Supplementary Information for

Carbon export from leaves is controlled via ubiquitination and phosphorylation of sucrose transporter SUC2

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Materials and Methods

Plant Materials and Growth Conditions
The T-DNA insertion lines of ubc34 (N674382), rlc0vii (N563868), cdka1;1 (N681294) and wakl8 (N661981) were obtained from the Eurasian Arabidopsis Stock Centre at University of Nottingham, UK (51). Genotyping using primers against the gene of interest and the inserted T-DNA (T-DNA Primer Design, Salk Institute Genomic Analysis Laboratory) was used for the identification of homozygous lines (Suppl. Table S1). Loss-of-function of these mutants was further confirmed by qRT-PCR analysis. Methods for seed sterilization and conditions for plant growth were used as described previously (7). All Arabidopsis plants used here belonged to the accession Col-0. High light conditions meant that light intensity increased from 90 μmol photon m⁻² s⁻¹ to 400 μmol photon m⁻² s⁻¹. If not specified otherwise, samples were taken after 4 h exposure to high light. N. benthamiana plants were kept at a 16-h-light/8-h-dark period, with 300 μmol photon m⁻² s⁻¹ and a temperature of 25 °C during the day and 23 °C at night.

Measurement of photosynthesis, leaf sugar levels, and phloem exudate sugars
Measurement of photosynthesis, leaf sugar levels, and phloem exudate sugar levels were conducted as described previously (7) with minor changes. For the quantification of phloem exudate sugars, a vacuum concentrator (Eppendorf, Germany) was used to increase the concentration of sugars in the sample. Sugar analysis was then performed by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI, Shimadzu, Japan). A NH₂ column (4.6×250 mm, 5 mm, Shimadzu) was used as separation column at 40 °C with acetonitrile/deionized water, 7:3 (v/v) as mobile phase and a flow rate of 1 mL/min with 20 μL injection volume. The relative concentrations of the different sugars were analyzed quantitatively by the peak area normalizing method implemented in LabSolutions Essentia software (Shimadzu, Japan).

Leaf starch extraction and quantification
Arabidopsis leaves were harvested and ground to powder in liquid nitrogen using the high throughput tissue grinder (SCIENTZ-48, Scientz, China). Then, 5 mL 80% vol/vol ethanol were added to about 0.5 g leaf powder and incubated in a boiling water bath for 5 min, and then centrifuged at 3,000 g for 10 min at room temperature and the supernatant discarded. This ethanol extraction was repeated twice more, each time discarding the supernatants and the ethanol allowed to evaporate. Pellets were transferred to a mortar, homogenized thoroughly using 1 mL of water, and the homogenate transferred to a Falcon tube. Five samples were pooled to obtain a homogenate volume of 5 mL. Of the homogenate, 0.2 mL were transferred to a new tube and heated to 100 °C for 15 min to gelatinize starch granules and then cooled as recommended by Maness (52). After addition of 0.5 mL 200 mM Na acetate (pH 5.5), 6 units of a-amyloligosidase and 0.5 units of a-amylase, samples were incubated at 37 °C for 4 h and then centrifuged at 12,
Glucose concentration was determined using an enzymatic assay (Boehringer Mannheim/R-Biopharm, Germany) according to the manufacturer's protocol. The content of glucose was calculated as follows: \( c = \frac{V \times MW}{(\varepsilon \times d \times v \times 1000) \times \Delta A \times 10 \text{ mg} } \), where (V=final volume, v=sample volume, MW=molecular weight of glucose, d=light path, \( \varepsilon \)=extinction coefficient of NADPH at 340 nm, \( \Delta A \)= A after enzyme addition - A prior to enzyme addition. From this value, the starch content of the tissue was calculated: \( c / (\text{vol of incubation assay}) \times 10 / (\text{wt of tissue (g)}) \) (mg/g FW) \( {52, 53} \). The values of three biological replicates were averaged.

**Polysome extraction from Arabidopsis leaves**
To extract polysomes from Arabidopsis leaves, about 200 mg leaf material was collected under normal and high light conditions by grinding in mortar and pestle in presence of liquid nitrogen. Precooled polysome buffer (160 mM Tris-HCl pH 8.4, 80 mM KCl, 40 mM MgCl\(_2\), 5.26 mM EGTA, 0.5% (v/v) Octylphenoxy poly(ethyleneoxy)ethanol, 50 μg·mL\(^{-1}\) cycloheximide, 50 μg·mL\(^{-1}\) chloramphenicol) was added to the crude extract, samples incubated 10 min on ice and centrifuged at 16,000 g for 15 min at 4 °C to obtain the supernatant. Samples were then loaded on a 6 mL 20 to 50% Suc density gradient and ultracentrifuged at 175,000 g for 165 min at 4 °C in a SW 41 Ti rotor (Beckman Coulter, CA, USA). After centrifugation, tubes were carefully removed from the rotor and gradient fractions obtained by puncturing the bottom of a tube and collecting the effluent in 150 centrifuge tubes. The absorbance profile of the gradient fractions was measured using a UV-Vis Spectrophotometer at 254 nm.

**RNA Isolation and Quantitative RT-PCR**
RNA isolation from Arabidopsis leaves and quantitative RT-PCR were conducted as described by \( {7} \). RNA isolation from different sucrose gradient fractions was conducted as described by \( {54} \). Since the polysome extraction buffer contained heparin, a lithium chloride (LiCl) precipitation was performed instead of ethanol precipitation to remove any trace of heparin that could interfere with downstream analysis. After extraction, LiCl was added to a final concentration of 2.5 M. For precipitation, samples were kept for 30 min at -20 °C or overnight at 4 °C before centrifugation at 15,000 g for 15 min at 4 °C. Differences in gene expression between wild-type and mutant plants were calculated using the \( 2^{-\Delta\Delta C_{t}} \) method \( {55} \). \( UBPQ10 \) and \( Actin12 \) (Suppl. Table S1) were used as reference genes.

**Antibody preparation, membrane isolation and Western Blot**
The anti-SUC2 polyclonal rabbit antibody was raised and affinity-purified against a peptide of AtSUC2 corresponding to the amino acid residues 7 to 28 (EKAANGASALETQTGELDQPER) and 309–327 (YGGNSDATATAASKKLYND) by the ABclonal Biotechnology Company-WuHan (China). The final concentration of affinity-purified antibodies used for immunoblot analysis was about 40 ng/mL. Plant-specific Anti-ACTIN rabbit polyclonal antibody, used as reference for Western Blots, was purchased from Sangon Biotech (China). Specificity was tested by Western Blot, with occurrence of a single band of the right size as positive result. Membrane protein isolation and
quantification by Western Blot were conducted as described previously (7). As in the other experiments, rosette leaves of 3-week-old plants were used. For inhibitor studies, CHX (10 μM; Sigma-Aldrich, MO, USA), MG132 (10 μM; Merck Millipore, MA, USA) working solutions were prepared in DMSO. For control experiments, a 0.1% DMSO solution was used. Solutions were infiltrated into leaves of Arabidopsis plants using a syringe without needle.

**Engineering of gene constructs and transformation of N. benthamiana**

The multiple-copy plasmid vector pGWB444, which contains 35S promoters and C-terminal enhanced YFP was used for constructing FRET acceptor fusion proteins. For donor fusion proteins, CFP (pGWB441) was replaced with mTurquoise2, which offers higher quantum efficiency compared to the traditionally-used CFP, and referred to pGWB-mT2 in this study. Genes of interest were amplified and cloned into pENTR™/D-TOPO® vectors (Invitrogen, CA, USA). After verification of correct insertion by sequencing genes of interest were recombined into the desired Gateway destination vectors via Gateway® LR Clonase™II (Invitrogen, CA, USA). All primers used in this study are listed in Supplemental Table S1. Finally, the successfully constructed vectors were transformed into Agrobacterium tumefaciens strain GV3101 (C58 (rifR) Ti pMP90 (pTiC58DT-DNA)) using the freeze-thaw method, as described previously (56). The vectors containing SUC2-mT2, SUC3-YFP, STP1-YFP, UBC34-YFP, WAKL8-YFP, RLCKVII-YFP and CDKB1;1-YFP were used for FRET analysis.

The Agrobacterium-mediated infiltration method was adopted from Sparkes et al. (57) with minor modification. Agrobacterium suspensions were prepared by growing the bacteria containing suitable antibiotics at 28 °C overnight on a shaker and used freshly on the day infiltration. The bacteria were resuspended to an optical density of 0.5 at OD 600 nm in solution of 10 mM MgCl2 and 10 mM MES (pH 5.5), centrifuged twice, and incubated for at least 2-3 hours at room temperature with 200 μM acetosyringone (Sigma-Aldrich, MO, USA) before infiltration. The third or fourth leaf counted from the apical meristem of N. benthamiana plants were infiltrated using a needle-less syringe.

**Quenching assay**

A vector for the expression of HDEL-YFP fusion protein serving as ER-specific marker was constructed based on the pBI121-mCherry vector using one-step cloning according to the manufacturer’s protocol (TransGen Biotech, China). SUC2-YFP, UBC34-mT2 and WAKL8-mT2 were constructed using Gateway technology and the A. tumefaciens-mediated transient expression in N. benthamiana leaves was conducted as described above. Trypan Blue (Merck Millipore) was used as the quencher at a final concentration of 40 μM. The images were collected about 5 min after quencher application using a Thorlabs confocal (Thorlabs, NJ, USA) using a 40× water-immersion objective. The excitation laser wavelength was 488 nm. Emission filters were chosen to detect fluorescence between 520 nm and 550 nm. Software supplied by the microscope manufacturers was used for image acquisition. The quenching assay was repeated at least three
times for each sample. The intensity of the fluorescence was quantified using the ImageJ and the fluorescence reduction ratio was quantified as: \( \frac{Q_{\text{pre}} - Q_{\text{post}}}{Q_{\text{pre}}} \) (\( Q_{\text{post}} \) is the fluorescence intensity of the YFP after adding the quencher, and \( Q_{\text{pre}} \) is the fluorescence intensity of the YFP before adding the quencher.

**Fluorescence resonance energy transfer analysis**

FRET analysis of the fusion proteins transiently expressed in *N. benthamiana* leaf epidermal cells was carried out using a TCS SP8 laser-scanning confocal microscope (Leica Microsystems, Germany). Laser lines at 405 nm and 514 nm were used for excitation of mT2 and YFP, respectively. Emission windows were defined at 420 to 480 nm for mT2 and at 525 to 580 nm for YFP. The procedure followed the FRET Acceptor PhotoBleaching wizard of the Leica confocal software (LAS-AF, Leica Microsystems, Germany). Acceptor bleaching was used as it represents one of the most robust varieties of intensity-based FRET (58). In cells expressing the donor and acceptor, the YFP of the acceptor was bleached by scanning the cell for 15 to 30 seconds using the 514 argon laser line at 100% intensity. Images in the mT2-specific channel were collected before and after the bleaching. The intensity of the fluorescence was quantified using the ImageJ software and the energy transfer efficiency was quantified as: FRET eff = \( \frac{D_{\text{post}} - D_{\text{pre}}}{D_{\text{post}}} \) (\( D_{\text{post}} \) is the fluorescence intensity of the Donor after acceptor photobleaching, and \( D_{\text{pre}} \) is the fluorescence intensity of the Donor before acceptor photobleaching. The FRET efficiency is considered positive when \( D_{\text{post}} > D_{\text{pre}} \) (58).

**Expression and purification of GST Fusion Proteins**

*UBC34* was cloned into the pDEST-15 gateway vector under control of the T7 promoter and overexpressed in *Escherichia coli* strain BL21 AI. To increase protein solubility, a UBC34 mutant lacking a transmembrane domain (amino acids from 213 to 235, UBC34\( \Delta \text{tmd} \)) was produced as a fusion protein with a GST tag (59). Protein was expressed and purified as described by Stone et al. 2005 (60). Purification of GST-tagged proteins was performed using PureCube Glutathione Agarose (Cube Biotech, Germany) according to manufacturer instructions.

*WAKL8* was cloned into the pDEST15 gateway vector under control of the T7 promoter and overexpressed in *Escherichia coli* strain BL21-pLysS. Protein was expressed and purified using PureCube Glutathione Agarose (Cube Biotech, Germany) according to manufacturer instructions.

**Expression and purification of SUC2**

*SUC2* was purified from yeast cells with the help of an HA-tag. The vector for the expression of the HA-SUC2 fusion protein in yeast cells was constructed based on the pYES2 and pXCS-HA-Strep vectors via homologous recombination and named pYES2-HA. *SUC2* was cloned into the pYES2-HA vector under the GAL1 promoter using one-step cloning according to the manufacturer’s protocol (TransGen Biotech, China). HA-SUC2 and empty vector pYES2-HA were transformed into *Saccharomyces cerevisiae* strain BY4741 as described previously (61) and plated on selective glucose (2%, w/v) medium without uracil (SD/Ura\(-\)). Correct incorporation of the plasmid was
checked by sequencing after yeast colony PCR. For HA-SUC2 purification, cells grown to logarithmic phase were harvested and resuspended using induction medium containing galactose (2%, w/v) to a final OD of 0.5-0.6. After incubation for 10 h, total membrane proteins extracted as described previously (62). SUC2 was purified using anti-HA beads according to the manufacturer’s protocol (Smart Lifescience, China) after validating expression of recombinant fusion protein by western blot using anti-SUC2 and anti-HA antibodies.

**Pull-Down Assay**

SUC2 and UBC34 protein interactions were investigated by performing the GST pull-down assay. Purified GST-UBC34Δtmd fusion protein was incubated with glutathione-sepharose beads for 2 h to enable binding of GST-UBC34Δtmd-fusion protein to the beads. GST-UBC34Δtmd-bound beads and empty beads for control, were incubated with leaf membrane protein extracts overnight at 4 °C in a rotary shaker. The supernatant was removed and the beads were washed three times using PBS. Finally, proteins were eluted by adding 20 μL sample buffer and the eluate incubated at 100 °C for 5 min before analysis by SDS-PAGE and immunoblot using anti-SUC2 antibody.

**Immunoprecipitation and Immunoblot analysis**

Affinity-purified antibody raised against SUC2 was used to perform immunoprecipitation test reactions and experiments using the Pierce Co-Immunoprecipitation kit according to the manufacturer’s instructions (Pierce Biotechnology, ThermoFisher, IL, USA). Leaf membrane proteins were extracted as described above. Leaf membrane proteins were incubated with cross-linked anti-SUC2 protein-A agarose beads overnight at 4 °C while shaking. The wash buffer to elute the protein was adjusted to pH 7 in order to reduce binding and obtain a suitable elution yield. Immunoblot analysis was conducted following the protocol for Western Blot.

**Thioester Assay**

GST-UBC34Δtmd fusion protein was expressed and purified as described above. The thioester assays reaction was performed in a reaction volume of 30 μL, including 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 mM ATP, 100 ng UBE1 (Boston Biochemicals, Cambridge, MA, USA), 2 μg recombinant GST-UBC34Δtmd, and 10 μg recombinant plant ubiquitin (Boston Biochemicals, Cambridge, MA, USA). Reactions were split after incubation for 5 min at 37 °C and terminated by SDS sample buffer with or without dithiothreitol (DTT). The samples were separated on a 12% SDS-PAGE gel after boiling at 100 °C for 10 min and then detected by Western Blot with anti-ubiquitin antibody (P4D1; Santa Cruz Biotechnology, CA, USA).

**In vitro ubiquitination assay**

GST-UBC34Δtmd and HA-SUC2 fusion proteins were purified as described above. The ubiquitination assays were performed *in vitro*. Approximately 500 ng of GST-UBC34Δtmd and 250 ng of HA-SUC2 fusion proteins were mixed with 100 ng of UBE1 (Boston Biochemicals, Cambridge, MA, USA) and 1 μg ubiquitin (‘plant recombinant’, Boston Biochemicals, Cambridge, MA, USA). The reaction mixture was incubated in 50 mM Tris-HCl (pH 7.4), 2 mM ATP, 5 mM MgCl₂ and 2
mM DTT for 2 h at 30 °C. Samples were then incubated at 100 °C for 10 min before separation by SDS-PAGE and immunoblot analysis using anti-SU2 antibody.

**In vivo ubiquitination assay**

Total membrane proteins isolated from wild-type and *ubc34* mutant leaves were subjected to immunoprecipitation and immunoblot analysis as described above. Monoclonal antiubiquitin antibody (P4D1; Santa Cruz Biotechnology, CA, USA) was used for detecting ubiquitinated proteins.

**Phos-Tag™ SDS-PAGE**

Extraction of membrane protein from wild-type and *wakl8* mutant leaves detection of SUC2 by immunoblot was performed as describe above. Phos-tag SDS-PAGE was performed accoding to the kit manufacturer instructions (Wako Chemicals, Japan). 30% acrylamide and 20 μM Mn²⁺-PhosTag™ were used for the sufficient separation the phosphorylated and non-phosphorylated proteins. In contrast to regular SDS-PAGE, after electrophoretic separation, the gels were incubate in transfer buffer containing 10 mM EDTA for at least 10 min for three times and then in transfer buffer without EDTA for an additional 10 min.

**In vitro phosphorylation assay**

HA-SUC2 and GST-WAKL8 were incubated in 1X kinase reaction buffer (50 mM Hapes, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 2 mM DTT, 5 mM EGTA and 50 μM ultra pure ATP) for 30 min at room temperature. ADP produced from kinase reactions was quantified using the ADP-Glo kinase kit (Promega Corporation, USA) according to the manufacturer’s manual. Luminescence was recorded using a GloMax 20/20 luminometer (Promega Corporation, USA). As negative controls, GST alone and GST-WAKL6 were used.

**Esculin uptake assays**

A vector for the expression of SUC2-mCherry fusion protein was constructed based on the pBI121-mCherry vector using one-step cloning according to the manufacturer’s protocol (TransGen Biotech, China). The A. *tumefaciens*–mediated transient expression in *N. benthamiana* leaves was conducted as described above. Esculin (Sigma-Aldrich, MO, USA) was applied by soaking the 2cm x 2cm large leaf cutouts for 10 min in 1 mM esculin dissolved in water. Fluorescence imaging was performed using a Thorlabs confocal (Thorlabs, NJ, USA). Esculin was excited at 488 nm and its emission detected at 495 nm to 545 nm. mCherry was excited at 561 nm and its emission detected at 570 nm long-pass filter. The intensity of esculin fluorescence was quantified in ImageJ (63). UBC33-YFP and WAKL6-YFP were used as control, as they represent proteins closely related to the proteins of interest UBC34 and WAKL8. mCherry was used as negative control to determine the background level of esculin fluorescence.

**Yeast complementation assays**

SUC2 and WAKL8 cDNA were subcloned into the yeast shuttle vector pDR196 with the *Saccharomyces* PMA1 promoter. pDR196-SUC2 and pDR196-WAKL8 were transformed into
Saccharomyces cerevisiae strain SUSY7/ura3 for sucrose uptake assays and kinetic analysis. Assessment of yeast growth was performed on minimal selective medium at pH 5.0 supplemented with sucrose (2%, w/v) as the sole carbon source without uracil. The same medium with glucose as the carbon source was used for control. Plates were incubated at 30 °C and pictures were taken after the emergence of single colonies. Yeast cells transformed with the empty vector pDR196 were used as negative control. In addition to SUC2, also the closely related sucrose transporter SUC1 was included in the growth assays as negative control.

**Kinetic analysis of SUC2 activity**

Sucrose uptake assays were performed as described by Krügel et al. (10). Single yeast colonies expressing SUC2 alone or coexpressing SUC2 and WAKL8 were grown in liquid SD/Ura- medium containing glucose (2%, w/v) to OD600 of 0.6. Cells were harvested by centrifugation, washed twice in sterile water and 25 mM sodium phosphate buffer, pH 5.0, and resuspended at a final OD600 of 20 in the same buffer. Uptake assays were initiated by adding glucose to a final concentration of 10 mM and incubation for 1 min at 30 °C, after which 13C-labeled sucrose was added at concentrations of 0.05, 0.1, 0.3, 0.7, 1.0, 3.0, and 10.0 mM in 25 mM sodium phosphate buffer, pH 5.0. After shaking incubation at 30 °C for 5 min, 10 mM cold glucose was added to stop the reaction. Cells were collected and washed once in sodium buffer and twice in sterile water and the 13C content analyzed using isotope ratio mass spectrometry and elemental analysis (performed by Huake Stable Isotope Laboratory, Shenzhen, China) after vacuum freeze-drying the yeast cells. Three biological replications were performed. Data for kinetic analysis were analyzed using Origin 9 (OriginLab, MS, USA). Nonlinear regression was performed using the Michaelis–Menten equation and values for $K_m$ and $V_{max}$ presented as mean ± SE.

Esculin uptake assays were performed as described for sucrose above. Esculin was used at concentrations of 0.01, 0.03, 0.05, 0.07, 0.1, 0.2, 0.5, 1, 2 and 3 mM in sodium phosphate buffer, pH 5. To determine the pH dependency of esculin uptake, the yeast cells were washed and resuspended in sodium phosphate buffer at different pH values ranging from 4 to 7. The fluorescence intensity was measured on a spectrofluorometer with excitation at 367 nm and detection of emission at 454 nm. Culture density was determined as OD$_{600}$. The fluorescence per OD$_{600}$-unit was calculated to determine the relative fluorescence. Data analysis was performed as described for sucrose uptake above.

**Measurement of proton flux in yeast plasma membrane vesicles**

Single yeast colonies expressing SUC2 alone or coexpressing SUC2 and WAKL8 were grown in liquid SD/Ura- medium containing glucose (2%, w/v) to an OD$_{600}$ of 1-1.5. Cells were collected and washed three times with distilled water. The isolation and purification of plasma membrane vesicles were performed as previously described using a sucrose density gradient (64). The protein concentration was estimated using the bicinchoninic (BCA) method. The proton flux was measured by acridine orange quenching at 495 nm as described previously (65, 66). Briefly, the assay
medium consisted of 20 μM acridine orange, 2 mM MgCl₂, 10 mM Mops-BTP (pH 7.0), 40 mM KCl, 2.5 mM MgSO₄, 1 mM EGTA-BTP, 1 mM DTE, 50 μM PMSF, and 50 μg protein in a total volume of 1 mL. After 5 min preincubation, the reaction was started by addition of ATP-BTP (pH 6.5), giving a final concentration of 5 mM ATP. The rate of H⁺-accumulation was estimated from the initial slope of absorbance quenching of acridine orange.

**Accession Numbers**

Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR; 68) under the following accession numbers: AtSUC1 (AT1G71880), AtSUC2 (AT1G22710), AtSUC3 (AT2G02860), AtSTP1 (AT1G11260), AtUBC34 (AT1G17280), AtWAKL8 (AT1G16260), AtCDKB1;1 (AT3G54180), AtRLCKVII (AT1G07860), AtUBC33 (AT5G50430), AtWAKL6 (AT1G16110). Germplasm numbers are as follows: *ubc34* (SALK_033872C), *wakl8* (SALK_029502C), *cdkb1;1* (SALK_073457C), *rclkvii* (SALK_063868).
Supplemental Figures and Tables

**Fig. S1.** (A and B) Comparison of sugar content in leaf extract (A) and sugar content in phloem exudate (B) in rosette leaves of three-wk-old wild-type Arabidopsis exposed to normal light (NL) or high light (HL) conditions. (C and D) Western blots indicating SUC2 protein level in relation to application period of the protein synthesis inhibitor cycloheximide (CHX) alone (C) or CHX plus the inhibitor of late endosome trafficking MG132 (D). Membrane proteins were extracted from rosette leaves of 3-wk-old plants treated with cycloheximide (CHX) for 4 h and 8 h or treated with CHX and MG132 for 2 h, 4 h, 6 h and 8 h under NL and HL condition. Actin was used as a loading control. (E) Western Blot analysis of SUC2 protein abundance under normal light (NL) or high light (HL) conditions. Actin was used as a control. (F) Western blot showing the SUC2-specific band at about 55 kD. E6497 and E6444 were two different peptides used for polyclonal antibody preparation. (G)
Immunoblot with SUC2 antibody using standard SDS-PAGE. (H) Immunoblot with SUC2 antibody using a Phos-Tag SDS-PAGE gel after 1 h treatment with alkaline phosphatase (AP). Molecular masses in kilodalton (kDa) are indicated on the right side of each blot. Asterisks indicate significant differences (P<0.05). All error bars depict standard deviation of the mean (n= 3). WT, wild type.
Fig. S2. (A, B and C) FRET images showing fluorescence of the donor SUC2-mT2 before and after bleaching of the acceptor STP1-YFP (A), SUC3-YFP (B) and UBC34-YFP (C). Rectangles indicate the bleaching regions. Bar plots indicate difference in SUC2-mT2 fluorescence intensity after photobleaching in the unbleached and bleached regions indicated in the images. Pixel-wise values were averaged. (D) FRET efficiency of SUC2-mT2 and STP1-YFP and SUC2-mT2 and SUC3-YFP. Asterisks indicate significant differences (P<0.05). All error bars depict standard deviation of the mean (n= 7 [C]). Scale bar, 50 μm.
Fig. S3. Protein purification. (A-C) Blots indicating expression of the fusion proteins GST-UBC34Δtmd (A) and GST-WAKL8 (B) in E. coli and HA-SUC2 (C) in yeast. Lane 1 shows the protein extract before the addition of inducers of protein expression (IPTG in E. coli and galactose in yeast). The other lanes show the protein extract after addition of IPTG or galactose (upper panel) at 4 h, 8 h and 12 h for A and B, at 12 h and 24 h for C. The lower panel of A, B, C shows the immunoblot analysis with corresponding antibodies. Arrowheads indicate bands of target fusion proteins. (D) Western Blot analysis of GST-UBC34Δtmd, GST-WAKL8 and HA-SUC2 after affinity purification with the corresponding antibodies (‘purified proteins’). As controls, total extracted protein (‘protein extract’), total protein after elution from the GST- or HA-binding agarose column (‘unbound proteins’) and proteins in the washing solution (‘bound proteins’) before elution were included. (E) Coomassie-stained gel showing bands for purified GST-UBC34Δtmd, GST-WAKL8 and HA-SUC2. Molecular masses, in kilodalton (kDa), are indicated on the side of each gel or blot.
Fig. S4. T-DNA insertion in the exon of *UBC34* (AT1G17280) in SALK_033872C (A), promoter of *WAKL8* (AT1G16260) in SALK_029502C (B), promoter of *RLCKVII* (AT1G07860) in SALK_063868 (C) and exon of *CDKB1;1* (AT3G54180) in SALK_073457C (D). Primers used to screen the mutant lines indicated by black arrows LB, LP and BP, were derived from the SALK DNA primer design web tool (http://signal.salk.edu/tdnaprimers.2.html) and listed in Suppl Table S1. (E and F) Identification of homozygous mutants by three primers PCR (E) and qRT-PCR (F). PCR amplification of wild type allele band using LP, RP and T-DNA band (LB). Homozygous wild type allele was seen as single bands at about 1000 bp whereas a T-DNA homozygous allele band at
around 600-800 bp. Black rectangles indicate the exon regions, gray rectangles indicate the promoter regions and black lines indicate introns. (G) Immunoblots indicating SUC2 protein abundance in leaves of plants grown under NL or exposed to HL. Actin was used as a loading control. One representative image out of 3 replicates is shown. (H) Immunoblot analysis of SUC2 protein isolated from leaves treated with cycloheximide (CHX) for 4 h, 8 h and 12 h of NL-grown wild-type and ubc34 plants. Actin was used as a loading control. One representative image out of 3 replicates is shown. (I) Quantification of SUC2 protein abundance according to immunoblot analysis in G for 8 h. Molecular masses, in kilodalton (kDa), are indicated on the right side of each blot. Significant difference (P<0.05) is indicated by letters. Letters were arranged alphabetically starting from the highest values with similar letters indicating no significant difference. All error bars depict standard deviation of the mean (n= 3). WT, wild type.
Fig. S5. Phenotype of ubc34 and waki8 mutant plants. (A) Phenotypic differences between wild type and ubc34 mutant at different growth stages. (B) Phenotypic differences between wild type and waki8 mutant at different growth stages. (C) Morphology of detached leaves of ubc34 and waki8 mutants. Shown are the 5th and 6th leaf (numbered after appearance), representing the juvenile stage, and the 10th and 11th leaf, representing the mature stage. (D and E) Glucose and fructose content of leaf extracts (D) and phloem exudate (E) of wild-type and ubc34 mutant plants under normal light (NL) and high light (HL) conditions. (F) Seedling fresh weights of wild-type and ubc34 mutant plants. (G and H) Glucose and fructose content of leaf extracts (G) and phloem
exudate ($H$) of wild-type and wakl8 mutant plants exposed to NL or HL conditions. (I) Seedling fresh weights of wild-type and wakl8 mutant plants. Significant difference (P<0.05) is either indicated by asterisk or letters. Letters were arranged alphabetically starting from the highest values with similar letters indicating no significant difference. All error bars depict standard deviation of the mean (n=3). WT, wild type.
Fig. S6. In vitro ubiquitination and in vitro phosphorylation of SUC2. (A) Immunoblots with anti-ubiquitin antibody as the results of a DTT-sensitive thioester assay of GST-UBC34Δtmd. The open arrowhead indicates the band of the DTT-sensitive thioester linkage that results from ubiquitin-binding by a ubiquitin ligase. (B) In vitro ubiquitination of HA-SUC2 by GST-UBC34Δtmd. Samples were separated by 12% SDS-PAGE and detected by anti-SUC2 immunoblot analysis. The free and polyubiquitinated HA-SUC2 are indicated. (C) Standard Curve for the conversion of ATP to ADP.
using the ADP-Glo™ assay of kinase activity. (D) WAKL8 activity indicated by ADP-dependent luminescence in the presence of HA-SUC2 at various concentrations. HA-SUC2 alone was used as negative control. ATP was supplied at 1 mM concentration. (E) WAKL8 activity indicated by ADP-dependent luminescence in relation to WAKL8 concentration in the presence of 8 μM SUC2. ATP was supplied at 1 mM concentration. Abbreviation: RLU, relative light unit. Molecular masses are indicated in kilodaltons (kDa) on the side of each blot. All error bars depict standard deviation of the mean (n= 3).
Fig. S7. Control experiments for the esculin uptake assay. (A and C) Uptake of the sucrose-analogue esculin in *N. benthamiana* epidermal cells expressing mCherry as negative control, SUC2-mCherry alone or SUC2-mCherry together with UBC33-YFP (A) and WAKL6-YFP (C) visualized by fluorescence microscopy. (B and D) Intracellular esculin signal in relation to the level of the negative control for A, C, separately. All error bars depict standard deviation of the mean (n= 5). Scale bars: 50 μm.
**Fig. S8.** Analysis of quenching efficiency using the quencher Trypan Blue. (A) Images showing fluorescence of SUC2-YFP before and after adding the quencher either expressed alone or co-expressed with UBC34-mT2 and WAKL8-mT2. HDEL-YFP was used as the negative control. (B) Relative fluorescence reduction ratio is calculated based on the images from A. Significant difference (P<0.05) is indicated by letters. Letters were arranged alphabetically starting from the highest values with similar letters indicating no significant difference. All error bars depict standard deviation of the mean (n= 5). Scale bar, 20 μm.
Fig. S9. Comparison of SUC2 protein levels and sucrose content in phloem exudate in different kinase mutant. FRET images showing fluorescence of the donor SUC2-mT2 before and after bleaching of the acceptor WAKL8-YFP (A), RLCKVII-YFP (C), CDKB1;1-YFP (E). Rectangles indicate the bleaching regions. (B, D and F) Difference in SUC2-mT2 fluorescence intensity after photobleaching in the unbleached and bleached regions indicated in A, C, E, separately. Pixel-wise values were averaged. (G) Western Blot analysis of SUC2 protein in the leaf of 3-wk-old wild-type and wakl8, rickvii, cdkb1;1 mutant plants grown under normal light conditions. Actin is used as a loading control. (H) Average intensity of SUC2-specific bands. (I) Phloem exudate sucrose content in wild-type and wakl8, rickvii, cdkb1;1 mutant plants exposed to normal light conditions. (J)
Western blot analysis of SUC2 protein in the leaf of 3-wk-old wild-type and wakl8 mutant under high light conditions. Actin is used as a loading control. Molecular mass is indicated on the right side of each blot. Significant difference (P<0.05) is either indicated by asterisk or letters. Letters were arranged alphabetically starting from the highest values with similar letters indicating no significant difference. All error bars depict standard deviation of the mean (n= 3). WT, wild type.
**Fig. S10.** Complementation of the sucrose uptake-deficient yeast strain SUSY7/ura3-. (A) Transformants growing on SD/Ura- medium with glucose as sole carbon source. (B) Transformants growing on SD/Ura- medium with sucrose as the sole carbon source. Images show growth after 3 to 5 d at 30°C. 1: empty vector control (pDR196), 2: WAKL8, 3: SUC2, 4: SUC2+WAKL8, 5: SUC1, 6: SUC1+WAKL8.
Fig S11. Effect of WAKL8 on the kinetics of SUC2-mediated transport. (A) Proton flux from inside out plasma membrane vesicles expressed as the relative absorbance of acridine orange at 495 nm per mg of plasma membrane protein. Plasma membrane vesicles were derived from yeast cells expressing SUC2 alone or coexpressing SUC2 and WAKL8. Addition of ATP starts the proton pumping, while it is stopped by addition of the proton pump inhibitor nigericin. (B, C) $K_m$ (B) and $V_{max}$ (C) of sucrose or esculin uptake into yeast cells expressing SUC2 alone or coexpressing SUC2 and WAKL8. Uptake was measured over 5 min at pH 5. (D) Esculin uptake of yeast cells expressing SUC2 alone or coexpressing SUC2 and WAKL8, measured at pH 5. (E) Relative transport activity of SUC2 at different pH values using 1 mM esculin or 1 mM sucrose as substrate. Data for sucrose was extracted from Sauer and Stolz (67). Asterisks indicate significant differences (P < 0.05). Error bars depict standard deviation of the mean, except D, which displays standard error. Number of replicates n = 3 (A), n = 4 (B-E).
Table S1. Sequences of gene-specific primers used for qPCR, genotyping and vector construction.

| Gene name | Primer sequence (5’ to 3’) |
|-----------|----------------------------|
| SUC2      | Forward: GCAGACGGGTGAGTTAGA  |
|           | Reverse: GGAGATTGGGACCACAGAG |
| UBC34     | Forward: ACAGTTGGCTAAGTCACTCTCT |
|           | Reverse: GCTAGTGGCTTGCTGTTGTT |
| CDKB1;1   | Forward: AGGTCGGAGAAGGAACATACG |
|           | Reverse: CGAAGAGCAGTGGTGGGAATAC |
| RLCKVII   | Forward: CTCCCAGTCTTCTTCTTCT |
|           | Reverse: ATCCAAATATCGGATAACCG |
| WAKL8     | Forward: CGATATGGAGAAAGGGTCTCC |
|           | Reverse: CCCACACGAATTGTCATTTC |
| UBC34     | Forward: GGCCTTGTATAATCCCTGATGATTAAG |
|           | Reverse: AAAGAGATAACAGGAACGGAAACATAGT |
| Actin12   | Forward: GCCAACAGAGAAGATGAC |
|           | Reverse: ACCAGAATCCAGCAAAATAC |

| Gene name | Primer sequence (5’ to 3’) |
|-----------|----------------------------|
| LBb1.3    | ATTTTGCCGATTTCGGAAC |
| UBC34     | LP: GCCACTGAACACATCTCTTC |
|           | BP: CCAATTGGCAATTTCAAGCTTC |
| CDKB1;1   | LP: CTGGTGTTGACATGTGGTCTG |
|           | BP: TGTTGTAAACCATTTGTGGGC |
| RLCKVII   | LP: GAGTGGGAGAAATCCAGAGG |
|           | BP: CAAAGACAGGGAACATTCCC |
| WAKL8     | LP: CGATATGGAGAAGGGTCTCC |
|           | BP: CCCACACGAATTGTCATTTC |

| Gene name | Primer sequence (5’ to 3’) |
|-----------|----------------------------|
| SUC3      | Forward: CACCATGAGTGAATCGGTG |
|           | Reverse: GCCAGATGAGAACAGCCTT |
| STP1      | Forward: CACCATGCTGAGCCTGTT |
|           | Reverse:AACATGCTTCCTCACAGC |
| SUC2      | Forward: CACCATGGTACGGCATCCTAG |
|           | Reverse: AGTGAATCCCATAGTACGTC |
| UBC34     | Forward: CACCATGGCAGAAGGCTCTATA |
|           | Reverse: CAGTTGAGAGCAGAGGCA |
| WAKL8     | Forward: CACCATGGTATGAGGTAAGAAG |
|           | Reverse: GAACTGAGACTTTGTCAAG |
| CDKB1;1   | Forward: CACCATGGGAAGTACGAGAAG |
|           | Reverse: GAACTGAGACTTTGTCAAG |
| RLCKVII   | Forward: CACCATGGGAAGTACCTTCTT |
|           | Reverse: GACTGCGGTGCTGGCTT |

| Gene name | Primer sequence (5’ to 3’) |
|-----------|----------------------------|
| UBC34     | Forward: CACCATGGCAGAAGGCA |
|           | Reverse: TCATTATTGTTTCCTCTC |

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| Gene        | Primer sequence (5’ to 3’) | Gene     | Primer sequence (5’ to 3’) |
|-------------|----------------------------|----------|----------------------------|
| WAKL8       | Forward: CACCATGGGTGTTGATGTGAAACG  
              Reverse: TCACTGTGTCCTTGTGAGGCATTAG | SUC2     | Forward: GGATCGGACTACTAGCAGCTGAATACGACTCAGTATAGGGCGAGC  
              Reverse: TGATGGATATCTCAGAATTTCTAATTGAAAATCCCATGAGCTTGGAA |
| Gene-specific primers for vector reconstruction | | | |
| Gene name       | Primer sequence (5’ to 3’) | | Primer sequence (5’ to 3’) |
| PGWB-mT2-  | Forward: ACAGCCAGTCTCGACGGGTACCATATGTGAATATGTGTTTGAATTACA  
              Reverse: ACAGCTCTAGGCTGACTCACTAGTGTGAGGCATTAG | PGWB-mT2-  | Forward: TAACAGCAGTGTGAGGCGAGGCGAGGCTGT  
              Reverse: GACGGGGAATTTGAGTGAATATGTGAATATGTGTTTGAATTACAAGAAGTGGTGA | |
| insert1       | PGWB-mT2-       | Forward: ACAGCCAGTCTCGACGGGTACCATATGTGAATATGTGTTTGAATTACA  
              Reverse: ACAGCTCTAGGCTGACTCACTAGTGTGAGGCATTAG | PGWB-mT2-       | Forward: TAACAGCAGTGTGAGGCGAGGCGAGGCTGT  
              Reverse: GACGGGGAATTTGAGTGAATATGTGAATATGTGTTTGAATTACAAGAAGTGGTGA | |
| insert2       | PGWB-StrepII-  | Forward: ACAGCCAGTCTCGACGGGTACCATATGTGAATATGTGTTTGAATTACA  
              Reverse: ACAGCTCTAGGCTGACTCACTAGTGTGAGGCATTAG | PGWB-StrepII-  | Forward: TAACAGCAGTGTGAGGCGAGGCGAGGCTGT  
              Reverse: GACGGGGAATTTGAGTGAATATGTGAATATGTGTTTGAATTACAAGAAGTGGTGA | |
| insert1       | PGWB-StrepII-  | Forward: ACAGCCAGTCTCGACGGGTACCATATGTGAATATGTGTTTGAATTACA  
              Reverse: ACAGCTCTAGGCTGACTCACTAGTGTGAGGCATTAG | PGWB-StrepII-  | Forward: TAACAGCAGTGTGAGGCGAGGCGAGGCTGT  
              Reverse: GACGGGGAATTTGAGTGAATATGTGAATATGTGTTTGAATTACAAGAAGTGGTGA | |
| UBC33       | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | WAKL6     | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | |
| pGADT7-     | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | pGADT7-     | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | |
| 7-HA        | pGADT7-        | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | pGADT7-        | Forward: CACCATGGCAGAAAAAGCTTGTATA  
              Reverse: CAGCTGAAGCAGAAAAAGCTTGTATA | |
| pYES2-GAL1- | Forward: GGATCGGACTACTAGCAGCTGTAATACGACTCAGTATAGGGCGAGC  
              Reverse: GGTACTATGAGCAGCTGTAATACGACTCAGTATAGGGCGAGC | pYES2-GAL1- | Forward: GGATCGGACTACTAGCAGCTGTAATACGACTCAGTATAGGGCGAGC  
              Reverse: GGTACTATGAGCAGCTGTAATACGACTCAGTATAGGGCGAGC | |
| 6-HA        | pYES2-GAL1-    | Forward: GGATCGGACTACTAGCAGCTGTAATACGACTCAGTATAGGGCGAGC  
              Reverse: GGTACTATGAGCAGCTGTAATACGACTCAGTATAGGGCGAGC | pYES2-GAL1-    | Forward: GGATCGGACTACTAGCAGCTGTAATACGACTCAGTATAGGGCGAGC  
              Reverse: GGTACTATGAGCAGCTGTAATACGACTCAGTATAGGGCGAGC | |
| pDR19-6-    | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | pDR19-6-    | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | |
| SUC1        | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | |
| WAKL8       | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | |
| 6-SUC1      | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | pDR19-6-       | Forward: TCCCCCGGGCTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA  
              Reverse: GGGCCCCCGCGCGGTGAGGAGAATCTGTGAGGACCTATGAAAACAGAAAA | |
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