRK1α1-GFP::tSnRK1α1/siz1-2* (referred as $SnRK1\alpha1\text{-}GFP_{siz1\text{-}2}$). BASTA-resistant transformants were selected based on their segregation ratio (T2) and homozygosity (T3). Homozygous T3 or T4 generation transgenic lines were used.

For the generation of plants with constitutive GFP expression (*35S::GFP*), Col-0 plants were transformed with a pBA vector (Duque and Chua 2003) for expression of GFP under the Cauliflower Mosaic Virus *35S* promoter.

Recombinant Protein Production and Purification

Recombinant His-ΔC ABF2 (residues 1–173) was produced and purified as previously described (Rodrigues *et al.* 2013). Successful protein production and purification was verified by immunoblotting with an anti-T7 antibody.

Endogenous protein quantification

Leaves of 5-week-old Col-0 and *siz1-2* plants were grounded in liquid nitrogen and resuspended in Buffer C. After two successive centrifugations (20,000g, 4°C, 10min), the supernatant was recovered and filtered (0.45µm), and total protein was quantified using the Bradford protein assay (Bio-Rad; #5000006). Equal protein amounts from the three extracts were solubilized with Laemmli buffer (Laemmli 1970), resolved by SDS-PAGE (8%), transferred to a PVDF membrane at 15V for 1h (semi-dry transfer, Bio-Rad) and immunodetected using antibodies against specific SnRK1 subunits.

Quantification of immunoblot results

Membranes were analyzed by immunoblotting and then stained with Coomassie Brilliant Blue R250. Band intensity was quantified using Image J (http://imagej.nih.gov/ij/) and GelQuantNet (http://biochemlabsolutions.com/GelQuantNET.html) softwares. The immunoblot intensities were normalized to the Coomassie staining intensity (referred as "loading"). In Figures 3, 4 and 5 quantifications were normalized to the t=0 of each kinetics or to Col-0 for Figure 3B and C.

Statistical Analyses

All statistical analyses were performed with the GraphPad Prism 6 software (GraphPad softwares). For analyses of qPCR data, the statistical significance of the indicated changes was assessed employing $\log_2$-transformed relative expression values (Rieu and Powers 2009).

Chemicals

Cycloheximide (Sigma, C7698; ethanol stock), MG132 (Sigma, C2211; DMSO stock) and Salicylic Acid (Aldrich: 105910; ethanol stock) solutions were always freshly prepared and used at a final concentration of 100µM, 50µM and 5µM, respectively.

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LEGENDS FOR SUPPLEMENTARY FIGURES

Figure S1. SnRK1α1 interacts with the SUMO E2 Conjugating Enzyme 1 (SCE1) in a Yeast two-Hybrid assay

The full-length coding sequence (FL), C-terminal Regulatory Domain (RD) encompassing the KA1 domain, or N-terminal Kinase Domain (KD) of SnRK1α1 (represented on the left), were cloned into pGADT7 in fusion with the GAL4 activation domain and co-transfected with pGBKT7 harboring either the GAL4 binding domain alone (empty) or in fusion with the full-length SCE1. The growth of transformed AH109 yeast cells was assessed in permissive [-Leucine (L), -Tryptophan (W)], selective [-L-W,-Histidine (H)], or more stringent [-L-W-H,-Adenine (A)] media. A representative experiment of a minimum of two independent assays is shown.

Figure S2. SnRK1γ is SUMOylated in *E. coli* and enriched in *siz1-2*, but is not part of the SnRK1α1 complex in Arabidopsis leaves

(a) SnRK1γ subunits containing 6*His and T7 tags were co-expressed in *E. coli* with SUMO3 together with the SUMO-activating (AtSAE1a/AtSAE2) and SUMO-conjugating

(AtSCE1) enzymes. After production, the protein from total lysate was immunoblotted against its T7 tag. GG and AA refer to conjugatable and non-conjugatable SUMO3 variants, respectively. Black and grey arrowheads mark non-SUMOylated and SUMOylated SnRK1γ, respectively. Equal protein loading is shown by Coomassie Blue (CB) staining of membranes. (b) SnRK1γ accumulates to higher levels in the *siz1-2* mutant. Total leaf protein extracts (10, 17, or 24 µg) of Col-0 and *siz1-2* plants were analyzed by Western-blot (WB) using antibodies against SnRK1γ. The signals were quantified and normalized to loading. The average quantification in *siz1-2* normalized to Col-0 is presented. Stars denote statistical significance, as determined by ratio paired *t*-test prior to normalization (n=3; error bars=SEM; *p<0.05). (c) Anti-GFP immunoprecipitation (IP) of SnRK1α1-GFP as in Figure 1b. The presence of SnRK1γ was assessed by immunodetection with SnRK1γ antibodies. Co-immunoprecipitation of SnRK1β1 was used as a positive control. The input corresponds to the soluble protein extracts that were used for IP. The black arrowheads indicate the SnRK1 subunits.

Figure S3. Generation of *SnRK1α1-GFP* transgenic lines

(a) Structure of the *SnRK1α1* gene (At3g01090.2 gene model). All indicated positions have the start codon (noted +1) as a reference. The promoter (2kb upstream of the start codon) and terminator (1 kb downstream of the stop codon) regions used, as well as the kinase and KA1 domains, are indicated. The location of the T-DNA insertion defining the *snrk1α1-3* mutant line (GABI-KAT: 579E09) is indicated as well as the position of the three genotyping primers (LP, Left Primer; RP, Right Primer; LB, Left Border; Supplementary Table 1). The sequence providing the exact location of the insertion (2583) in indicated inside the red box (”lost” denotes the 11 bases lost due to the insertion). White boxes correspond to exons. (b) Genotyping PCR using primers LP, RP, and LB, shown in (a). The expected sizes of the two products are indicated on the left (LP: LP-RP reaction, 386bp indicates WT allele; LB: LB-RP reaction, 190bp indicating a mutant allele). (c) Total proteins from adult Arabidopsis leaves of Col-0 or the *snrk1α1-3* mutant were analyzed by Western blot (WB) using antibodies against SnRK1α1, SnRK1α2 or P-AMPK (recognizing the phosphorylated T175/176 of SnRK1α1/α2, respectively). The black and grey arrowheads indicate phospho-SnRK1α1 and phospho-SnRK1α2, respectively. (d) Supporting data for Figure 1b. *SnRK1α1::GFP* transgenic lines were generated by transformation of the *snrk1α1-3* mutant with the pBm43GW,0

MultiSite Gateway Binary vector, containing the promoter (2 kb upstream of the start codon), the genomic coding region (exons-introns) and the terminator (1 kb downstream of the stop codon) of the *SnRK1α1* gene (At3g01090.2; indicated in (a). Several independent transgenic lines were tested for the presence of SnRK1α1-GFP by Western-blot (WB) using antibodies against SnRK1α1 and GFP. The selected lines (presenting a SnRK1α1 signal close to Col-0) are indicated with arrows. (e) *SnRK1α1ΔKA1::GFP* transgenic lines were generated using the CDS of *SnRK1α1* and analyzed as in (d). (f) Supporting data for Figure 2b. *SnRK1α1::GFP*$_{siz1-2}$ transgenic lines were generated by transformation of the *siz1-2* mutant with the pBm43GW,0 MultiSite Gateway Binary construct described in (d). Several transformants were tested for the presence of SnRK1α1-GFP by Western-blot (WB) using antibodies against SnRK1α1 and GFP. The selected lines are indicated with arrows. Equal protein loading in (c-f) is shown by Coomassie Blue (CB) staining of membranes.

Figure S4. SnRKα1 residues found SUMOylated in the *E. coli* assay

(a) Schematic representation of SnRK1α1 showing the regions referred thereafter. K48 (catalytic phospho-transfer) and T175 (activating T-loop phosphorylation) are the two residues crucial for SnRK1 enzymatic activity later mutated to generate inactive SnRK1α1 variants. The residues predicted [(Elrouby and Coupland 2010); results in (b)], found by MS/MS analyses [from samples shown in (d)], and confirmed to be crucial for SUMOylation in *E. coli* [results in (e and f)] are indicated. Numbering corresponds to gene model At3g01090.1 according to TAIR. (b to f), SUMOylation assay using the Arabidopsis SUMO machinery reconstituted in *E. coli*. The indicated SnRK1α1 variants harboring 6*His and T7 tags were co-expressed in *E. coli* with the indicated SUMO isoform together with the SUMO-activating (AtSAE1a/AtSAE2) and SUMO-conjugating (AtSCE1) enzymes. GG and AA refer to conjugatable and non-conjugatable SUMO variants, respectively. SUMOylation was assessed by Western-blot using antibodies against the T7-tag. (b) Mutation of predicted SUMOylation sites [“predicted” in (a)]. does not abolish SnRK1α1 SUMOylation. (c) Several residues are SUMOylated in SnRK1α1, as shown by the positive SUMOylation signal with truncated SnRK1α1 variants harboring only the KD or RD. Black and grey arrowheads designate non-SUMOylated and SUMOylated proteins, respectively. (d) SnRK1α1-KD and SnRK1α1-RD samples used to identify the SUMOylated lysine residues [“MS/MS” in (a); their relative position in the

predicted SnRK1 structure is also shown]. SnRK1α1 was purified *via* the His tag by IMAC and immunoblotted against the T7 tag. Bands corresponding to SUMOylated SnRK1α1 (grey arrowhead) were excised and analyzed by MS/MS. RGG denotes mature SUMO3 mutated at the C-terminus (S91R) to generate smaller tryptic peptides more amenable MS/MS analyses. The lysine residues indicated in the table correspond to the ones found to be SUMOylated in MS analyses of SnRK1α1-KD and SnRK1α1-RD using WT mature SUMO3 (GG) or its RGG variant. The structure of the SnRK1 complex in cartoon representation was modeled with the Swiss model portal using the AMPK structure as a template (2Y94). SnRK1α1 is in blue [colored as in (a) with the KA1 domain in dark blue; residues 14 to 395 coupled to a model of the KA1 (Rodrigues *et al.* 2013) from 396 to 512], SnRK1β1 is in yellow (209-281), and SnRK1βγ is in wheat (152-486). SnRK1α1 lysine residues found to be SUMOylated by MS/MS [(a), "MS/MS")] are shown with their sidechains in stick representation in red. The three lysines numbered in red correspond to the three lysines confirmed to be SUMOylated ["confirmed" in (a); see panel (e)]. (e) Same analysis as in (c) on total soluble protein extract from bacteria expressing the indicated SnRK1α1 domains mutated or not for the shown lysines. An area pointed out with a blue arrowhead indicates where the SUMOylated protein should be in the mutated variant (compare to grey arrowhead in the WT control of the same panel). (f) Validation of the mutational analysis in (e) using affinity-purified protein from bacteria expressing a full length SnRK1α1 mutated or not for the three confirmed lysines (K34/63/390). KD, Kinase domain; RD, Regulatory Domain; KA1, Kinase Associated 1 domain; UBA, Ubiquitin-Associated domain. Equal protein loading in (e-f) is shown by Coomassie Blue (CB) staining of membranes.

Figure S5. Salicylic acid (SA) has no effect on SnRK1 signaling

(a) SA does not alter SnRK1 reporter gene induction in protoplasts. The *pDIN6::LUC* reporter for SnRK1 signaling is strongly induced by SnRK1α1 expression, but this induction is similar in mock- and SA-treated cells. Protoplasts were transfected with a plasmid expressing SnRK1α1 or control DNA, incubated for 4h and thereafter treated with SA (5µM) or ethanol (mock control) and the t0 was collected. After 2h or 15h the cells were collected for luciferase activity assays and western-blot (WB). Equal protein loading in (e-f) is shown by Coomassie Blue (CB) staining of membranes. Data presented are means and error bars are SEM (n=3). (b) Various SA treatments induce the

expression of two marker genes of Systemic Acquired Resistance (SAR; *PR1*, At2g14610 and *PR5*, At1g75040), but not the induction of the SnRK1 marker genes *DIN6* (At3g47340), *AXP* (At2g33830), and *TPS8* (At1g70290). The data are from four independent studies and were obtained by using Genevestigator (https://genevestigator.com/gv/).

Figure S6. Specificity of the anti-SnRK1βγ antibody

Several SnRK1βγ-containing protein preparations were used to assess the specificity of the antibody (Agrisera AS09 463) in western-blot ("WB"). The antibody recognized the recombinant protein produced in *E. coli* ("His-SnRK1βγ", presented in Figure 1a) and the HA-tagged protein overexpressed in protoplasts ("SnRK1βγ-HA"). This antibody was also able to recognize SnRK1βγ from SnRK1α1 immunoprecipitation ("IP-SnRK1α1"). However, this antibody fails to recognize SnRK1βγ in crude extracts ("CE"), where only unspecific bands of higher or smaller molecular weight are detected. Arrowhead indicates SnRK1βγ.

Figure S7. Conservation in SnRK1α1 of the residues implicated in SNF1 SUMOylation

(a) Partial ClustalW alignment of the α-subunits of SnRK1 (α1/α2 from Arabidopsis), AMPK (α1/α2 from Human), and SNF1 (*S. cerevisiae*). The two partially overlapping SIMs from SNF1 (Simpson-Lavy and Johnston 2013) are boxed. Only SIM1 (black box, containing I128, pointed) was shown to be important for the inhibition of Snf1 by SUMOylation. (b) Structural alignment of the KA1 domain from Arabidopsis SnRK1α1 [model, pale green, (Rodrigues *et al.* 2013)] and the crystal structure of SNF1 (light blue, PDB: 2QVLA: 506-591). RMS=0.45Å (69 to 69 atoms) generated by Pymol.