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# Mapping of the plant SnRK1 kinase signaling network reveals a key regulatory role for the Class II T6P synthase-like proteins

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# ABSTRACT

The central metabolic regulator SnRK1 controls plant growth and survival upon activation by energy depletion, but detailed molecular insight into its regulation and downstream targets is limited. Here, we used phosphoproteomics to infer the sucrose-dependent processes targeted upon starvation by kinases as SnRK1, corroborating the relation of SnRK1 with metabolic enzymes and transcriptional regulators, while also pointing to SnRK1 control of intracellular trafficking. Next, we integrated affinity purification, proximity labeling and cross-linking mass spectrometry to map the protein interaction landscape, composition and structure of the SnRK1 heterotrimer, providing insight in its plant-specific regulation. At the intersection of this multi-dimensional interactome, we discovered a strong association of SnRK1 with Class II T6P synthase (TPS)-like proteins. Biochemical and cellular assays show that TPS-like proteins, similar intricate connections were found with known regulators, suggesting that plants utilize an extended kinase complex to fine-tune SnRK1 activity for optimal responses to metabolic stress.

#### INTRODUCTION

A well-balanced coordination between energy supply and expenditure is essential for viability in all organisms. When energy levels decrease, eukaryotes rely on an ancient regulatory mechanism to maintain metabolic homeostasis, involving a conserved family of protein kinases, comprising mammalian AMP-activated kinase (AMPK), yeast Sucrose Non-Fermenting 1 (SNF1), and plant SNF1-related kinase 1 (SnRK1)<sup>1</sup>. Upon activation, these kinases trigger a metabolic switch, generally promoting catabolic processes, while repressing energy-consuming anabolic processes. In plants, SnRK1 not only responds to carbon and energy depletion that arises during adverse growth conditions<sup>2,3</sup>, but it also coordinates nutrient allocation between source and sink tissues as well as developmental transitions that are associated with altered metabolic needs and fluxes<sup>4-6</sup>.

Similar to the AMPK and SNF1 kinases, the plant SnRK1 kinases function as heterotrimeric complexes, with a catalytic  $\alpha$ -subunit (SnRK1 $\alpha$ 1 or SnRK1 $\alpha$ 2 in Arabidopsis) and two regulatory ( $\beta$  and  $\gamma$ ) subunits<sup>1</sup>. Despite this shared configuration and their conserved role as cellular energy sensor, the SnRK1 kinases diverged from their opisthokont counterparts, adapting to the unique lifestyle of plants<sup>1,7,8</sup>. For example, plants possess a unique hybrid  $\gamma$ subunit (SnRK1 $\beta\gamma$ ) with a N-terminal carbohydrate-binding module (CBM) typically only present in the  $\beta$ -subunits (SnRK1 $\beta$ 1 or SnRK1 $\beta$ 2 in Arabidopsis), and a plant-specific truncated β-subunit isoform (SnRK1β3) lacking this N-terminal CBM<sup>1,9</sup>. Importantly, these larger structural differences and additional minor amino acid changes are accompanied by plantspecific regulatory mechanisms<sup>1,7</sup>. Whereas AMPK and SNF1 are allosterically activated by a decreasing adenylate charge, with AMP and ADP competing with ATP for binding to the  $\gamma$ -subunit four-CBS (cystathionine  $\beta$ -synthetase) domain, the plant kinases apparently are not<sup>8</sup>. Phosphorylation of a conserved threonine residue in the T-loop of the catalytic domain is a general prerequisite for AMPK/SNF1/SnRK1 kinase activity, but, although upstream SnRK1 activating kinases (SnAK1/2) have been identified, SnRK1 also significantly autophosphorylates<sup>10</sup>. Consistently, overexpression of the catalytic subunit is sufficient to confer high and specific SnRK1 activity in plants<sup>10</sup>. This suggest that, rather than being activated upon energy deficit, SnRK1 is repressed under conditions of energy abundance, in line with its inhibition by sugar-phosphates, such as trehalose-6-phosphate (T6P)<sup>11,12</sup>.

In comparison with the yeast and mammalian systems, detailed mechanistic insight into the structure of the plant SnRK1 kinases, their upstream regulation, and the downstream processes they target is only starting to emerge<sup>1,2,7</sup>. So far, SnRK1-interacting proteins have been identified mainly through small-scale yeast two-hybrid (Y2H) screening<sup>13</sup>, often awaiting further biochemical and *in planta* confirmation. Nonetheless, these and more dedicated studies support the conserved role of SnRK1 as a central regulatory hub that acts primarily through

transcriptional reprograming and direct modulation of key metabolic enzyme activities<sup>1,13</sup>. These findings were reinforced in two phosphoproteomics screens, identifying SnRK1dependent phosphoproteins during energy deprivation triggered either by extended night treatment<sup>14</sup> or hypoxia<sup>15</sup>.

By comparing phosphoproteomics data from sucrose-starved and sucrose-replenished Arabidopsis cell cultures, we inferred a diverse set of candidate SnRK1 substrates at the heart of primary metabolism, further extending the signaling network downstream of SnRK1. In parallel, we combined affinity purification, proximity labeling, and cross-linking mass spectrometry to explore the SnRK1 protein interaction landscape in a proteome-wide setting, uncovering novel substrates as well as regulatory proteins. Integration of these analyses provides novel insight in the stoichiometry and structure of the core SnRK1 complex and reveals a remarkably tight connection with the enigmatic Class II T6P synthase (TPS)-like (TPS II) proteins. Through in vitro kinase and transient luciferase assays, we show that the TPS II proteins repress SnRK1 kinase activity and nuclear SnRK1 signaling. Moreover, transient colocalization analyses reveal that the TPS II proteins enhance the subcellular localization of the SnRK1α1 catalytic subunit at the endoplasmic reticulum (ER) in young tobacco leaves. Similar to the TPS II proteins, also the land plant-specific FLZ (FCS-like zinc finger) SnRK1 scaffold or adaptor protein family<sup>16</sup> and homologues of the rice SKIN (SnRK1A-Interacting Negative Regulator) protein<sup>17</sup> were co-purified abundantly with multiple SnRK1 subunits. These intricate interactions illustrate that plants have evolved a complex toolbox of regulatory proteins to finetune SnRK1 activity. Overall, the obtained SnRK1 signaling network significantly advances the current knowledge of SnRK1 functioning and provides an excellent base to further explore how SnRK1 senses internal and external cues and translates this information into the control of plant growth and survival.

#### RESULTS

## Sucrose triggers rapid dephosphorylation of SnRK1 targets

Previously, we mapped phosphoproteins regulated by the Target Of Rapamycin (TOR) kinase, which mostly functions antagonistically to SnRK1<sup>3</sup>. These targets were identified in a dynamic phosphoproteomics screen, analyzing phosphorylation events affected by specific TOR inhibitors in Arabidopsis cell cultures upon sucrose synchronization (Fig. 1a)<sup>18</sup>. Further examination of the sucrose-dependency of these phosphoproteomics data shows that the phosphorylation states of the group C bZIP transcription factor bZIP63 and the nitrate reductase NR2, two well-established SnRK1 substrates<sup>14,19</sup>, rapidly decrease upon sucrose addition to sucrose-starved cells, independently of TOR (Fig. 1b). A similar phosphorylation profile was found for bZIP25, another group C bZIP member that has been linked to SnRK1<sup>20</sup>. These observations prompted us to search for proteins with a similar phosphorylation profile

as this dataset might be enriched with SnRK1 substrates. In total, 137 phosphosites on 109 proteins showed significant sucrose responsiveness (Supplementary Table 1a). In contrast, no significant differences could be observed for these proteins at their accumulation level in a parallel proteome analysis (Extended Data Fig. 1a and Supplementary Table 1b). Hierarchical clustering of the sucrose-dependent phosphorylation profiles revealed two main clusters in which phosphorylation was either up- (cluster I, 49 phosphosites) or down-regulated (cluster II, 88 phosphosites) upon sucrose addition to the sucrose-starved cultures (Fig. 1c and Extended Data Fig. 1b). As expected, cluster I possesses several TOR-dependent phosphoproteins such as the ribosomal protein RPS6b, which is indirectly regulated by TOR through the S6K kinase, or the SUPPRESSOR OF GENE SILENCING3<sup>18</sup>. However, most phosphorylation events in both clusters showed a TOR-independent sucrose response (82% of cluster I and 89% of cluster II).

When analyzing the conservation of the sucrose-dependent phosphorylation sites in plants, the evolutionary constrains on the sucrose down-regulated sites were more pronounced than those on the sucrose up-regulated or TOR-dependent sites<sup>18</sup>, highlighting their functional importance (Extended Data Fig. 1c and Supplementary Table 1c). De novo motif enrichment analysis (MEME)<sup>21</sup> of the phosphosites that are down-regulated upon sucrose replenishment identified a motif that matches the known SnRK1 consensus phosphorylation motif (Fig. 1c), which starts and ends with a hydrophobic residue (M, L, V, I, or F) at positions P-5 and P+4 and has a basic residue at position P-3 or P-4 relative to the phosphorylated serine<sup>1</sup>. Based on the MEME motif analysis and a second, independent motif analysis in Motif-X<sup>22</sup> (Supplementary Table 1d), we found that 30% of the phosphosites in cluster II perfectly match the known SnRK1 consensus motif. When loosening the requirement of the hydrophobic residues to only one of the two positions (P-5 or P+4), we found that 51% of the phosphosites closely resemble the SnRK1 motif (Fig. 1d and Supplementary Table 1d). Nevertheless, this enrichment should be interpreted carefully, as this motif is similar to the phosphorylation motif of calcium dependent protein kinases (CDPKs)<sup>1,14</sup>. In that regard, it has been shown that a proline residue at P-4 selectively inhibits phosphorylation by CDPKs relative to SnRK1, highlighting proteins such as the SnRK1 transcriptional marker SEN5<sup>23</sup> (and its homologs AT3G22850.1, AT4G27450.1 and AT5G43830.1), the CO<sub>2</sub> Responsive CCT protein AtCRCT1, whose homologue positively regulates starch synthesis in rice<sup>24</sup>, and AtBRCC36A, a protein involved in homologous recombination<sup>25</sup>, as highly plausible SnRK1 substrates. In support of the proposed enrichment of SnRK1 substrates in cluster II, we also observed a strong preference for valine or serine at P-2 and for aspartic acid or asparagine at P+3, which perfectly fits with the human AMPK consensus motif<sup>26</sup>.

To further substantiate that cluster II is enriched for true SnRK1 targets, we next investigated the overlap with known SnRK1-dependent phosphoproteins<sup>14,15</sup> (Fig. 1d and Supplementary

Table 1a), uncovering significant overlap with SnRK1-regulated phosphoproteins identified either during prolonged darkness (p < 2.72e-15) or in hypoxic conditions (p < 3.03e-13), directly relating 32% of cluster II with SnRK1. Moreover, multiple proteins of cluster II (Fig. 1e) have been related to SnRK1 in other studies (see Supplementary Note), such as i) the cytosolic invertase CINV1, which mediates breakdown of sucrose into glucose and fructose and directly interacts with SnRK1 $\beta\gamma$  in Y2H<sup>27</sup>, ii) three homologs (AtO3L4/KCP, AtO3L1 & AtOXS3) of the SKIN protein, which negatively regulates SnRK1 in rice<sup>17</sup>, or iii) AtVPS9a and AT5G52580.1, homologs of human AMPK substrates that function in intracellular trafficking<sup>28,29</sup>.

Despite the clear enrichment of the SnRK1 consensus motif and the overlap with known SnRK1 targets or proteins related to SnRK1 signaling, our phosphoproteomics screen only indirectly infers putative SnRK1 targets. To further support that cluster II is enriched for SnRK1 substrates, we performed in vitro kinase assays testing phosphorylation of seven novel substrates by the catalytic SnRK1a1 subunit (Fig. 1f). As negative control, kinase assays were performed without substrates or with a catalytically inactive SnRK1a1 Lys48 >Met (K48M) mutant protein<sup>1</sup> (Extended Data Fig. 2). For four out of the seven tested substrates, a strong phosphorylation signal was detected on a 1-h-exposed autoradiogram. Weaker, but significant phosphorylation was detected for the remaining three substrates on a 6-h-exposed autoradiogram, while the 'no substrate' control only showed the expected SnRK1 $\alpha$ 1 autophosphorylation. The observed differences in phosphorylation levels might be provoked by a varying number of phosphorylation sites or differential recognition by SnRK1, enabling downstream effectors to respond differentially to changes in SnRK1 activity. Taken together, there is ample evidence that the proteins in cluster II (see Supplementary Note) are enriched for SnRK1 substrates. The presence of multiple transcription factors and a diverse set of metabolic enzymes corroborate the known downstream functions of SnRK1 in transcriptional and metabolic reprogramming. In addition, an intimate crosstalk was found with proteins functioning in intracellular trafficking, consistent with the emerging picture in mammalia where AMPK controls intracellular trafficking and organelle dynamics during metabolic stress<sup>30,31</sup>.

## Mapping the SnRK1 interactome by AP-MS and proximity labeling

To complement our phosphoproteomics analysis in search for SnRK1 substrates, we performed a dual, proteome-wide interactome screen in Arabidopsis, assessing protein interactions by affinity purification coupled to mass spectrometry (AP-MS)<sup>18</sup> and by TurboID-based proximity labeling (PL) (Fig. 2a). While SnRK1 substrates should appear at the intersection of the phosphoproteome and interactome, the interactome analyses will extend the SnRK1 signaling network towards upstream regulators. To ensure sufficient sensitivity, multiple representative SnRK1 subunits were used as bait with both methods, and baits were fused N- and/or C-terminally to the GS<sup>rhino</sup> tag for AP-MS<sup>18</sup> or to the TurboID tag for PL<sup>32</sup>. To

obtain a comprehensive view of the SnRK1 signaling network at the core of primary cell metabolism, all transgene fusions were constitutively expressed in Arabidopsis cell cultures and biomass was sampled under growth-promoting conditions (i.e. three days after subculturing in nutrient-rich medium) or during starvation induced by sucrose removal<sup>18</sup>. For AP-MS, SnRK1 protein complexes were also isolated from starved Arabidopsis seedlings (3 h extended night) using the unique SnRK1 $\beta\gamma$  subunit as bait, providing a complementary source for protein complex analysis. In the AP-MS experiments, proteins were extracted under near-physiological conditions to prevent complex disassembly<sup>33</sup>. Moreover, in several experiments, we applied *in vitro* cross-linking with dithiobis(succinimidyl propionate) (DSP) during extraction to stabilize weak protein-protein interactions (PPIs)<sup>18</sup>. In total, we performed 18 different AP-MS experiments following the protocol used to build the plant TOR interactome<sup>18</sup>. Briefly, SnRK1 protein complexes were purified based on the high-affinity interaction between the Protein G moiety of the GS<sup>rhino</sup> tag and immunoglobulins covalently coupled to magnetic beads, and purified proteins were analyzed by LC-MS/MS. To identify specific interactors, normalized spectral abundance factors (NSAF) were calculated for each identified protein, averaged over the different replicates, and quantitatively compared to a representative large in-house control dataset covering 379 equivalent AP-MS experiments with 76 baits functionally not related to SnRK1. After removal of non-specific proteins, an AP-MS network was obtained with 245 PPIs among 132 proteins, involving 44 interactors found with multiple baits and 88 bait-specific interactors (Supplementary Table 2). In parallel, SnRK1 interactors were assessed in seven PL experiments on sucrose-starved cell cultures following the TurbolD-based protocol that we recently established in plants cells<sup>32</sup>. During PL, proximal SnRK1 interactors were in vivo biotinylated and enriched under denaturing conditions using streptavidin beads. After LC-MS/MS, non-specific PL proteins were removed by a dual filtering strategy, integrating a NSAF-based large dataset approach and MaxQuant label-free quantification<sup>34</sup>, resulting into a PL network of 152 PPIs among 120 proteins (Supplementary Table 3). Finally, the AP-MS and PL networks were combined, giving rise to a SnRK1 interactome of 361 PPIs among 233 proteins (Supplementary Table 4).

## Quality and biological significance of the SnRK1 interactome

For detailed exploration of the interactome data, we selected all known and novel highconfidence PPIs and presented them in a quantitative dot plot matrix (Fig. 2b and Extended Data Fig. 3). Moreover, we visualized the whole integrated dataset as a PPI network generated in Cytoscape (Fig. 3). When analyzing the overlap between both interactome datasets, only 19 proteins were found in common (Fig. 2a), supporting earlier findings that AP-MS and PL are complementary methods that each survey a specific subspace of the interactome<sup>35</sup>. Nonetheless, literature and database screening confirmed that both subnetworks harbor many known SnRK1 interactors or proteins related to SnRK1 signaling (42 out of 233 interactors) (Fig. 2b and Supplementary Table 4), highlighting the quality of both subnetworks and the novelty of the interactome with 191 proteins not linked to SnRK1 before. Next to interactions among the different subunits of the core SnRK1 complex (see below), several known SnRK1associated proteins were biochemically validated through one or both methods. First of all, we found remarkably stable and intricate interactions with the Class II T6P synthase-like proteins TPS5-11, which co-purified with all tested SnRK1 subunits<sup>36</sup>. Although several TPS II proteins are phosphorylated by SnRK1<sup>14,37</sup>, the detected interaction strength seems to exceed that of mere kinase-substrate interactions, suggesting a different type of relation. Phylogenetically, the TPS II proteins form a separate clade in the trehalose metabolism protein family<sup>36</sup>. Despite that they contain both a T6P synthase (TPS) and T6P phosphatase (TPP) domain, they have no apparent enzymatic activity<sup>38</sup>. Transcriptionally, the TPS II proteins are extensively regulated, showing differential tissue-specific expression and responsiveness to carbon availability and hormones<sup>38</sup>. This suggests an important regulatory role for the TPS II proteins in plant development and growth, but so far, their function remains largely elusive<sup>6</sup>. A similar tight interaction profile was found with multiple members of the FLZ SnRK1 scaffold/adaptor protein family<sup>16</sup>, as well as with homologs of the rice SKIN protein<sup>17</sup>. Notably, three members of the SKIN family (AtOXS3, AtO3L4/KCP, AtO3L1) were also present in cluster II of our phosphoproteomics screen, supporting an earlier report that identified these proteins as SnRK1 substrates<sup>39</sup>. However, in analogy with the TPS II proteins, also here the observed interaction strength suggests a different type of relation, consistent with the function of the rice SKIN protein as negative regulator of SnRK1. Next to the three SKIN-related proteins, our interactome screen uncovered only four other proteins whose phosphorylation was downregulated upon sucrose resupply, pinpointing them as highly plausible SnRK1 substrates (Fig. 2). These four proteins comprise i) the cytosolic invertase CINV1, ii) the acetyl-CoA synthetase ACS, which is involved in the recovery of carbon during hypoxia<sup>40</sup>, iii) P5CS2, a rate-limiting enzyme of proline biosynthesis, and iv) ASG1 (Altered Seed Germination 1), a poorly characterized plant-specific protein that has been linked to seed germination<sup>41</sup>. Likewise, the interactome has only few proteins in common with the SnRK1-dependent phosphoproteins identified under extended night<sup>14</sup> (8 proteins) or hypoxic<sup>15</sup> conditions (4 proteins) (Supplementary Table 4c). This limited overlap between the interactome and phosphoproteome datasets clearly illustrates the challenge to identify kinase-substrate interactions by interactomics, in agreement with prior observations made during mapping of the plant TOR signaling network<sup>18</sup>.

Next to the TPS II, FLZ and SKIN-related proteins, the interactome harbors multiple other proteins that have been linked to SnRK1 signaling, including i) the heterotrimeric TOR kinase complex, which is negatively regulated by SnRK1 through phosphorylation of the regulatory

subunit RAPTOR1B<sup>14</sup>, ii) the HSPRO1/2 proteins, known interactors of SnRK1βγ that are implicated in biotic stress<sup>42</sup>, iii) the AGC kinase AGC1-3, an ortholog of the tomato cell death suppressor Adi3 which interacts with the SnRK1 α-subunit and phosphorylates the β-subunit GAL83<sup>43</sup>, iv) STARCH-EXCESS4 (SEX4), a dual-specificity protein tyrosine phosphatase that interacts with SnRK1α2 and plays a role in starch degradation<sup>44</sup>, v) TIME FOR COFFEE (TIC), a circadian clock protein for which a genetic interaction has been reported with SnRK1α1<sup>45</sup>, vi) Acetyl-CoA carboxylase 1 (ACC1), a known substrate of yeast SNF1 and mammalian AMPK<sup>1</sup>, vii) the translation initiation factor eIFiso4E, which is phosphorylated by SnRK1 to inhibit protein translation<sup>46</sup>, viii) the autophagy-related protein ATG6, which is phosphorylated by SnRK1 providing an alternative route to activate autophagy during prolonged carbon starvation<sup>13</sup>, and ix) three proteins that were identified as SnRK1 interactors in a large scale Y2H screen<sup>27</sup> (CINV1, the lectin EULS3 and the trihelix transcription factor ASIL2).

Furthermore, we also mapped a diverse and highly reliable set of novel interactions, identified either by different methods and/or in multiple conditions (Fig. 2b). These include i) the three sucrose-dependent phosphoproteins mentioned before (ACS, ASG1 & P5CS2), ii) four poorly characterized proteins found both through AP-MS and PL, iii) 18 interactors specific for one of both methods that were confirmed with multiple SnRK1 bait proteins, such as KUODA1 (KUA1), a MYB transcription factor involved in dark-induced leaf senescence and cell expansion<sup>47,48</sup>, and iv) 31 bait-specific interactors found specifically with AP-MS or PL but that were isolated under multiple experimental conditions (Extended Data Fig. 3). For example, the electron transfer flavoproteins ETFA and ETFB were co-purified with SnRK1<sub>β</sub>3 under multiple conditions, establishing a direct relation between SnRK1 signaling and alternative mitochondrial energy metabolism, two pathways that were recently linked more indirectly<sup>49</sup>. The remainder of the interactome represents bait- and condition-specific interactors, including for instance seedling-specific interactors that are absent in cell cultures or more weak or transient interactors, which are typically more challenging to identify. For instance, through AP-MS with SnRK1 $\beta$ 2 as bait, we specifically isolated three subunits of the WAVE complex (SRA1, GRL and ABIL3) from sucrose-starved cells. The latter interactions might provide insight into how the WAVE complex is stimulated in the dark when it helps to restructure actin filaments during stomatal closure<sup>50</sup>, or into its functioning during autophagy<sup>51</sup>. However, caution is needed when interpreting dynamic interactions found specifically under starved or non-starved conditions, as reliable quantitative comparison of conditions would for instance require chemical labeling with isobaric mass tags. Moreover, many interactors were retrieved from both conditions, possibly reflecting a certain degree of basal SnRK1 activity in non-starved cells inherent to the cell culture system. Finally, extra confidence can be attributed to the 35 proteins whose transcripts are highly co-expressed<sup>52</sup> with one or more of the SnRK1 subunit transcripts (Figure 3 and Supplementary Table 5).

## Charting the composition and stoichiometry of the SnRK1 complex

When zooming in onto the composition of the core SnRK1 complex, the interactome screen confirmed earlier reports that the more prototypical y-like protein AT3G48530, previously unduly called SnRK1 $\gamma$ 1, does not behave as a functional  $\gamma$ -subunit<sup>8</sup> as it was not isolated with any of the SnRK1 subunits. This was further validated through tandem affinity purification (TAP) using SnRK1γ1 as bait (Fig. 3 and Supplementary Table 1). Based on the observed interactions with heavy chain myosin XI proteins (MYA1, MYA2, XIG, XIK) and calmodulin-like proteins (CML13 and CML14), which are homologous to yeast and mammalian myosin light chains, we postulate that AT3G48530 functions in intracellular trafficking<sup>53</sup>. To further investigate the composition and stoichiometry of the core SnRK1 complex, we determined the relative abundance of each SnRK1 subunit in the AP-MS experiments through label-free quantification, taking into account the intensity-based absolute quantification (iBAQ) ratios of each subunit over the whole complex (Fig. 4a, Extended Data Fig. 4 and Supplementary Table 6)<sup>54</sup>. Overall, low standard deviations were observed for the different replicates, highlighting the reproducibility of the AP-MS procedure. Moreover, with SnRK1 $\beta\gamma$  and the three  $\beta$ -subunits as bait, similar levels were found for each subunit when comparing the different experimental conditions. The stoichiometry analyses with the three  $\beta$ -subunits as bait illustrate that the  $\beta$ subunits are almost completely mutually exclusive, consistent with their differential subcellular localizations and the formation of a heterotrimeric SnRK1 complex<sup>55-57</sup>. When the relative abundances of the different subunit isoforms were added up, the stoichiometries with SnRK1 $\beta\gamma$ and SnRK1ß1 as bait approximate the expected heterotrimeric 1:1:1 nature of the SnRK1 complex. With the other two  $\beta$ -subunits as bait, suprastoichiometric levels were detected for the bait itself, likely reflecting overexpression of the bait. Nonetheless, for the fraction of the SnRK1 $\beta$ 2 and SnRK1 $\beta$ 3 bait that is complexed, similar levels of  $\alpha$ - and  $\beta\gamma$ -subunits were found, in support of the proposed heterotrimeric 1:1:1 stoichiometry. Finally, using SnRK1a1 as bait, major differences were detected depending on the orientation of the tag, most likely indicating interference of the tag with proper complex assembly. Notably, with SnRK1a1 as bait, we found interaction with the other catalytic subunit SnRK1 $\alpha$ 2. This interaction might reflect the existence of higher-order heterotrimeric protein complexes in which both catalytic subunits are present, as has been reported before for SNF1 and AMPK<sup>1</sup>.

#### Structural modeling reveals plant-specific features of the SnRK1 complex

To gain deeper insight into the structure of the SnRK1 complex and how the different SnRK1 subunits and their respective domains are positioned towards each other, we next implemented an integrative structural approach <sup>58</sup>. Hereto, we first pinpointed peptides that are in close proximity in the SnRK1 complex through cross-linking MS (XL-MS), following the protocol that we recently applied on the endocytic TPLATE complex<sup>59</sup> (Fig. 4b). The unique SnRK1<sub>By</sub> subunit was selected as bait for triplicate XL-MS experiments because it enabled isolation of the SnRK1 complex in a 1:1:1 stoichiometry, covering all six SnRK1 subunits. Complexes were isolated at high purity by TAP from cell cultures expressing GS<sup>rhino</sup>-SnRK1<sub>βγ</sub>, cross-linked with bissulfonsuccinimidyl suberate (BS<sup>3</sup>), and on-bead digested for LC-MS/MS analysis. After identification of cross-linked peptides using the pLINK 2 search engine<sup>60</sup> and further filtering (E-values <0.05), 2512 cross-linked spectra were identified covering 121 interand 123 intra-protein cross-linked peptides (Supplementary Table 7a-b). The XL-MS method was highly reproducible as a similar number of cross-linked spectra (837±27) was detected in each replicate and 93% of the cross-linked spectra involved cross-linked peptides found in all three replicates. Notably, 71% of the cross-linked spectra mapped exclusively to SnRK1 subunits. The remaining cross-linked spectra mainly derived from TPS II (19%) or SKIN-related (10%) proteins, demonstrating their direct association with SnRK1.

For further robust elucidation of the architecture of the core SnRK1 complex using the integrative modeling approach, we considered only SnRK1 specific cross-links identified in all three replicates (Fig. 4b and Supplementary Table 7c). Based on the stoichiometry and XL-MS analyses, we focused on two possible heterotrimers, one composed of SnRK1 $\alpha$ 1, SnRK1 $\beta$ 2 and SnRK1 $\beta$  $\gamma$ , the other of SnRK1 $\alpha$ 2, SnRK1 $\beta$ 2 and SnRK1 $\beta$  $\gamma$ , because these two heterotrimers produced the most cross-links between individual subunits. Input information to calculate a structure for the SnRK1 heterotrimers included chemical cross-links, complex stoichiometry and protein structures obtained from the AlphaFold database<sup>61</sup>. Protein structures (highly confident regions in the Alphafold database, in total covering 78 % of the SnRK1 heterotrimers) were represented as rigid bodies (Extended Data Fig. 5). In contrast, linker regions (unstructured low confidence structures) were represented as flexible beads of different sizes ranging from 1 to 20 amino acid residues per bead. After randomization of the position of all subunits, the Metropolis Monte Carlo algorithm was used to search for structures satisfying the input restraints. In the case of the SnRK1 $\alpha$ 1 $\beta$ 2 $\beta\gamma$  heterotrimer, an ensemble was obtained containing 9,026 models satisfying excluded volume restraints, sequence connectivity and at least 89% of cross-links (good-scoring models) (Extended Data Fig. 6). For the heterotrimer with SnRK1a2, an ensemble of 15,016 good-scoring models satisfied excluded volume restraints, sequence connectivity and at least 93% of cross-links (Extended Data Fig. 7). The ensembles of both heterotrimers passed all statistical tests provided by Viswanath et al.<sup>62</sup> (Extended Data Fig. 6 & 7) and the precision of the generated models was 23 Å for the SnRK1 $\alpha$ 1 $\beta$ 2 $\beta\gamma$  heterotrimer and 20 Å for the SnRK1 $\alpha$ 2 $\beta$ 2 $\beta\gamma$  heterotrimer. The precision was defined by the root mean square fluctuation of the dominant cluster containing 98% or 82% of all good scoring models, respectively. The resulting structures of both heterotrimers were highly similar (Fig. 4c-e and Extended Data Fig. 7), further corroborating convergence of modeling. Comparison of the obtained models with a structure of the evolutionarily related AMPK complex<sup>63</sup> revealed overall similar interactions between individual subunits (Extended Data Fig. 8a). The kinase domain (KD) of SnRK1a1/2 interacts with the CBM of SnRK1 $\beta$ 2, and the C-terminal domain of SnRK1 $\alpha$ 1/2 ( $\alpha$ -CTD) interacts with the Cterminal domain of SnRK1 $\beta$ 2 ( $\beta$ -CTD). Both  $\alpha$ -CTD and  $\beta$ -CTD are then in close contact with the structurally conserved four-CBS domain of SnRK1<sub>β</sub>γ. Notably, also differences were found compared to the AMPK structure, because in the plant heterotrimers the SnRK1 $\alpha$ 1/2 KD and the SnRK1 $\beta$ 2 CBM were also in close proximity of the SnRK1 $\beta\gamma$  four-CBS domain. Moreover, closer inspection of the interaction between the SnRK1 $\beta\gamma$  four-CBS domain, SnRK1 $\alpha$ 1/2 and SnRK1<sub>β</sub>2 indicate that, different from AMPK, CBS3 and CBS4 mediate contacts with the other subunits. Although there is no direct cross-link between the CBM of SnRK1 $\beta\gamma$  and the other subunits, this domain is located on the same side of the complex as the SnRK1 $\alpha$ 1/2 KD and the SnRK1<sub>B</sub>2 CBM, in agreement with the previously suggested homology model of the plant SnRK1 complex<sup>1</sup>. For both heterotrimers, two intramolecular cross-links located in the four-CBS domain of SnRK1 $\beta\gamma$  are inconsistent with the generated model of the plant SnRK1 complex (Fig. 4f-g, Extended Data Fig. 7). The representation of the four-CBS domain as a single rigid body likely causes this inconsistency as it does not allow intramolecular flexibility of the domain. Two intermolecular cross-links are violated in the structure of the SnRK1 complex comprised of SnRK1 $\alpha$ 1, SnRK1 $\beta$ 2 and SnRK1 $\beta\gamma$ , particularly between subunits SnRK1 $\alpha$ 1 and SnRK1 $\beta\gamma$  (Fig. 4g). This inconsistency might be caused by the rigid-body description of interacting domains or by the existence of higher-order oligomers of the SnRK1 complex. In support of such higher-order oligomers, one of the cross-linked peptides that connects the SnRK1 $\alpha$ 1 and SnRK1 $\alpha$ 2 subunits lies within the amphipathic  $\alpha$ G-helix (Extended Data Fig. 8b), which is known to mediate oligomerization of AMPK and dimerization of SNF1 complexes through hydrophobic interactions<sup>64</sup>.

## Class II TPS-like proteins function as negative regulators of SnRK1

At the intersection of the AP-MS, PL and XL-MS screens, the TPS II proteins clearly emerged as direct, stable SnRK1 interactors that are intricately connected to the heterotrimeric core SnRK1 complex. In the AP-MS analysis, all seven TPS II proteins (TPS5-TPS11) were identified from cell cultures, of which six members were confirmed by AP-MS in seedlings or by PL, illustrating that these interactions occur in vivo (Fig. 2b). This tight connection was further strengthened in the XL-MS analysis, which revealed direct interaction of TPS7 and TPS11 with both catalytic SnRK1  $\alpha$ -subunits (Supplementary Table 7d). Because of their enigmatic function, we selected the TPS II proteins for further functional analysis. First, we performed reciprocal AP-MS experiments using several TPS II proteins as bait (Fig. 5a and Supplementary Table 2), not only confirming the stable interaction with the SnRK1 complex as witnessed by the co-purification of all SnRK1 subunits, but also revealing that the different TPS II proteins interact with each other. In addition, the reciprocal AP-MS experiments link the TPS II with the FLZ proteins<sup>16</sup>, as three FLZ proteins were co-purified with TPS7 as bait. Other robust interactors included the flowering time regulator FVE and the thioredoxin protein ACHT2, which were isolated with both TPS5 and TPS7 as bait. Furthermore, TPS7 also copurified the UDP-glucose transporter UTR3, which is required for import of the T6P precursor UDP-glucose into the ER<sup>65</sup>, whereas in mammals, its ortholog SLC35B1 functions as part of an ER-to-cytosol low energy response regulatory axis linked to AMPK<sup>66</sup>. Notably, also the TPS7 interactor FLZ10, which regulates stability of the SnRK1 catalytic α-subunits, locates at the ER<sup>67</sup>, suggesting that the TPS II proteins might function at the ER.

The direct interaction observed between the TPS II proteins and the catalytic SnRK1 subunits in the XL-MS analysis incited us to test if they could directly regulate the catalytic kinase activity of SnRK1. To this end, we implemented a non-radioactive kinase assay that was recently developed to evaluate SnRK1 activity<sup>68-70</sup>. For this assay (Fig. 5b), we recombinantly produced a tagged SnRK1 activity reporter peptide that harbors a tandem repeat of the rat Acetyl-CoA Carboxylase 1 (ACC1) AMPK recognition motif. As a readout for SnRK1 kinase activity, we used a commercial antibody that recognizes the phosphorylated Ser79 of rat ACC1. Upon incubation of the ACC reporter with recombinant SnRK1a1, clear phosphorylation could be detected through immunoblotting with the phospho-specific anti-ACC1 antibody, whereas no signal was found in the different negative controls where i) SnRK1 $\alpha$ 1 was omitted, ii) the two residues corresponding to rat Ser79 were mutated to alanine (S79A), or iii) the kinase-dead SnRK1α1 K48M protein was used (Fig. 5b). Remarkably, addition of either of the five tested TPS II proteins strongly reduced SnRK1 kinase activity in vitro (Fig. 5c). Importantly, this effect was highly specific for the Class II TPS-like proteins as no significant reduction in phosphorylation of the ACC reporter was observed when SnRK1a1 was combined with the Class I T6P-synthase TPS1 or with the T6P phosphatase TPP-A (Fig. 5d). These in vitro results indicate that the TPS II proteins function as SnRK1 repressors. To analyze if the TPS II proteins also repress SnRK1 activity in vivo, we next investigated their effect on SnRK1 activity in a cellular luciferase reporter assay in which we probed promoter activity of the

SnRK1 transcriptional target genes *SEN5* and *DIN6/ASN1*<sup>10</sup> (Fig. 5e). Consistent with earlier results obtained in Arabidopsis mesophyll protoplasts<sup>10</sup>, both promoters were clearly activated in tobacco BY2 protoplasts upon co-transfection of SnRK1 $\alpha$ 1 with its upstream activating kinase SnAK1. In line with the observed repression of SnRK1 $\alpha$ 1 kinase activity in the *in vitro* kinase assays, co-transfection with (untagged) TPS5 or TPS7 significantly diminished the activation of either promoter, pinpointing the TPS II proteins as repressors of nuclear SnRK1 signaling.

To obtain deeper insight on the functioning of the TPS II proteins, we finally analyzed their subcellular localization and possible co-localization with SnRK1 in the plant cell. An earlier report showed that TPS5 localized at the ER<sup>71</sup>, which is consistent with the localization of some of the interactors found by AP-MS using TPS7 as bait. To obtain a broader view of the subcellular localization of the Class II TPS-like family, TPS5, TPS8 and TPS9 were fused to the mCherry tag, transiently expressed in Nicotiana benthamiana (tobacco) leaves from the 35S promoter and analyzed by confocal microscopy. Co-localization analysis with the ER-GFP marker protein CD3-955 (WAK2-GFP-HDEL)<sup>72</sup> demonstrated that, next to TPS5, also the other tested TPS II members localize at the ER, as witnessed by the intense ring-shaped fluorescent structures around the nuclei, corresponding to the perinuclear ring, a distinctive hallmark of the ER (Fig. 5f). To shed more light onto the relationship between the TPS II proteins and SnRK1, we subsequently investigated their effect on the subcellular localization of SnRK1, which is known to be tightly controlled<sup>23,73-75</sup>. Based on the XL-MS and *in vitro* kinase analyses, we selected the catalytic SnRK1a1 subunit for co-localization analysis. Without TPS II protein co-transfection, SnRK1a1-GFP showed its expected dual localization pattern inside and outside of the nucleus (Fig. 5g), which is typically observed upon transient overexpression of the catalytic SnRK1 $\alpha$  subunits in leaf cells<sup>1</sup>. Conversely, when SnRK1 $\alpha$ 1-GFP was cotransfected with either of the three tested TPS II proteins, the nuclear GFP signal was significantly reduced, and SnRK1a1 strongly co-localized with the TPS II proteins in the perinuclear ring of the ER (Fig. 5g). Intriguingly, this effect was only observed when transfecting young leaves, whereas in mature leaves the TPS II proteins did not influence SnRK1 $\alpha$ 1-GFP localization (Fig. 5h). These results suggest that the TPS II proteins enhance SnRK1a1 localization at the ER in young tissues, thereby diminishing its nuclear localization. Jointly, these results thus offer a first glimpse on the role of Class II TPS-like proteins, which appear to function as negative regulators of nuclear SnRK1 signaling. Although the exact molecular mechanism how TPS II proteins regulate SnRK1 in planta remains elusive, our observations will guide follow-up research on how to unravel their precise mode-of-action.

#### DISCUSSION

In this work, we established a comprehensive view of the plant SnRK1 signaling network that functions at the heart of primary metabolism. By analyzing the sucrose-dependency of our phosphoproteome screen, we indirectly inferred a variety of novel candidate SnRK1 substrates. Although this screen was originally focused on the discovery of TOR substrates, we provide ample evidence that this dataset in parallel enabled the discovery of SnRK1 substrates. Nonetheless, as these were mapped through indirect means, further validation is required to confirm their SnRK1-dependent phosphorylation, especially when considering that other kinases such as the YAK1<sup>76</sup> or ATG1<sup>18</sup> kinases might cooperate with SnRK1 to ensure an optimal starvation response. As the YAK1 and ATG1 kinases are directly regulated by TOR, phosphorylation of their substrates will be TOR-dependent<sup>18,76</sup>, while most of the phosphoproteins in cluster II, including known SnRK1 substrates, respond in a TORindependent manner. The latter suggests that SnRK1 is not directly regulated by TOR in plants, in contrast to recent observations made in humans and yeast<sup>77</sup>. Moreover, the observed reduction in phosphorylation upon sucrose-replenishment occurs very rapidly, indicating a molecular switch in which sucrose triggers activation of phosphatases independently of TOR, supporting prior observations that phosphatases are required for induction of sucrosedependent signaling<sup>78</sup>. Intriguingly, our SnRK1 network harbors four poorly characterized PP2c phosphatases (PP2c21, PP2c40, PP2c60, PP2c76). In future research, it will be interesting to examine if these phosphatases control SnRK1 activity79, or if they are involved in the dephosphorylation of SnRK1 substrates upon sucrose-replenishment. In the latter scenario, these phosphatases might be phosphorylated by SnRK1 as a negative feedback mechanism. Altogether, our phosphoproteomics analysis complements earlier SnRK1 phosphoproteomics studies in plants, providing a deeper view of the downstream processes targeted by SnRK1 (see Supplementary Note). In addition to the well-established function of SnRK1 in transcriptional regulation and in modulation of metabolic enzyme activities, multiple novel candidate SnRK1 substrates indicate an important role for SnRK1 in intracellular trafficking, suggesting for instance that SnRK1 regulates Rab5-mediated endosomal trafficking to maintain carbon homeostasis<sup>80</sup>.

To complement this phosphoproteome screen and also obtain insight into upstream SnRK1 regulatory mechanisms, we next performed a multi-layered interactome screen. In the overlap of the phosphoproteome and interactome, seven proteins were highlighted as SnRK1 substrates. While the stable interactions with three SKIN-related proteins suggest a function for these proteins in SnRK1 regulation, the other four are transient and low abundance interactions, which are typically challenging to detect by AP-MS or PL. This explains the overall low overlap between the interactome and phosphoproteome, while underscoring the higher

sensitivity of phosphoproteomics in detecting kinase substrates. Next to these SnRK1 substrates, the interactome screen biochemically validated numerous known SnRK1 interactors and revealed a diverse array of novel SnRK1 protein interactors. Among these, there might be putative SnRK1 substrates present that were missed in this or prior phosphoproteomics analyses. For instance, the regulatory TOR subunit Raptor1B, a known substrate of SnRK1, was detected with multiple SnRK1 baits by AP-MS and PL, whereas phosphorylation of its predicted SnRK1 site was not found by phosphoproteomics, possibly because of the large size of the corresponding tryptic peptide<sup>14</sup>. In addition to Raptor1B, also TOR itself and LST8-1 were co-purified with SnRK1 by AP-MS, indicating that SnRK1 represses TOR activity through phosphorylation of Raptor1B when it resides inside the TOR complex. SnRK1 and TOR might not always act antagonistically as both kinases are also found to cooperate, for instance in stomatal development<sup>81</sup>. To obtain more insight into their complex interplay, it will be worthwhile to study proteins shared between the TOR and SnRK1 signaling networks. For example, based on our phosphoproteome data, TOR regulates β-amylase1 activity in guard cells through phosphorylation of Ser31<sup>82</sup>. We now also found two phosphosites that are repressed by sucrose (Ser55 & Ser59), implying that both TOR and SnRK1 are needed to adjust guard cell starch degradation in response to carbon availability.

When zooming in onto the core SnRK1 complex, the interactome screen provided an unprecedented view of its composition and stoichiometry, reinforcing its heterotrimeric nature and suggesting the existence of higher-order SnRK1 complexes. This information led us to perform an integrated structural analysis, revealing that SnRK1 acquired plant-specific structural modifications, in line with its plant-specific regulation. For example, structural differences in the interaction of SnRK1 $\alpha$ 1 and SnRK1 $\beta$ 2 with the SnRK1 $\beta\gamma$  four-CBS domain are consistent with the apparent absence of regulation by nucleotide charge in plants, whereas in mammals and yeast AMP and ADP compete with ATP for binding to the four-CBS domain<sup>1,8</sup>. A distinguishing feature of SnRK1 is that, rather than being activated by a reduced nucleotide charge, its default activity is repressed under conditions of carbon and energy abundance by sugar phosphates, such as glucose-6-phosphate, glucose-1-phosphate and T6P<sup>1,9,11</sup>. Especially T6P has emerged as an important signaling molecule, which, as a proxy for sucrose levels, controls plant growth and development<sup>6</sup>. Over the years, an increasing number of studies revealed that the SnRK1 and T6P signaling pathways have converged and that T6P functions at least in part through inhibition of SnRK1<sup>12,83</sup>. Biochemical assays suggested the involvement of an intermediate proteinaceous factor that is present only in young, actively dividing tissues<sup>11</sup>, but the precise mechanism remains elusive. While T6P was more recently reported to directly bind the catalytic α-subunits, interfering with upstream kinase interaction and T-loop phosphorylation<sup>84</sup>, additional players are likely required<sup>12</sup>. The TPS II proteins have

been postulated to mediate T6P-dependent SnRK1 regulation because they maintained key residues for T6P binding in their phosphatase-like domain and because they are transcriptionally and post-translationally regulated by SnRK1<sup>1,36</sup>. TPS II expression is not only regulated by carbon- and nutrient-availability, their differential cell or tissue type-dependent expression suggests diverse key functions during plant growth and development<sup>38</sup>. Consistently, genetic studies have linked TPS II proteins with plant development and stress responses<sup>12</sup>, however, as they are part of a multimember protein family with likely functional redundancy, further elucidation of their precise role will require the generation of higher-order mutants. Through our interactome analyses, we now discovered that the TPS II proteins are amongst the most stable interactors of SnRK1, suggesting a key regulatory function. Through in vitro kinase activity and cellular assays, we demonstrate that TPS II proteins regulate SnRK1 kinase activity, nuclear SnRK1 signaling and the subcellular localization of SnRK1. The TPS IIdependent dynamic localization of SnRK1a1 is in agreement with the known dynamic subcellular localization of SnRK1 $\alpha$ 1, which changes in function of the metabolic status of the cells<sup>23,74</sup>, its interaction with the SnRK1 regulatory  $\beta$ -subunits<sup>23</sup>, in response to abscisic acid<sup>75</sup>, or in relation to the developmental context<sup>73</sup>. Similar to the T6P-dependent repression of SnRK1, the enhanced ER-localization of SnRK1α1 occurs specifically in young and not in mature leaves. Therefore, it will be worthwhile to study whether the TPS II proteins mediate T6P regulation of SnRK1, possibly by direct binding of T6P. Moreover, as our results were mainly obtained *in vitro* or upon overexpression of TPS II proteins and SnRK1 $\alpha$ 1 in tobacco, more dedicated biochemical and in planta experiments are needed to confirm these results and further unravel the precise molecular mechanism(s) how TPS II proteins regulate SnRK1. Follow-up research should clarify i) if the TPS II proteins directly sequester SnRK1a1 at the ER upon interaction, thereby diminishing its nuclear localization, or if they indirectly regulate SnRK1a1 localization, ii) if the reduction in nuclear signaling observed in the luciferase assay is related to the enhanced ER localization, the direct inhibition of SnRK1 kinase activity, or both, iii) if, next to their role as repressors of nuclear SnRK1 signaling, the TPS II proteins also regulate cytosolic SnRK1 activity, and iv) how direct interaction between SnRK1α1 and TPS II proteins leads to inhibition of kinase activity at the structural level?

Despite that several TPS II members have been reported as SnRK1 substrates<sup>14,37</sup>, the detected interaction exceeded that of mere kinase-substrate interactions, supporting a different type of relation in which the phosphorylation of TPS II proteins by SnRK1 might be a feedback mechanism<sup>1</sup>. Also for other known SnRK1 regulatory proteins, putative feedback mechanisms have been reported, such as phosphorylation of the SnAKs upstream kinases by SnRK1<sup>85</sup> or the SnRK1-dependent transcriptional regulation of FLZ6 and FLZ10<sup>86</sup>. Notably, the SKIN-related proteins show similar characteristics, suggesting that they also function as SnRK1

regulators in Arabidopsis. This illustrates how plants apparently deploy a whole arsenal of regulatory proteins which are feedback regulated to tightly control and fine-tune SnRK1 activity. The functioning of SnRK1 as an extended complex with tightly bound regulatory proteins might compensate for the apparent loss of regulation by adenylate charge, a general readout of energy status in heterotrophic organisms, and enables the dynamic regulation by and integration of very diverse complementary environmental and developmental signals, thereby also preventing potentially detrimental persistent SnRK1 activity.

Finally, the SnRK1 network was mainly mapped in Arabidopsis cell cultures. It will therefore be important to also explore the spatiotemporal dynamics of the core SnRK1 complex and its interactions with regulatory proteins and substrates during development and in different environmental conditions. A more detailed investigation of these mechanisms and their biological relevance will further advance our understanding of how plants cope with metabolic stress during key developmental transitions and in a rapidly changing environment.

#### METHODS

## Molecular cloning

Oligo's used for plasmid construction are listed in Supplementary Table 8. All sequences of SnRK1 $\alpha$ 1, SnRK1 $\beta$ 2, SnRK1 $\beta$ 3, SnRK1 $\beta\gamma$ , SnRK1 $\gamma$ 1, TPS IIs, TPS1, bZIP16, SnAK1, as well as the promoters of DIN6 and SEN5 were PCR-amplified from cDNA or gDNA and cloned through Gateway BP reactions into pDONR221 (Invitrogen). Coding sequences of ACS10, SEN5, CRCT1, PP2c40, MTHFR2, and bZIP68 were synthesized flanked by Gateway attL1 and attL2 sites for cloning into pDONR221. The resulting Gateway entry vectors were sequence-verified and used for Gateway LR reactions to construct plant expression vectors encoding GS<sup>rhino</sup>, linkerTurboID-3xHA, GFP, or mCherry fusion proteins under control of the cauliflower mosaic virus 35S promoter, as described earlier<sup>18,32,33,87</sup>. To generate SnRK1<sub>β</sub>1 fusions, SnRK1<sub>β1</sub>, GS<sup>rhino</sup> and linkerTurbo-3xHA sequences were cloned into Greengate empty entry vectors<sup>88</sup> for further Golden Gate cloning with 35S promoter and 35S terminator entry vectors into pGGK-AG<sup>89</sup>. For the luciferase assay, 35S overexpression and promoter:LUC vectors were Gateway-cloned into p2GW7 and pGWL7, respectively<sup>90</sup>. Constructs for recombinant production in Escherichia coli BL21 were Gateway cloned into pDest-HisMBP<sup>18</sup>. For the HisMBP-TPP-A fusion, an entry vector was used encoding TPP-A fused to the 2xHA-tag<sup>91</sup>. To generate the synthetic *in vitro* SnRK1 kinase activity reporter, part of the coding sequence of rat acetyl-CoA carboxylase peptides (ACC1<sup>74-84</sup>, MRSSMSGLHLV) was tandemly repeated and fused with a GFP and a double HA-tag (Fig. 5e) and introduced in the pGEX-T4-1 (GST fusion) vector. All vectors for recombinant protein production were transformed in E. coli BL21 cells.

#### Analysis of the sucrose-dependent phosphoproteomics data

For statistical analysis of the sucrose-dependency of the phosphoproteomics data, Log<sub>2</sub>LFQ phosphopeptide intensities were extracted from Van Leene *et al.*<sup>18</sup> and analyzed in a similar manner as for the TOR-dependency<sup>18</sup>, now estimating the following contrasts with Wald tests: t10\_Ctrl vs t0\_Ctrl, t20\_Ctrl vs t0\_Ctrl and t40\_Ctrl vs t0\_Ctrl, with Ctrl being the samples where no AZD8055 or rapamycin was applied. t0 corresponds to the 24-h sucrose-starved samples and t10, t20 and t40 were sampled 10, 20 and 40 min after sucrose addition, respectively. For the proteome analysis, proteome samples corresponding to the t0\_Ctrl and t20\_Ctrl samples were analyzed as previously described<sup>18</sup>. For the phosphopeptide cluster analysis, the average estimated Log<sub>2</sub> LFQ phosphopeptide intensity fold changes were loaded into the multiple array viewer (<u>http://mev.tm4.org</u>) and hierarchical clustering was performed on both the phosphosite and sample tree with both leaf orders optimized using Euclidean

distance and average linkage clustering. For the *de novo* motif analysis, the sequence windows around the phosphorylated residues were analyzed with Motif-X<sup>22</sup> and MEME<sup>21</sup>, using standard settings. Evaluation of the phosphopeptide conservation was done as before<sup>18</sup>, with a sequence window of +6,-6 around the phosphorylated residue and taking into account following reference genomes: *Arabidopsis thaliana, Arabidopsis lyrata, Brassica rapa, Eucalyptus grandis, Glycine max, Populus trichocarpa, Vitis vinifera, Solanum lycopersicum, Oryza sativa* ssp. *Japonica, Amborella trichopoda,* and *Physcomitrella patens.* For analysis of the overlap with the SnRK1-dependent phosphoproteins regulated under submergence<sup>15</sup>, we considered the 38 phosphoproteins that were upregulated in Col-0 and not in the SnRK1 K48M mutant, and the 34 phosphoproteins that were downregulated in the SnRK1 K48M mutant but not in Col-0. For the overlap with the phosphoproteins identified under extended night<sup>14</sup>, we took into account the SnRK1-regulated phosphoproteins as reported by Jamsheer and colleagues<sup>13</sup>. To analyze the statistical significance of overlaps, a hypergeometric function was used.

## Arabidopsis transformation and biomass generation

All GS<sup>rhino</sup> and linkerTurboID-3xHA fusion constructs were transferred to *Agrobacterium tumefaciens* C58C1 Rif<sup>R</sup> (pMP90), transformed into the dark-growing Arabidopsis PSB-D cell culture, upscaled, and harvested three days after subculturing in our standard nutrient-rich conditions, or after 1-h, 6-h or 24-h sucrose starvation (see Fig. 1a for more details), as described<sup>18,32,33</sup>. For protein complex analysis in seedlings, the GS<sup>rhino</sup>-SnRK1βγ fusion was transformed into Arabidopsis Columbia wild-type background. Transgenic seedlings were grown on half strength Murashige and Skoog plates for 6 days at 21°C in a 12-h light/12-h dark regime and harvested after an extended night period of 3 h (15 h dark in total). For proximity labeling in cell cultures, 50  $\mu$ M biotin treatment was started directly at the initiation of the sucrose starvation, and biotin labeling was performed together with sucrose starvation for 6 h or 24 h at 28°C.

## Isolation of protein complexes by GS<sup>rhino</sup>-based affinity purification

Total protein extracts from transgenic cell cultures or from  $GS^{rhino}$ - $SnRK1\beta\gamma$  seedlings were prepared in our standard extraction buffer (25 mM Tris-HCI (pH 7.6), 15 mM MgCl<sub>2</sub>, 150 mM NaCl, 15 mM *p*-nitrophenyl phospate, 60 mM β-glycerophosphate, 0.1% NP-40, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 mM PMSF, 1 µM E64, EDTA-free Ultra Complete tablet (Roche), 5% ethylene glycol) as described<sup>18,33</sup>. Standard GS<sup>rhino</sup>-based affinity purification experiments were performed at least in triplicate, as reported before<sup>18</sup>. Briefly, protein complexes were trapped through the Protein G moiety of the GS<sup>rhino</sup> tag by incubating 25 mg total protein extract for 45 min with 50 µL in-house prepared magnetic IgG bead suspension. Beads were washed three times with 500  $\mu$ L extraction buffer, once with 500  $\mu$ L extraction buffer without detergent, and once with 800  $\mu$ L 50 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0). The wash buffer was removed and beads were incubated in 50 µL 50 mM NH<sub>4</sub>HCO<sub>3</sub> with 1 µg Trypsin/Lys-C (Promega) for 4h at 37°C. Next, the digest was separated from the beads and incubated overnight with 0.5 µg Trypsin/Lys-C at 37°C. Finally, the digest was centrifuged at 20800 rcf for 5 min, and supernatant was dried in a SpeedVac and stored at -20°C until MS analysis. For experiments with DSP cross-linking, Tris-HCl in the extraction buffer was replaced by 50 mM HEPES buffer (pH 7.5) and proteins were cross-linked for 45 min with 3 mM DSP (ThermoFisher Scientific Pierce, cat. no. 22585) during protein solubilization. Prior to centrifugation of protein extracts, non-reacted DSP was neutralized by addition of 1 mL 1 M Tris-HCI buffer (pH 7.5). For the N-terminal GS<sup>rhino</sup>-SnRK1β3 fusion, a high-molecular weight band was visible during protein expression analysis by immunoblotting, which disappeared upon protein extraction in the presence of 1 % (v/v) digitonin. Therefore, in Exp. 18 (see Fig. 1a), NP40 in the extraction buffer was replaced by 1% (v/v) digitonin during extraction and binding, and by 0.2% (v/v) digitonin during washing. For DSP and digitonin experiments, proteins were eluted by incubating washed IgG beads three times with 150 µL 0.2M glycine/HCI (pH2.5) at 4°C. The pooled eluate was neutralized with 100 µL (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>. Proteins were reduced for 30 min in 5 mM TCEP at 37°C, alkylated for 30 min in 10 mM iodoacetamide at room temperature, and overnight digested with 1 µg Trypsin/Lys-C at 37°C. Peptides were acidified to 1% (v/v) TFA, desalted on C18 Omix tips (Agilent, cat. no. A57003100), dried in a SpeedVac and stored at -20°C until MS analysis. TAP experiments on SnRK1y1 GS<sup>rhino</sup> expressing cell cultures were performed using 100 mg total protein input as reported before<sup>33</sup>.

# **TurboID-based PL**

Proximity labeling experiments were performed as described recently in detail for Arabidopsis plant cells<sup>32</sup>, using denaturing extraction buffer (100 mM Tris (pH 7.5), 2% (w/v) SDS, 8 M urea) and with the additional acid elution step following the Trypsin/Lys-C digest.

# Mass spectrometry analyses of the AP-MS and PL interactome data

Affinity purification and proximity labeling experiments were analyzed by LC-MS/MS on an LTQ Orbitrap VELOS or Q Exactive (Thermo Fisher Scientific), as previously reported<sup>18,32</sup>. Proteins were identified with either Mascot Distiller software (version 2.5.0.0; Matrix Science) combined with the Mascot search engine (versions 2.5.1 and 2.6.2; Matrix Science) using the Mascot Daemon interface (version 2.5.1) or with MaxQuant (version 1.6.10.43). To identify specific protein interactors from the resulting protein lists of the affinity-purified samples, a large dataset approach was followed. In this approach, NSAF values were calculated for each identified

protein and compared between the SnRK1 bait experiments and a representative control AP-MS dataset generated in-house by 379 AP-MS experiments on 76 baits that were not related to SnRK1 and that were functionally grouped in 29 different bait groups. Based on these NSAF values, a dual filtering strategy was applied, analyzing NSAF values with the KNIME Analytics Platform (versions 4.0.2 and 4.4.0). First, the dataset was screened in a qualitative manner, retaining proteins that were identified with at least two peptides in at least two out of three SnRK1 AP-MS replicates and in no more than one control bait group. In a second step, quantitative filtering was employed to find more promiscuous proteins that were however strongly and significantly enriched with the SnRK1 bait compared to the control dataset. Thereto, mean NSAF values were Ln-transformed and compared between the SnRK1 bait and the control dataset by a two-tailed *t*-test. Identifications were considered significantly enriched with the SnRK1 bait versus the control dataset, if they passed one of the following criteria: i) two-peptide identifications present in at least two out of three replicates are significantly enriched with a mean NSAF ratio  $\geq$  10 AND a -Log<sub>10</sub>(*p*-value)  $\geq$  10 or with a mean NSAF ratio  $\geq$  20 AND a -Log<sub>10</sub>(*p*-value)  $\geq$  8, ii) one-peptide identifications present in at least three replicates, which were detected in at least one replicate with two peptides, are significantly enriched with a mean NSAF ratio  $\geq$  20 AND a -Log<sub>10</sub>(p-value)  $\geq$  50. For AP-MS experiments with DSP in cell cultures, an extra filtering was applied to remove DSP-specific background by comparing with a smaller control dataset containing only experiments with DSP. Only proteins passing the filtering versus the large dataset and the smaller dataset with DSP were finally retained. For the SnRK1βy AP-MS experiments in seedlings, only two replicates were done. Proteins identified with at least two peptides that were present in both experiments and in not more than one control bait group were retained. Secondly, also proteins identified with at least two peptides that were present in both experiments and showing high enrichment with a mean NSAF ratio ≥ 15 versus the control dataset were retained. For removal of non-specific PL proteins, a dual filtering was applied. First, a similar NSAF-based strategy was applied as for the AP-MS filtering, now comparing with an in-house control dataset of 210 PL experiments with 31 baits unrelated to SnRK1 signaling. Thereto, an enrichment factor was calculated as the product of the mean NSAF ratio and -Log<sub>10</sub>(p-value). Proteins identified in at least two replicates, with an enrichment factor  $\geq$  20 were retained. In addition, a second filtering was implemented to further remove non-specific PL proteins. Thereto, specific proteins were determined through a MaxQuant Label Free Quantification analysis<sup>32,34</sup>, comparing with a small representative negative control PL experiments obtained from sucrose-starved cell cultures (wild-type or transgenic cultures expressing an unrelated TurboID fusion protein). LFQ intensities were analyzed in Perseus<sup>92</sup> (version 1.6.15.0) to determine the significantly enriched proteins with the SnRK1 baits versus the control experiments. Different thresholds (S0, FDR) were used in the volcano plot analysis to retrieve the top-enriched protein set for

the individual PL experiments, as indicated in Supplementary Table 3. For robust identification of specific interactors, only proteins that passed both filtering methods were retained. All protein interactions were visualized in a dot plot matrix by ProHits-viz<sup>93</sup> or as a network generated in Cytoscape<sup>94</sup>.

## Stoichiometry analyses

To evaluate the stoichiometry of the SnRK1 complex, all raw MS files from the AP-MS experiments were analyzed with MaxQuant version 1.6.10.43 using standard settings and iBAQ selected. iBAQ values were calculated based on unique peptides. iBAQ values of each SnRK1 subunit were extracted from the resulting proteinGroups.txt file and per experiment, relative iBAQ abundances were calculated by dividing the iBAQ value of each individual SnRK1 subunit or per subunit type by the sum of the iBAQ values of all identified SnRK1 subunits. Next, relative iBAQ ratios were averaged over the different replicates and visualized in bar graphs using GraphPad Prism (version 9.4.0).

## Cross-linking mass spectrometry

For XL-MS, protein complexes were purified in triplicate by TAP, following our established TAP cell culture protocol<sup>33</sup> with minor adaptations, and purified proteins were on-bead cross-linked as recently described<sup>59</sup>. Per replicate, proteins were extracted from 15 g  $GS^{rhino}$ -SnRK1 $\beta\gamma$ expressing cell suspension cells, harvested three days after subculturing. During TAP, 360mg total protein input was used per replicate and complexes were bound on 75 µL IgG and streptavidin beads during both binding steps. After TAP, streptavidin beads were washed four times with PBS buffer. Next, beads were resuspended in 175 µL PBS supplemented with 1 mM BS<sup>3</sup> (Thermo Fisher, cat. no. A39266) and cross-linked for 45 min at room temperature. After cross-linking, the reaction was quenched in 50 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.0) for 30 min at room temperature. Proteins were reduced in 5 mM DTT and alkylated with 15 mM iodoacetamide. Next, beads were washed with 50 mM NH<sub>4</sub>HCO<sub>3</sub> and incubated overnight at 37°C with  $1\mu g$ Trypsin/Lys-C. The next day, an additional incubation was done with 0.5 µg Trypsin/Lyc-C. The digest was removed from the beads and desalted with Monospin C18 columns, dried using a SpeedVac and stored at -20°C. Peptides were analyzed by LC-MS/MS as described<sup>59</sup>. Raw MS files were processed with pLINK2<sup>60</sup> (version 2.3.5) with settings listed in Supplementary Table 7e, using a customized protein database of 49 proteins focused towards SnRK1. For analysis of the XL-MS data, the cross-link spectra output file was further filtered retaining only spectra with E-value, Alpha\_E-value and Beta\_E-value <0.05. Cross-links were visualized by xVis<sup>95</sup>, integrating SnRK1 protein domain information extracted from Broeckx *et al.*<sup>1</sup>. The width of the cross-links was manually adjusted in Illustrator according to the amount of PSMs of each cross-link.

## Integrative structure modeling of the plant SnRK1 complex

The integrative modeling platform (IMP) package<sup>58</sup> (version 2.12) was used to generate the structure of the plant SnRK1 complex. Structures of individual SnRK1 subunits were taken from the AlphaFold Structure Database<sup>61</sup>. Beads of varying size represent the domains of the SnRK1 subunits, 1 to 20 residues per bead, arranged into either a rigid body or a flexible string of beads (loop regions). For the SnRK1 complex containing either the SnRK1a1 or the SnRK1 $\alpha$ 2 subunits together with SnRK1 $\beta$ 2 and SnRK1 $\beta\gamma$ , respectively, 40 or 34 unique intraand intermolecular BS<sup>3</sup> cross-links identified in all three repeats were used to construct the scoring function that restrained the distances spanned by the cross-linked residues. For both heterotrimers, the excluded volume restraints were applied to each one-residue bead. The sequence connectivity restraints were used to enforce proximity between beads representing consecutive sequence segments. After randomizing a position of all domains, the Metropolis Monte Carlo algorithm was used to search for structures satisfying input restraints. For the SnRK1α1 heterotrimer, the sampling produced 500,000 models from 25 independent runs, each starting from a different initial conformation of the SnRK1 complex, and a total of 9,026 good-scoring models satisfying at least 89% of chemical cross-links were selected for further analysis. For the SnRK1 $\alpha$ 2 heterotrimer, the sampling produced 420,000 models from 21 independent runs, each starting from a different initial conformation of the SnRK1 complex, and a total of 15,016 good-scoring models satisfying at least 93% of chemical cross-links were selected for further analysis. The four-step protocol<sup>62</sup> was used to analyze sampling convergence, exhaustiveness, and precision. Protein structures were visualized with UCSF ChimeraX<sup>96</sup>.

## Subcellular localization analysis

Fusion proteins were transiently expressed with P19 in tobacco through *Agrobacterium*mediated (C58C1 Rif<sup>R</sup> pMP90) leaf infiltration, as previously described<sup>97</sup>. For staining of nuclei, DAPI (10 μg/mL in infiltration buffer) was infiltrated 1 h prior to imaging. All analyses were imaged on an Olympus FluoView 1000 or an inverted Zeiss LSM 710 confocal microscope equipped with an UPLSAPO 20X/0.75 or a Plan-Apochromat 20X/0.8 objective lens, respectively. DAPI, EGFP, and mCherry were sequentially visualized with laser excitation at 405 nm, 488 nm, or 559 nm, and spectral detection at 425-470 nm, 500-525 nm, or 575-620 nm, respectively. Nuclear GFP signal was normalized against the total GFP signal through quantification in ImageJ (version 1.8.0), and analyzed with GraphPad Prism (version 9.4.0).

## *In vitro* kinase assays

N-terminal HisMBP and GST fusion proteins were expressed in *E. coli* by IPTG induction at 18°C and purified as previously described<sup>18</sup>. The GST-tagged ACC reporter was purified by Gluthation sepharose 4B beads (Sigma, cat. no. 17-0756-01) and elution in GST elution buffer (50 mM Tris-HCI (pH 8.0), 0.4 M NaCI, 50 mM reduced L-Glutathione, 0.1 % (v/v) Triton-x-100,1 mM DTT). Radioactive kinase reactions were performed in kinase assay buffer (50 mM Tris-HCI (pH 8.0), 1 mM EGTA, 1 mM DTT, 5 mM MgCl<sub>2</sub>, 10  $\mu$ M cold ATP, 5  $\mu$ Ci  $\gamma$ -<sup>32</sup>P ATP, 1x PhosSTOP) for 1 h at 30°C, combining 2 µL kinase with 1.5-10 µL substrate. For the ACC kinase assays, reactions were performed in ACC kinase assay buffer (50 mM Tris-HCl pH 8.0, 1mM EGTA, 5mM DTT, 5mM MgCl<sub>2</sub>, 10 µM ATP, 1x PhosSTOP) for 1 h at 30°C, combining 1 μL kinase with 2-8 μL substrate and 8-16 μL of the TPS II proteins, TPS1 or TPP-A. Amiconpurified MBP or GST elution buffer was added to correct for varying amounts of recombinant proteins in each reaction. Reactions were stopped by addition of SDS sample buffer and incubation for 10 min at 95°C. For detection of radiolabeled phosphoproteins, proteins were separated by SDS-PAGE on TGX 4-15% gradient gels (Biorad) and stained with Coomassie brilliant blue R-250. Gels were dried and radioactivity was detected by autoradiography on a photographic film. For the ACC kinase assays, proteins were separated by SDS-PAGE on TGX 4-20% gradient stain-free (Biorad) gels. Kinase activity was determined by immunoblotting with Phospho-ACC (Ser79) Antibody (dilution 1/2000) (Cell Signaling Technology, cat. no. 3661S) for detection of the phosphorylated fraction and with anti-HA 12CA5 (dilution 1/2000) (Roche, cat. no. 11583816001) (for the ACC reporter) or anti-SnRK1a1 (dilution 1/1000) (Agrisera, cat. no. AS10919) for detection of the expressed fusion proteins. Anti-mouse-HRP (GE Healthcare, cat. no. NA931) or anti-rabbit-HRP (GE Healthcare, cat. no. NA934) were used as secondary antibodies (dilution 1/10000). Protein band intensities were determined in ImageJ (version 1.8.0) and analyzed with GraphPad Prism (version 9.4.0).

#### Transient luciferase assays in tobacco BY-2 cells

Transient luciferase assays were performed in tobacco BY2 protoplasts as previously reported<sup>90</sup>. Boxplots were generated with GraphPad Prism (version 9.4.0).

## DATA AVAILABILITY

The AP-MS and PL mass spectrometry data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifiers PXD029833 (AP-MS) and PDX030048 (PL). The protein interactions from this publication have been submitted to the IMEx

(http://www.imexconsortium.org) consortium through IntAct<sup>98</sup> and assigned the identifier IM-29283. All structure-related data files related to integrative modeling are deposited in the Zenodo repository (10.5281/zenodo.5552311). The data supporting the findings of this study are available at the Figshare digital repository (https://doi.org/10.6084/m9.figshare.20732371)<sup>99</sup>.

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# AUTHOR CONTRIBUTIONS

J.V.L wrote the manuscript. J.V.L., D.E., A.G., C.H., D.V.D, F.R. and G.D.J. conceived the research. J.V.L, A.G., C.M., C.H., N.D.W., G.P., E.V.D.S., F.P., T.M., W.S., N.C. and E.B. performed experiments. D.E. and J.V.L. analyzed the MS data. R.P. performed the structural analysis.

# **COMPETING INTERESTS**

The authors declare no competing interests.

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## **FIGURES**



Figure 1. Sucrose repletion to sucrose-starved cells induces a wave of dephosphorylation of SnRK1 substrates

**a**, Time-line illustrating the synchronization strategy to monitor sucrose-dependent phosphorylation events in Arabidopsis cell cultures. Sucrose starvation was induced in 3-day-old cell cultures by replacement of growth medium with medium lacking sucrose. To map TOR dependency, cell cultures were pretreated 2 h before sucrose addition with AZD8055 or rapamycin. **b**, Known SnRK1 substrates are dephosphorylated upon sucrose repletion in a TOR-independent manner. Quantified phosphopeptides (Y-axis: mean Log2(Phosphosite Intensity)  $\pm$  SEM; n = 4) are plotted in function of time (min) after sucrose addition (X-axis), comparing sucrose control (gray), AZD8055 (red) and rapamycin (blue) treated samples. Protein symbols, phosphorylated residues and their corresponding tryptic peptides are shown above each plot. **c**, Hierarchical cluster analysis of the sucrose-dependent phosphosite intensity fold changes. Fold changes were calculated comparing the sucrose-replenished conditions (t10, t20, t40) against the sucrose-starved condition (t0). Log2 fold changes of the

phosphosite intensities were colored according to the indicated color gradient. Cluster I/cluster II: specific clusters possessing respectively up- or downregulated phosphopeptides upon sucrose repletion. Phosphosites matching the SnRK1 motif or TOR-dependent phosphosites are indicated with an asterisk below the heat map. The enriched SnRK1 motif identified through *de novo* motif analysis (MEME) is shown. **d**, Venn diagram showing the overlap of cluster II phosphoproteins with two SnRK1-dependent phosphoproteome datasets. **e**, Overview of proteins in cluster II that match or resemble the known SnRK1 motif. Proteins were functionally grouped (see Supplementary Note). Amino acids were colored according to the motif shown in Fig. 1c. **f**, Validation of candidate SnRK1 substrates by *in vitro* kinase assays with <sup>32</sup>P-labeled ATP, upon 1 h or 6 h exposure of the autoradiogram. In the negative control (-), no substrate was added. CBB, Coomassie brilliant blue staining loading control. The bar graph (mean  $\pm$  S.D, n = 3) shows the quantification of the phosphorylation in arbitrary units (A.U.). For MWs, see Extended Data Fig. 2.



## Figure 2. SnRK1 protein interactome mapping

**a**, Overview of the AP-MS and PL experiments that were performed to construct the SnRK1 protein interaction network, including a schematic diagram of the used protocols and the filtering strategies. The number of specific and common interactors found with each subunit or each method are summarized in Venn diagrams. In the lower Venn diagram, the overlap with the cluster II phosphoproteins is shown, and the proteins found at the intersection of AP-MS

and PL are visualized as a subnetwork according to the legend of Fig. 3. +, 1 h sucrose starvation for AP-MS or 6 h for PL; ++, 24h sucrose starvation; EN, 3 h extended night starvation for seedling AP-MS; X, DSP crosslinking; X<sup>dig</sup>, DSP crosslinking in extraction buffer with 1% v/v digitonin. **b**, Quantitative dot plot matrix representing a selection of the SnRK1 interactome, covering known (top panel) or novel, robust (bottom panel) SnRK1 interactors. The color of the nodes reflects the abundance of each prey in a given experiment, calculated by subtracting the average normalized spectral abundance factor of each prey in a given experiment (NSAF<sub>bait</sub>) with its average NSAF in the control dataset (NSAF<sub>ctrl.</sub>). The size of the dots reflects the relative abundance of each prey over the different experiments. The AP-MS and PL data were separately analyzed for the dot plot visualization. The identification of each bait protein is shown by an asterisk. Phosphoproteins from cluster II are marked in bold. Protein identifies can be found in Supplementary Table 4b.



# Figure 3. Network visualization of the integrated SnRK1 interactome

Cytoscape visualization of the SnRK1 interactome combining all AP-MS and PL experiments on the core SnRK1 subunits, together with the SnRK1γ1 interactors. Integrated node and edge attributes are shown next to the network. Phosphoproteins from cluster II are marked in bold.



Figure 4. Composition, stoichiometry and structure of the core SnRK1 complex

**a**, Bar graphs (mean  $\pm$  S.D.) showing the relative abundance (iBAQ ratio) of each individual SnRK1 subunit or summed per subunit type over the whole SnRK1 complex, based on the AP-MS experiments with SnRK1 $\beta\gamma$  or SnRK1 $\beta1$  as bait protein. n=3, except for Exp 4 (n=12) and Exp. 6 (n=2). \*, no error bar is shown for Exp. 6, as only two repeats were performed in seedlings with SnRK1 $\beta\gamma$  as bait. **b**, Schematic overview of the cross-linking (XL) MS strategy and circular plot of the BS<sup>3</sup> cross-linked peptides. Only cross-linked peptides that were identified in all three repeats are shown. The width of the cross-links corresponds to the number of peptide spectral matches (PSMs). Proteins and protein domains were colored as shown in the legend below the circular plots. **c**, Structure of the core SnRK1 complex as

obtained by the integrative modeling approach. The structure presents a multiscale centroid structure, i.e. the structure with the minimal sum of root mean square deviations from all the good-scoring models in the dominant cluster 1. **d**, Input cross-links (gray dashed lines) mapped on the centroid structure. **e**, SnRK1 domains mapped on the centroid structure. **f**, Distance distribution of obtained cross-links in the centroid structure. The dotted red line represents the threshold for the consistent cross-links. **g**, The residue contact frequency map, calculated over ten best-scoring models, is depicted by colors ranging from white (0, low frequency) to blue (1, high frequency). A contact between a pair of amino acid residues is defined by the distance between bead surfaces below 35 Å. Cross-links are plotted as green dots (consistent XLs) or orange dots (inconsistent XLs).



Figure 5. Functional characterization of the relationship between TPS II proteins and SnRK1

**a**, Selection of robust interactors found by AP-MS using TPSII-GS<sup>thino</sup> fusions, represented in a dot plot matrix as in Fig. 2b. **b**, Schematic overview and proof of concept of the ACC/SnRK1 kinase assay. Phosphorylation was detected by immunoblotting with anti-Phospho-ACC (S79). The Stain-free loading control is shown below the immunoblot. S79, ACC1 Ser79 reporter;  $\alpha$ 1, SnRK1 $\alpha$ 1; S79A, ACC1 Ser79>Ala mutant; K48M, SnRK1 $\alpha$ 1 Lys48>Met mutant. The experiment was three times repeated with similar results. **c**, ACC/SnRK1 kinase assay showing negative effect of TPS IIs on SnRK1 $\alpha$ 1 activity. As loading control, ACC and SnRK1 $\alpha$ 1 levels were assessed with anti-HA or anti-SnRK1 $\alpha$ 1, respectively, and TPS II levels through the Stain-free loading control. The bar graph (mean ± S.D, n = 3) shows the quantification of phosphorylation in arbitrary units. **d**, ACC/SnRK1 kinase assay comparing the effect of TPS8 with that of TPS1 or TPP-A. Protein levels were assessed through the Stain-free loading controls are marked with an asterisk. Bar graph is as in Fig.

5c. Significance was analyzed by one-way ANOVA with adjusted p-values (Dunnett's test). ns, not significant, \*\* p-value = 0.01. **e**, Box plot (Tukey whiskers, n = 4, mean indicated with +) of the luciferase assay showing reduced activation of SnRK1 transcriptional markers upon cotransfection of TPS5/TPS7. SnAK1 was co-transfected to promote SnRK1 activity. Student's *t*-test (two-sided) p-values are shown. **f-g**, Confocal analysis of the subcellular localization of TPS5, TPS8, TPS9 and SnRK1 $\alpha$ 1 upon single (f) or co-transfection (g) in tobacco leaves. TPSIIs were fused to mCherry and SnRK1 $\alpha$ 1 to GFP. In the SnRK1 $\alpha$ 1-GFP analyses, DAPI was infiltrated to visualize nuclei. In the single transfections with the TPSIIs, an ER-GFP marker protein was co-infiltrated. The insets are zoomed-in views of DAPI-stained nuclei. Scale bars, 10 µm. The bar graph shows the quantification of nuclear GFP normalized against total GFP, analyzed per picture (mean ± S.E.M., n=4 independent infiltration experiments) with one-way ANOVA adjusted p-values (Dunnett's test). **h**, Bar graph comparing the effect of TPS5 and TPS8 on nuclear SnRK1 $\alpha$ 1-GFP in young (Y) versus mature (M) tobacco leaves (mean ± S.E.M., n=4). One-way ANOVA adjusted p-values (Šidák's test) are shown.