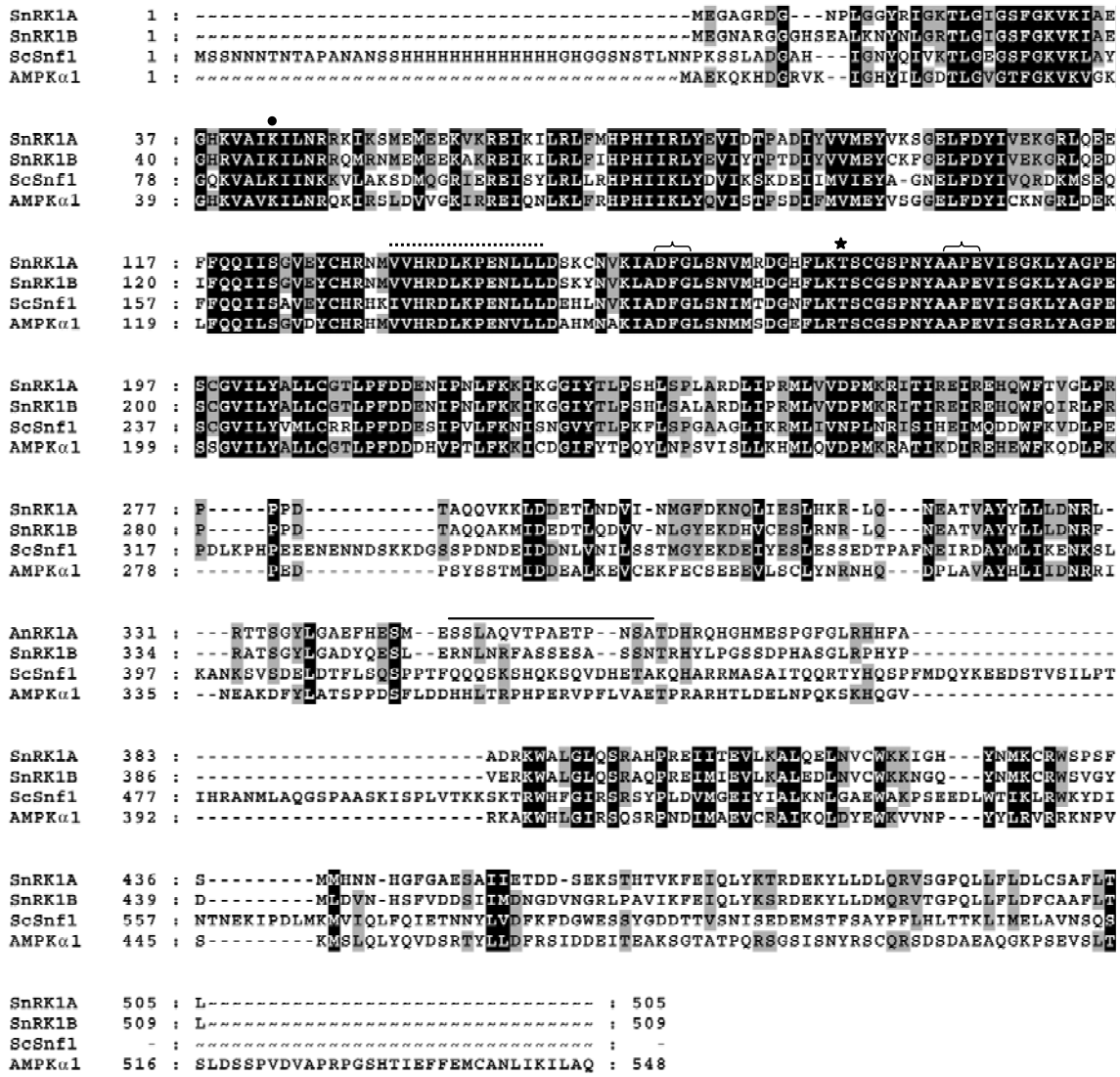
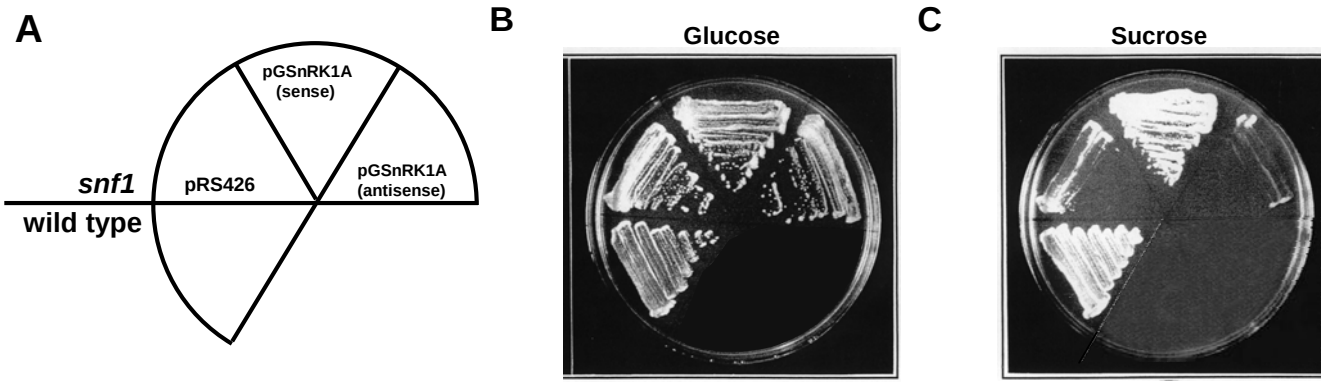
Supplemental Figure 1. Sequences of the Rice SnRK1, Yeast Snf1, and Mammalian AMPK1α Are Conserved.

Gaps have been introduced to maximize the alignment. Identical amino acid residues are highlighted in dark and the conserved residues are highlighted in grey. The ATP binding site is marked by a dot, the phosphorylation site is marked by an asterisk, the catalytic center is marked by a dash line, and the conserved Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) motifs that define the activation loop are marked by brackets. Polypeptides used for generation of SnRK1-specific antibodies is marked by a solid line. GenBank accession numbers: rice SnRK1A (BAA36298), rice SnRK1B (BAA36299), ScSnf1 (AAA35058) and AMPKα1 (P54645).



Supplemental Figure 2. SnRK1A Complements the Yeast *snf1* Mutant.
(A) Diagram showing the positions of yeast wild type (MCY 1093) and *snf1* mutant strains (MCY 1846) transformed with plasmid pRS426 (control), pGSnRK1A(sense) or pGSnRK1A(antisense), and their culture in medium containing **(B)** glucose or **(C)** sucrose .To determine whether SnRK1s have similar function as yeast Snf1 in catabolite derepression in yeast cells, a yeast mutant complementation test was performed. *SnRK1A* cDNA in sense and antisense orientations was fused downstream of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAP*) promoter, forming *GAP-SnRK1A* (sense) and *GAP-SnRK1A* (antisense) fusion constructs, respectively. These plasmids were delivered into yeast wild type and *snf1* mutant (MCY1846) strains (Supplemental Figure 2A). The *snf1* mutant grew as well as the wild type when glucose was a carbon source regardless of which plasmids they carried (Supplemental Figure 2B). The mutant cells carrying a control plasmid (pRS426) or *GAPSnRK1A* (antisense) construct were unable to grow when sucrose was the sole carbon source, while cells carrying *GAP-SnRK1A* (sense) construct grew as well as wild-type cells under the same condition (Supplemental Figure 2C). Similar results were obtained in a yeast mutant complementation test using rice *SnRK1B* cDNA (data not shown and (Takano et al., 1998). These results indicate that SnRK1s have the same catabolic derepression function in yeast as yeast Snf1.



Methods

Yeast Complementation Test

A glyceraldehyde 3-phosphate dehydrogenase (*GAP*) (Holland and Holland, 1980) promoter, excised from pGAP with *Eco*RI and *Hin*dIII, was subcloned into pRS426 (Sikorski and Hieter, 1989), generating pGAP-426. To make constructs for yeast transformation, *SnRK1A* cDNAs were excised from pSnRK1A with *Sac*II and inserted, in sense and antisense orientations, into pGAP-426, generating pGSnRK1(sense) and pGSnRK1(antisense), respectively. These two plasmids were used for transformation of yeast strains MCY 1093 and MCY 1846. Yeast transformation was performed as described (Gietz et al., 1995). The transformed yeast cells were cultured on minimal medium containing 2% sucrose or 2% glucose at 30 ºC.

References

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Holland, J.P., and Holland, M.J. (1980). Structural comparison of two nontandemly repeated yeast glyceraldehyde-3-phosphate dehydrogenase genes. J Biol Chem **255:** 2596-2605.
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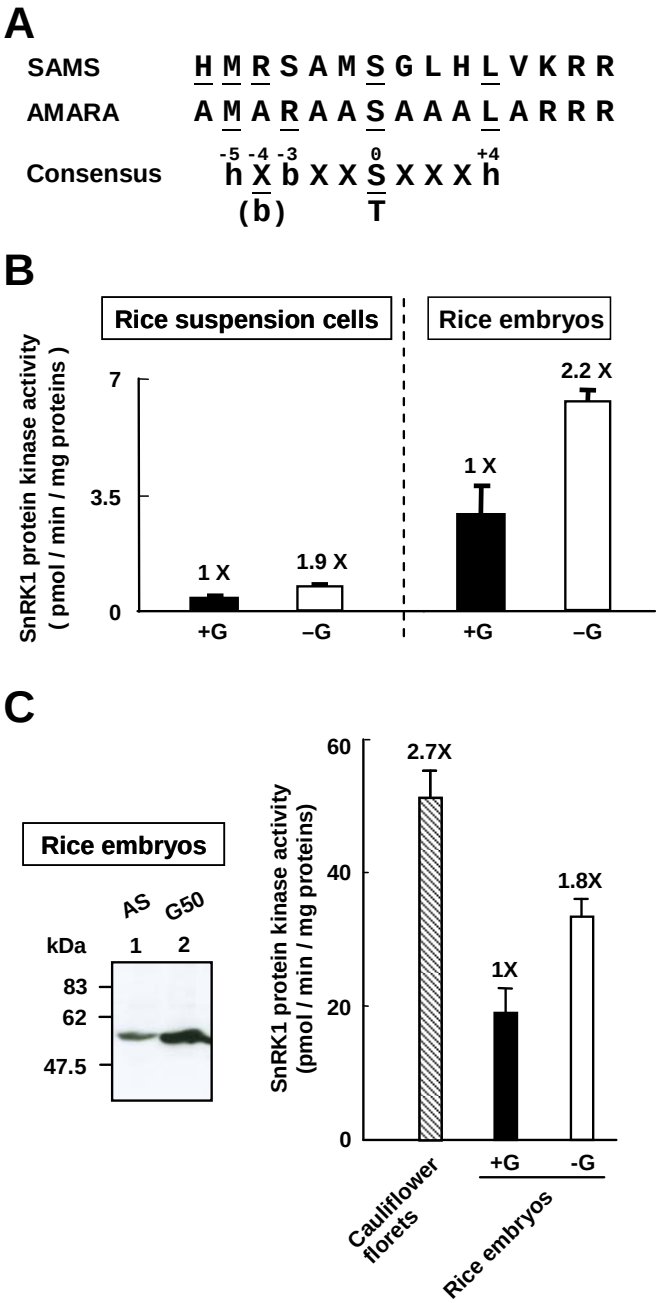
Supplemental Figure 3. The Protein Kinase Activity of SnRK1A Is Repressed by Sugar.

(A) SAMS and AMARA peptides contain substrate recognition motifs for SnRK1 and AMPK phosphorylation. The residues required for recognition are underlined as follows: phosphorylated Ser or Thr (position 0), hydrophobic residues (h) at positons -5 and +4, at least one basic residue (b) at position -3 or -4 (Hardie et al., 1998; Halford et al., 2003).

(B) Rice embryos were incubated in medium with (+G) or without (–G) 100 mM glucose for 24 h. Total proteins were extracted, without further purification, and subjected to SnRK1 protein kinase activity assay using the SAMS peptide as a substrate.

(C) Total proteins from rice embryos as prepared above were partially purified as described below. Left panel, rice embryo SnRK1 partially purified through ammonium sulfate precipitation (lane 1) and G50 column (lane 2). Right panel: SNF1 protein kinase activity assay for cauliflower florets (positive control) and rice embryos.

Error bars indicate standard errors from triplicate samples. The experiment was repeated twice with similar results. The value of SnRK1 protein kinase activity in +G condition was assigned a value of 1X, and the value in the absence of glucose was calculated relative to this value.



The SnRK1 protein kinase activity in –G medium was approximately 2-fold of that in +G medium; additionally, in –G medium, the activity was significantly higher in embryos (6.3 pmole/min/mg proteins) than in suspension cells (0.7 pmole/min/mg proteins) (Supplemental Figures 3B and 3C). The SnRK1 protein kinase activity in rice embryos in –G medium increased to 33.8 pmole/min/mg proteins after partial protein purification, which was comparable to that found in partially purified potato tuber and leaf extracts (Man et al., 1997), and was also 2-fold of that in +G medium (Supplemental Figure 3C). The SnRK1 protein kinase activity in cauliflower florets (51 pmole/min/mg proteins) was used as a positive control in the assay (Supplemental Figure 3C). Using AMARA (Supplemental Figure 3A), another substrate for SnRK1 and AMPK phosphorylation (Halford et al., 2003), although leading to a 4-fold higher SnRK1 protein kinase activity, no difference between +G and –G conditions was detected (data not shown).

AMPK, SNF1 and SnRK1 have very similar, but distinguishable, substrate recognition requirements. For example, AMARA peptide is a better substrate than the SAMS peptide for AMPK and a cauliflower HMG-CoA reductase kinase (HRK, an SnRK1), however, it was not a good substrate of SNF1 as the SAMS peptide (Dale et al., 1995). Two spinach HRKs also exhibit similar but non-identical substrate specificity for AMARA and SAMS peptides (Sugden et al., 1999). These studies suggest that optimization of substrate recognition motifs or specificity could be required to optimize the activity of rice SnRK1.

Supplemental Figure 3 Methods

SnRK1 Protein Kinase Activity Assay

Eight rice embryos (as prepared for transient expression assay) or 0.5 ml rice suspension cells were incubated in MS medium containing 100 mM glucose or 100 mM mannitol for 24 h, frozen in liquid nitrogen, and ground to a fine powder. Five hundred □l of extraction buffer (250 mM mannitol, 50 mM Hepes, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium orthorvandate, 1 mM sodium pyrophosphate, and 1X protease inhibitor cocktail, pH 8.2) was added to the cell powder and vigorously vortexed to thaw, and Triton-X 100 was added to 0.5 % (v/v). The extraction mixture was gently mixed by inversion and centrifuged at 13,500 x *g* at 4 °C for 15 min. The supernatant was concentrated by centrifugation at 4 °C through three passages of Microcon YM-30 centrifugal filter Units (Millipore, Billerica, MA). SnRK1 in cauliflower florets obtained from the market were similarly extracted except that volumes were scaled up and Amicon Ultra-15 Tltracel-30K centrifugal filter unit (Millipore) was used at the last step of purification. The protein extract was stored in an Eppendorf tube at -70 °C until use.

For partial purification of the protein extract for SnRK1 protein kinase activity assays, thirty-two rice embryos were collected and extracted as above described. After extraction and centrifugation twice, each with 1 ml and 500 µl cold extraction buffer, supernatants were collected and combined. Ammonium sulfate was then slowly added to the supernatant to 40 % saturation, with constant stirring for 20 min at 4 °C. After centrifugation at 13,500 x *g* at 4 °C for 15 min, the precipitated proteins were suspended in 250 µl fraction buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 100 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium orthorvandate, 1 mM sodium pyrophosphate, and protease inhibitor cocktail, 0.5%(v/v) Triton-X 100, 10 %(v/v) glycerol, pH 8.2), desalted with Sephadex G50 resin spin columns, and concentrated by YM-30 centrifugal filter units (Millipore). The protein extract was stored in an Eppendorf tube at -70°C until use.

The SnRK1 protein kinase activity assay, using the SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) as a substrate, was performed as described (Man et al., 1997) with minor modification. Protein extract (10 µl) was mixed with 6 µl kinase buffer (50 mM Hepes, 50 mM sodium fluoride, 1 mM DTT, pH 7.0), 6 µl SAMS peptide stock solution (500 mM), 6 µl sterile water, and 2 µl labeled ATP stock solution (0.5 µM [γ^{32}P] ATP, 0.5 µM cold free ATP, 75 mM magnesium chloride). The reaction was incubated at 30 °C for 10 min and a 20 µl aliquot was spotted onto a phosphocellulose P81 paper (Upstate, Charlottesville, VA). The paper was immersed in 1% (v/v) phosphoric acid solution to terminate the reaction and washed for 5 min. The washing step was repeated twice. Then the paper was washed once in acetone and air dried for 5 min. The dried paper was transferred to a scintillation vial containing 2 ml scintillation fluid (Amersham,

Buckinghamshire, United Kingdom) and radioactivity counted in a liquid scintillation counter. SnRK1 protein kinase activity was expressed as pmol phosphate incorporated into SAMS peptide per minute per mg of total proteins. Protein concentration was determined with Coomassie protein assay reagent (Pierce, Rockford, IL) based on the Bradford method with BSA as a standard.

Supplemental Figure 3 References

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Sugden, C., Donaghy, P.G., Halford, N.G., and Hardie, D.G. (1999). Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase, and sucrose phosphate synthase in vitro. Plant Physiol **120:** 257-274.

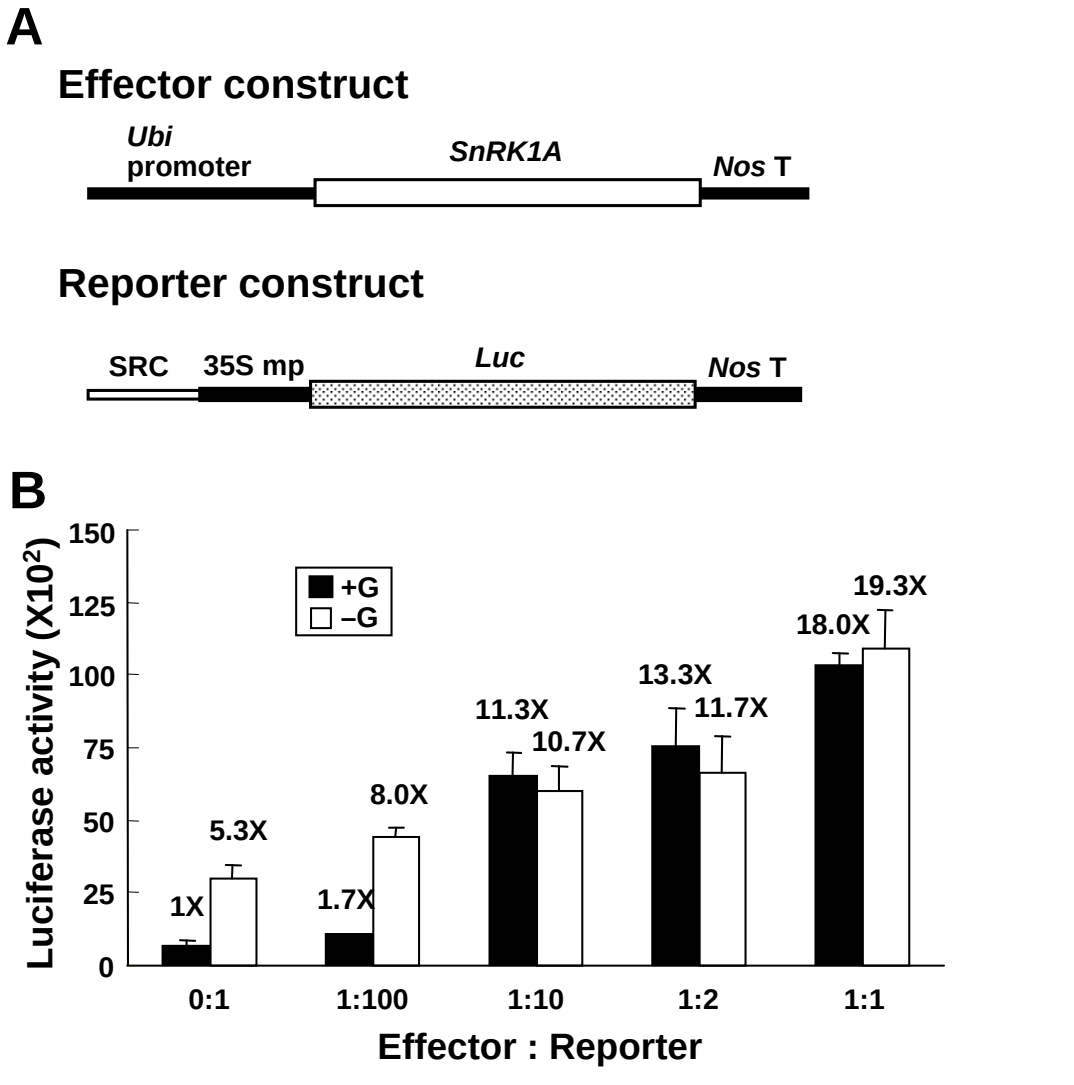
Supplemental Figure 4. SnRK1A Relieves Sugar Repression of *αAmy3* SRC in a Dose-dependent Manner.

Rice embryos were co-transfected with plasmids, incubated with glucose (+G) or without glucose (-G), and their luciferase activities assayed.

(A) The effector contains *Ubi* promoter-*SnRK1A* fusion construct. The reporter contains SRC-*35S*mp-*Luc* construct.

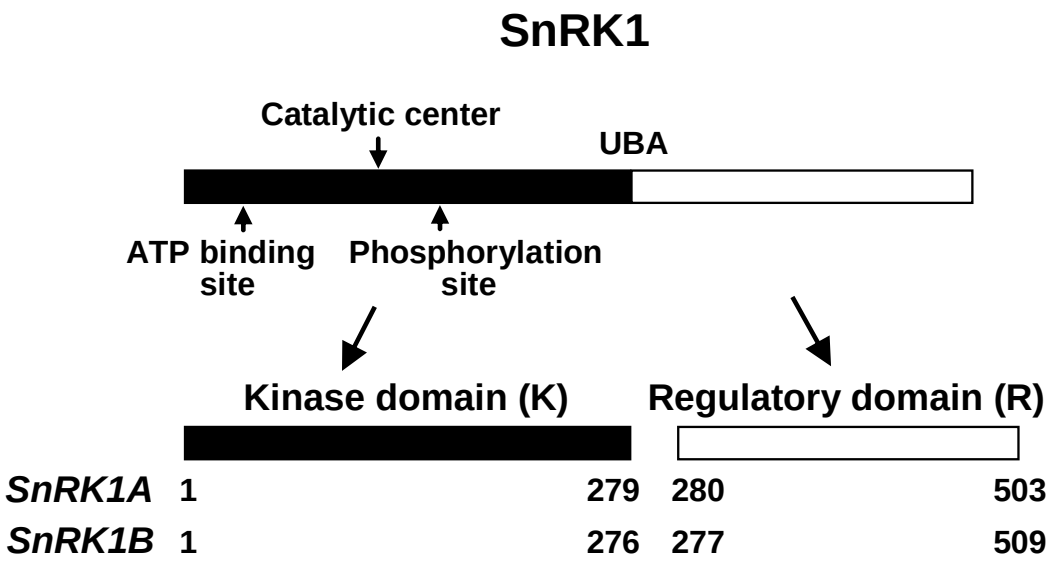
(B) Luciferase activity in rice embryos co-transfected with effector, control and reporter plasmids. The molecular ratio of effector and reporter plasmids is indicated on the x axis. The value of luciferase activity in rice embryos bombarded with reporter construct only and in the presence of glucose was assigned a value of 1X, and other values were calculated relative to this value.

To determine the effectiveness of SnRK1A for transactivation of SRC, a dosage-response experiment was performed with molar ratio of effector to reporter ranging from 1:100 to 1:1. The rice embryos were particle bombarded with different amounts of effector plasmid, divided into two halves and each half was incubated with 100 mM glucose or 100 mM mannitol for 18 h, and luciferase activity determined. When the reporter plasmid was bombarded alone, luciferase activity was repressed by glucose; co-transfection of the effector plasmid with the reporter at a molar ratio of 1 : 10 and 1 : 2 led to enhanced luciferase activity in the presence of glucose. All subsequent experiments were performed at a ratio of effector : reporter = 1 : 2.



Supplemental Figure 5. Diagram shows the relative position of the putative functional domains of SnRK1A and SnRK1B.
The putative ATP binding site, catalytic center, and phosphorylation site (Kleinow et al., 2000) are marked.

Kleinow, T., Bhalerao, R., Breuer, F., Umeda, M., Salchert, K., and Koncz, C. (2000). Functional identification of an Arabidopsis snf4 ortholog by screening for heterologous multicopy suppressors of snf4 deficiency in yeast. Plant J **23:** 115-122.

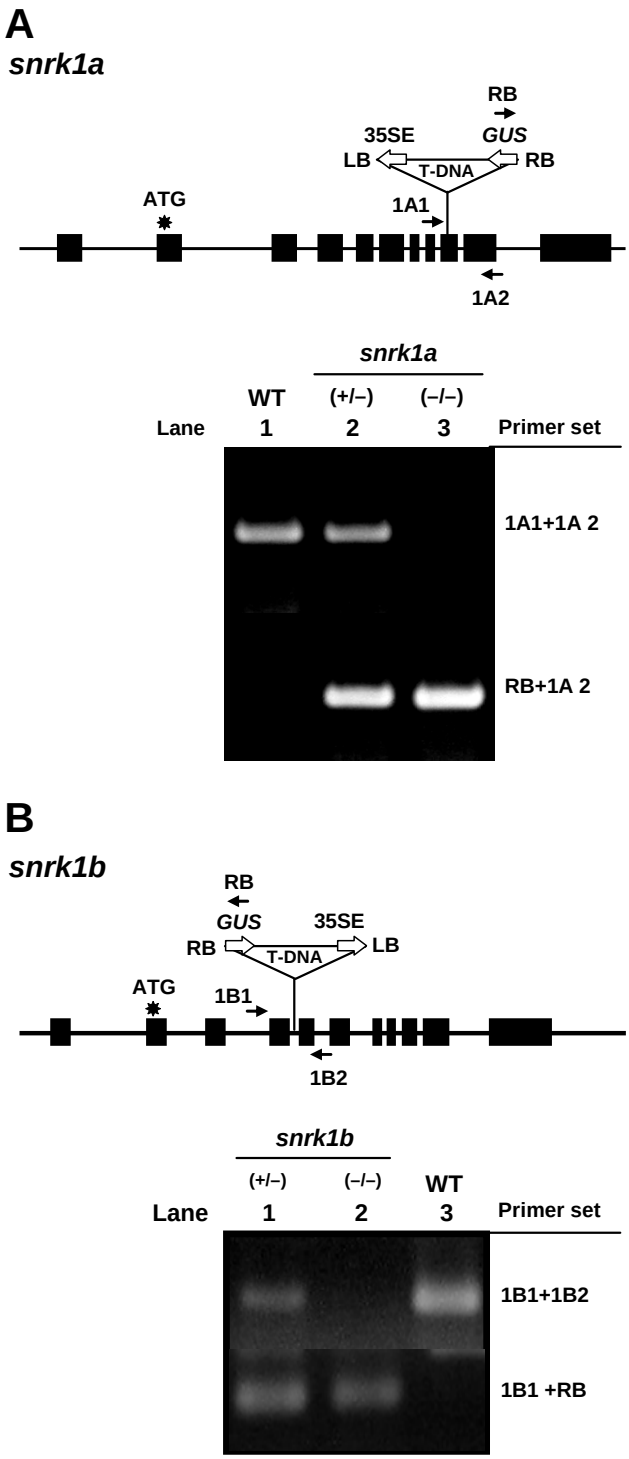


Supplemental Figure 6. Genotyping Identified *snrk1a* and *snrk1b* Homozygous (-/-) and Heterozygous (+/-) Mutants.

(A) and **(B)** Diagrams show positions of T-DNA insertions in two *SnRK1* genes. Boxes indicate exons, lines indicate introns and triangle indicates T-DNA which is inserted at the 9th exon of *SnRK1A* in *snrk1a* mutant and at the 4th intron of *SnRK1B* in *snrk1b* mutant. Bold arrows on T-DNA indicate positions and orientations of the *CaMV35S* enhancer (35SE) octamer and *GUS*. ATG indicates the translation initiation codon. Thin arrows indicate positions of DNA primers used for RT-PCR analyses.

(A) Genotyping of *snrk1a*. PCR with DNA primers 1A1 and 1A2 produced a product of 422 bp from wild-type (+/+) rice genomic DNA, and with DNA primers RB and 1A2 produced a product of 400 bp from the rice genomic DNA-T-DNA junction region in *snrk1a* mutant.

(B) Genotyping of *snrk1b*. PCR with DNA primers 1B1 and 1B2 produced a product of 580 bp from wild-type (+/+) rice genomic DNA, and with DNA primers 1B1 and RB produced a product of 420 bp from the rice genomic DNA-T-DNA junction region in *snrk1b* mutant.

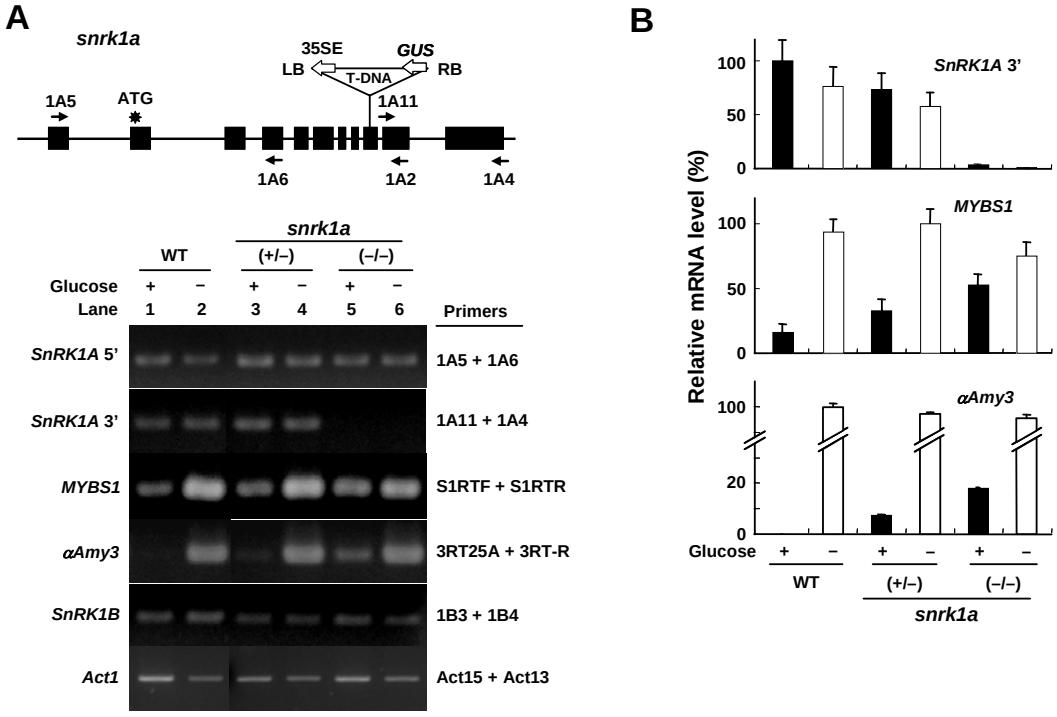


Supplemental Figure 7. Sugar Repression of *MYBS1* and *αAmy3* Is Partially Inhibited in *snrk1a* Mutant Missing the Regulatory Domain of SnRK1A.

(A) RT-PCR analysis for expression of various genes in wild type and *snrk1a* mutant. Embryonic calli from the wild type, heterozygous (+/-) mutant or homozygous (-/-) mutant were incubated with or without 100 mM glucose for 24 h. Total RNA was purified from cells and subjected to RT-PCR analysis using primers as indicated.

(B) Quantitative RT-PCR analysis for expression of *SnRK1A* 3' region, *MYBS1* and *αAmy3* using total RNAs and primers used in (A). RNA levels were quantified and normalized to the level of 18S rRNA. The highest mRNA level was assigned a value of 100, and mRNA levels of other samples were calculated relative to this value. Error bars indicate the standard error for three replicate experiments.

When using primers 1A5 and 1A6, the *SnRK1A* 5' mRNA (the region upstream of the T-DNA inserted site) was detected in heterozygous and homozygous *snrk1a* mutants, with levels higher than the wild type (Supplemental Figure 7A). However, when using primers 1A11 and 1A4, while *SnRK1A* 3' mRNA (the region downstream of the T-DNA inserted site) was detected in wild type and the heterozygous *snrk1a* mutant, it was not detected in the homozygous *snrk1a* mutant (Supplemental Figure 7A). These results indicated that *SnRK1A* mRNAs were transcribed but truncated by the insertion of T-DNA at the 9th exon. The loss of the *SnRK1A* 3' mRNA, which encodes the regulatory domain of SnRK1A, correlated well with the partial relief of glucose repression of *MYBS1* and *αAmy3* (Supplemental Figure 7A). The relative mRNA levels of above genes in wild type and *snrk1a* mutants were confirmed by quantitative RT-PCR analyses (Supplemental Figure 7B).



Supplemental Figure 7 Methods

RT-PCR Analysis

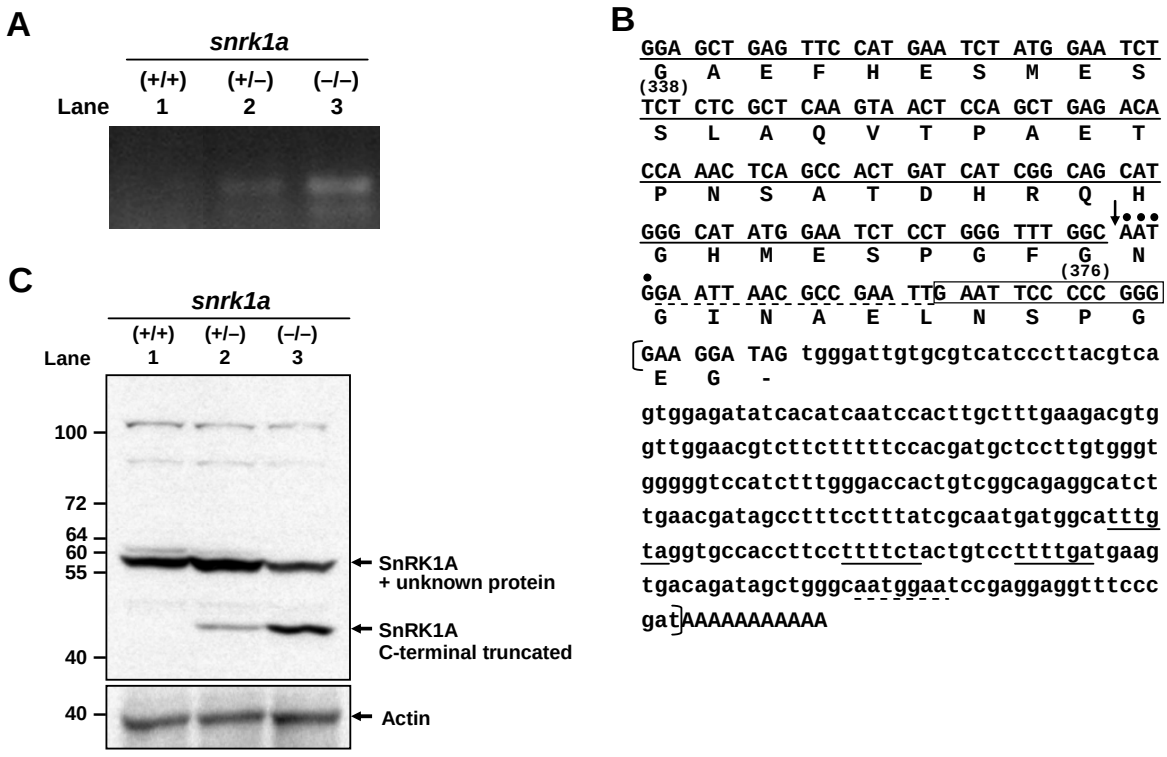
Total RNA was purified from rice suspension cells using Trizol reagent (Gibco BRL, Carlsbad, CA). Two and half micrograms of purified RNA was treated with 1 unit of RNase-free DNase I (Promega, Madison, WI) at 37 °C for 15 min. cDNA was reverse-transcribed with the Reverse-iT 1st strand synthesis kit (ABgene, Surrey, UK). PCR was carried out with Taq DNA polymerase (Promega). The amount of template cDNA for PCR and the number of PCR cycles were determined by several test experiments to ensure that the DNA amplification occurred in the linear range. The resulting PCR products were resolved by 2% agarose gel electrophoresis and visualized with ethidium bromide staining.

Supplemental Figure 8. The Truncated *SnRK1A* mRNA in the *snrk1a* Mutant Encodes a Constitutively Active Protein Kinase

(A) RT-PCR analysis of expression of the 5' region of truncated *SnRK1A* mRNA *in snrk1a*. Embryo calli from the wild type, heterozygous (+/-) *snrk1a* or homozygous (-/-) *snrk1a* rice were cultured in +G medium for 24 h. Total RNA was purified from cells and subjected to RT-PCR analysis using primers 1A7 and oligo dT anchor.

(B) Nucleotide sequence of 3'end of truncated *SnRK1A* mRNA. The upper-case sequence with underline indicates the 3' end of truncated *SnRK1A* cDNA, the arrow indicates the position where *SnRK1A* was truncated, the upper case sequence (4 bp) with dots on top was filler DNA generated during T-DNA insertion, the upper-case sequence with dotted underline is a part of T-DNA left border sequence, the box indicates *Eco*RI and *Sma*I restriction sites between the left border sequence and the *CaMV35S* enhancer, brackets delineate one copy of the *CaMV35S* enhancer in reverse orientation, and the lower-case sequences with underlines and dotted underline indicate two putative plant poly(A) signals (upstream elements and near upstream element, respectively). The mRNA cleavage site TA is followed by the poly(A) tail. Numbers in brackets indicate position of SnRK1 amino acid sequences relative to the translation start codon.

(C) Protein gel blot analysis of SnRK1A and C-terminal truncated SnRK1A. Suspension cells from wild type, heterozygous (+/-) *snrk1a* or homozygous (-/-) *snrk1a* rice were cultured in medium containing 100 mM glucose for 5 days. Total cellular proteins were extracted and subjected to gel blot analysis using anti-SnRK1A antibodies. Molecular weight markers (in kD) are shown on the left of the protein gel. Arrows indicate positions of the full-length SnRK1A, which overlapped with an unknown protein, the C-terminal truncated SnRK1A and actin (internal control).



The truncated *SnRK1A* mRNA was isolated by RT-PCR, using a 5' DNA primer derived from the 8th exon of *SnRK1A* and a 3' oligo dT primer. RT-PCR products with the predicted size were not produced from wild-type mRNA, but were produced from heterozygous *snrk1a* mRNA and at higher levels from homozygous *snrk1a* mRNA (Supplemental Figure 8A). DNA sequence analysis of these RT-PCR products revealed that the *SnRK1A* mRNA was truncated at the 3' end of a codon encoding amino acid residue 376 of SnRK1A (Supplemental Figure 8B). Most of the T-DNA left border sequence was lost during T-DNA insertion into the rice genome, and the truncated *SnRK1A* cDNA fused with a 4-bp newly generated filler DNA sequence, a 16-bp left border sequence, a 13-bp restriction site sequence, and one

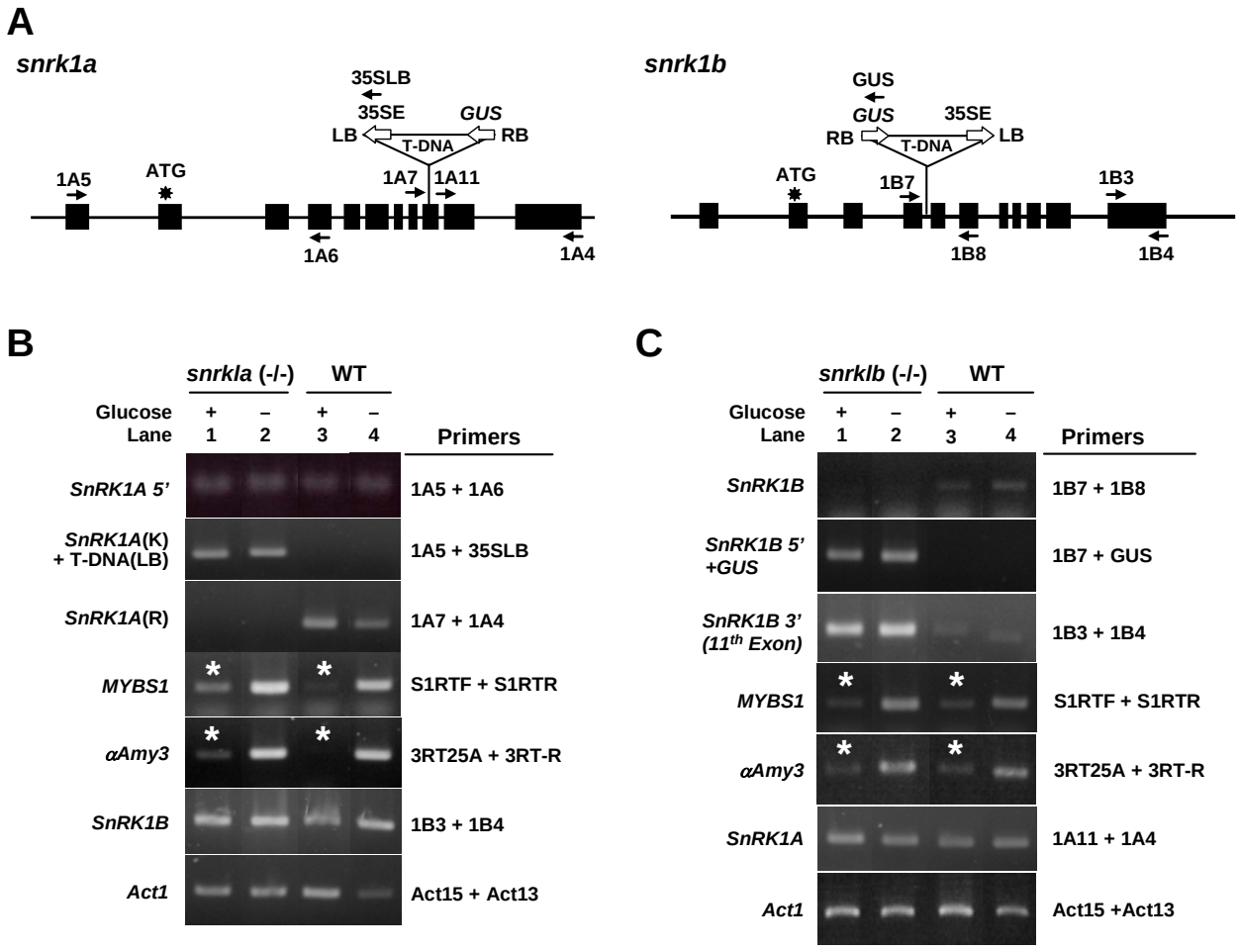
copy of the *CaMV35S* enhancer sequence (234 bp) (Hsing et al., 2007) in reverse orientation, which was then followed by the poly(A) tail (Supplemental Figure 8B). Formation of the chimeric gene led to an extension of 13 putative amino acids at the C-terminal of the truncated SnRK1A.

The protein gel blot analysis revealed that a protein, with the same molecular weight as SnRK1A, also reacted with anti-SnRK1A antibodies in the *snrk1a* homozygous mutant (Supplemental Figure 8C, lane 3). However, no full-length *SnRK1A* mRNA was detected in this mutant (Supplemental Figure 7A). A subsequent computer search identified neither a putative protein containing a sequence homologous to the SnRK1A polypeptide used for generating antibodies nor an extra copy of *SnRK1A* gene in the rice genome. The possibility that an unknown protein, along with two other proteins with molecular weights of 85 and 105 kD, reacted non-specifically with anti-SnRK1A antibodies has to be considered (Supplemental Figure 8C).

Supplemental Figure 9. SnRK1A Is an Upstream Regulator of *MYBS1* and *αAmy3* in the Sugar Signaling Pathway.

(A) T-DNA insertion in *SnRK1A* and *SnRK1B*. Boxes indicate exons, lines indicate introns and triangle indicates T-DNA which is inserted at the 9th exon of *SnRK1A* in mutant *snrk1a* and at the 4th intron of *SnRK1B* in mutant *snrk1b*. Bold arrows on T-DNA indicate positions and orientations of the *CaMV35S* enhancer (35SE) octamer and *GUS*. ATG indicates the translation initiation codon. Thin arrows indicate positions of DNA primers used for RT-PCR and quantitative RT-PCR analyses.

(B) and **(C)** Embryonic calli from the wild type and homozygous (-/-) *snrk1a* and *snrk1b* mutants were cultured in 100 mM glucose-containing medium for 24 h and transferred to glucose-containing or glucose-free medium for 24 h. Total RNA was purified from cells and analyzed by RT-PCR using primers as indicated. SnRK1A(K) and SnRK1A(R) contain kinase and regulatory domains of SnRK1A.



Supplemental Figure 10. Abnormal SnRK1A Expression Retards Germination and Seedling Growth.

Phenotypes of germinating seeds and seedlings of wild type (WT), transgenic lines 1A(Ri-2), and *snrk1a* and *snrk1b* mutant lines. Five seeds for each line were germinated for 2-4 days and photographed. The magnification of photographs at day 4 was reduced in order to cover all seeds with extended growth of shoots and roots.