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SnRK1A-Interacting Negative Regulators Modulate the Nutrient Starvation Signaling Sensor SnRK1 in Source-Sink Communication in Cereal Seedlings under Abiotic Stress

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In plants, source-sink communication plays a pivotal role in crop productivity, yet the underlying regulatory mechanisms are largely unknown. The SnRK1A protein kinase and transcription factor MYBS1 regulate the sugar starvation signaling pathway during seedling growth in cereals. Here, we identified plant-specific SnRK1A-interacting negative regulators (SKINs). SKINs antagonize the function of SnRK1A, and the highly conserved GKSKSF domain is essential for SKINs to function as repressors. Overexpression of SKINs inhibits the expression of *MYBS1* and hydrolases essential for mobilization of nutrient reserves in the endosperm, leading to inhibition of seedling growth. The expression of SKINs is highly inducible by drought and moderately by various stresses, which is likely related to the abscisic acid (ABA)–mediated repression of SnRK1A under stress. Overexpression of SKINs enhances ABA sensitivity for inhibition of seedling growth. ABA promotes the interaction between SnRK1A and SKINs and shifts the localization of SKINs from the nucleus to the cytoplasm, where it binds SnRK1A and prevents SnRK1A and MYBS1 from entering the nucleus. Our findings demonstrate that SnRK1A plays a key role regulating source-sink communication during seedling growth. Under abiotic stress, SKINs antagonize the function of SnRK1A, which is likely a key factor restricting seedling vigor.

INTRODUCTION

The plant life cycle is accompanied by source-sink transitions that modulate nutrient assimilation and partitioning during growth and development. The regulation of source-sink communication determines the pattern of carbon allocation in the whole plant and plays a pivotal role in determining crop productivity. Most studies have focused on the carbon supply-and-demand processes that regulate the expression of genes involved in carbohydrate production and reserve mobilization in source tissues (photosynthetic leaves and storage organs) and utilization in sink tissues (growing vegetative and reproductive tissues). However, components in underlying signal transduction pathways that regulate source-sink communication are largely unknown. Insight into the regulatory mechanisms is not only significant for understanding how sugar starvation/demand regulates plant growth and development, but also important for genetic manipulation of source-sink nutrient allocation for crop improvement.

The source-sink transition during germination and seedling growth in cereals can be viewed within a nutrient supply-demand paradigm and represents an ideal system to study the mechanism of nutrient demand/starvation signaling and gene regulation in source-sink communication. Germination followed by seedling growth constitutes two essential steps in the initiation of a new life cycle in plants, and completion of these steps requires coordinated developmental and biochemical processes, including mobilization of reserves in seeds (the source tissue) and elongation of the embryonic axis (the sink tissue). In these processes in cereals, the stored reserves in the endosperm are degraded, and mobilized, by a battery of hydrolases to sugars and other nutrients that are absorbed by the scutellum and transported to the embryonic axis to support seedling growth (Akazawa and Hara-Nishimura, 1985; Beck and Ziegler, 1989; Fincher, 1989; Woodger et al., 2004). Starch, which constitutes ~75% of cereal grain dry weight (Kennedy, 1980), provides the major carbon source for generating energy and metabolites during germination and seedling growth. Consequently, among all hydrolases, α-amylases are the most abundant and play a central role in the mobilization of starch and, thus, the rate of seedling growth. The expression of α-amylase is induced by both the hormone gibberellin (GA) and sugar demand/starvation (Yu, 1999a, 1999b; Lu et al., 2002, 2007; Sun and Gubler, 2004; Woodger et al., 2004; Chen et al.,

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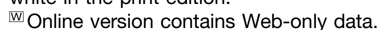 Online version contains Web-only data.
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2006; Lee et al., 2009), which has served as a model for studying the mechanism of sugar starvation signaling and crosstalk with the GA signaling pathway.

Our previous studies in rice (*Oryza sativa*) revealed that sugar starvation regulates α-amylase expression by controlling its transcription rate and mRNA stability (Sheu et al., 1994, 1996; Chan and Yu, 1998). The transcriptional regulation is mediated through a sugar response complex (SRC) in α-amylase gene promoters, in which the TA box is a key regulatory element (Lu et al., 1998; Chen et al., 2002, 2006). MYBS1 is a sugar-repressible R1 MYB transcription factor that interacts with the TA box and induces α-amylase gene promoter activity in rice suspension cells and germinating embryos under sugar starvation (Lu et al., 2002, 2007). GA also activates α-amylase gene promoters through the GA response complex, in which the adjacent GA response element and the TA/Amy box are key elements and act synergistically (Rogers et al., 1994; Gubler et al., 1999; Gómez-Cadenas et al., 2001). MYBGA (also called GAMYB) is a GA-inducible R2R3 MYB that binds to the GA response element and activates promoters of α-amylases and other hydrolases in cereal aleurone cells in response to GA (Gubler et al., 1995, 1999; Hong et al., 2012). Our recent study revealed that the nuclear import of MYBS1 is repressed by sugars, and GA antagonizes sugar repression by enhancing the conuclear transport of MYBGA and MYBS1 and formation of a stable bipartite MYB-DNA complex to activate α-amylase gene promoters (Hong et al., 2012). Furthermore, not only sugar but also nitrogen and phosphate starvation signals converge and crosstalk with GA to promote the conuclear import of MYBS1 and MYBGA and expression of hundreds of GA-inducible but functionally distinct hydrolases, transporters and regulators for mobilization of the full complement of nutrients to support active seedling growth (Hong et al., 2012).

The rice Snf1-related protein kinase 1 (SnRK1) family members SnRK1A and SnRK1B are structurally and functionally analogous to their yeast and mammalian counterparts, Sucrose non-fermenting 1 (SNF1) and AMP-activated protein kinase (AMPK), respectively (Lu et al., 2007). SNF1, AMPK, and SnRK1 are Ser/Thr protein kinases and are considered as fuel gauge sensors monitoring cellular carbohydrate status and/or AMP/ATP levels in order to maintain the equilibrium of sugar production and consumption necessary for proper growth (Halford et al., 2003; Hardie and Sakamoto, 2006; Rolland et al., 2006; Polge and Thomas, 2007). SNF1, AMPK, and SnRK1 are heterotrimeric protein complexes, consisting of a catalytic activating subunit (α or Snf1) and two regulatory subunits (β and γ or Sip1/Sip2/Gal83 and Snf4) (Polge and Thomas, 2007). These protein kinases contain an N-terminal kinase domain (KD) and a C-terminal regulatory domain (RD) (Dyck et al., 1996; Jiang and Carlson, 1996, 1997; Crute et al., 1998; Lu et al., 2007). In Glc-replete yeast cells, the SNF1 complex exists in an inactive autoinhibited conformation in which the Snf1 KD binds to the Snf1 RD (Jiang and Carlson, 1996). In Glc-starved yeast cells, Snf4 binds to the Snf1 RD and the Snf1 KD is released, leading to an active open conformation Snf1 (Jiang and Carlson, 1996). Sip1/Sip2/Gal83 acts as a scaffold protein binding to both Snf1 and Snf4, and this binding is also promoted by Glc starvation (Jiang and Carlson, 1996, 1997).

The conserved inter- and intrasubunit interactions and functions of SnRK1 protein kinases have also been demonstrated in the sugar starvation signaling pathway in rice, wherein SnRK1A acts upstream and plays a central role in the sugar starvation signaling pathway activating MYBS1 and α-amylase expression (Lu et al., 2007). Recently, we found that CIPK15 (for Calcineurin B-like-interacting protein kinase 15) acts upstream of SnRK1A and plays a key role in O_2 deficiency tolerance in rice (Lee et al., 2009). CIPK15 regulates the accumulation of SnRK1A protein, as well as interacting with SnRK1A, and links O_2 deficiency signals to the SnRK1A-dependent sugar starvation sensing cascade to regulate sugar and energy production and to program rice growth under flood conditions (Lee et al., 2009).

In plants, SnRK1s have been proposed to coordinate and adjust physiological and metabolic demands for growth, including regulation of carbohydrate metabolism, starch biosynthesis, fertility, organogenesis, senescence, stress responses, and interactions with pathogens (Polge and Thomas, 2007). SnRK1 regulates carbohydrate metabolism and development in crop sinks, such as potato (*Solanum tuberosum*) tubers (McKibbin et al., 2006) and legume (*Pisum sativum*) seeds (Radchuk et al., 2010). SnRK1 overexpression increases starch accumulation in potato tubers (Purcell et al., 1998; Halford et al., 2003), and SnRK1 silencing causes abnormal pollen development and male sterility in transgenic barley (*Hordeum vulgare*; Zhang et al., 2001). SnRK1 (KIN10/11) activates genes involved in degradation processes and photosynthesis and inhibits those involved in biosynthetic processes in *Arabidopsis thaliana* (Baena-González et al., 2007).

We are interested in understanding the mechanism regulating source-sink communication during plant growth and development. As a first step toward this goal, genes involved in sugar- and nutrient-demand signaling between source and sink tissues were studied. In this study, we identified an abiotic stress–inducible plant-specific gene family made up of SKIN1 and SKIN2, which interact with and repress the function of SnRK1A. We found that sugar-demand signals from the sink tissue (germinated embryo) were transmitted via SnRK1A to induce the expression of a full complement of enzymes necessary for the production of sugar and other nutrients in the source tissue (starchy endosperm). Using the hormone abscisic acid (ABA) as a stress signaling inducer, we further discovered that SKINs repress the SnRK1A-dependent sugar/nutrient starvation signaling by inhibiting the conuclear import of SnRK1A and MYBS1 and thus inhibit their functions in inducing enzyme expression facilitating nutrient mobilization under abiotic stress conditions.

RESULTS

A Previously Unidentified SKIN Family Interacts with SnRK1A

To identify components that interact with SnRK1A, we performed a yeast two-hybrid screen. The full-length cDNA of SnRK1A was fused to DNA for the Gal4 activation domain (*GAD-SnRK1A*) and used as bait for screening a rice cDNA library derived from Suc-starved rice suspension cells. One gene

A

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OsSKIN2          MSTAVA--R---GGMM-PAGHG------------FGKGKAAAVEEEE---DEVNGFFVEEEEEEEEEEEAAVSDAS----SI
ZmKCP            MSTAVA--RPAHGGFR-RSGSGKPDPDEADRMTRSANGYLVQEEEEEEHGEEAAARREDEDEEEEEEEAEAVSEAS----SI
OsSKIN1          MSTAVADVPPAAAYGFPGSAKR-------------GKP---EEVVVLMGKRRNEGFFIEEEE----EEEEVLTESS----SI
ZmMTD1           MSTAVAEARP--RYGFPGSGKGG-----------SGGGHGKEADMAAVGKRRSDGFFIEEV-----AEEEVLTDTS----SV
Sorghum02g028960 MPTAVAEVRP--AFGFPRSGKG------------SGGGHEKEE---AVGKRRSDGFFIEEVQEE--AEEEVLTDTS----SI
ZmMTD186T7R4     MSTAVAEVRP--PYGFPGSGKG------------SGGGYGKEAHLAAAGKRRSDGFFIEEEE----VEEDVLTDNS----SI
AtKCL1           MEVLVGS-----TF--------R-------------------------DRSSVTTHDQAVPASLSSRIGIRRCG---RS-
AtKCL2           MEVMVGS-----SFGIGMAAYVR----------------DHRGVSAQDKAVQTALFLADESGRGGSQIGIGIRMSNNNNKSP
AtKCP            MELMAKP-----TFSIEVSQYGT----------------TDLPATEKASSSSSSFETTNEEGVEESGLSRIWSGQ----TA
BnKCP1           M-AGGGP-----TFSIELSAYG--------------------SDLPTDKASGDIPNEEGSGLSRVGSGIWSGR----TV

                                                                                   KSD
OsSKIN2          GAASSDSSS-IGENSSSEKEGEE-EG--EEVESKAKEVAVEVEGGGLGFHGLGTLESLEDALPIKRGLSNFYAGKSKSFTSL
ZmKCP            GAASSDSSSSIGENSASDKEEEE-----EEVESKAQGLGM---------MGLATLESLDDALPSKRGLSSFYAGKSKSFTSL
OsSKIN1          GAPSPASSS-IGENSGEEEGGDD-E---EEVESKLKAEDE--------QVGLGCLDALEESLPIKRGLSNFYAGKSKSFTSL
ZmMTD1           GAPSPSGSS-IGENSSSEAGGDDGD---EEVESKLKEDDA------------LDCLDALEDSLPVKKGLSSFYSGKSRSFTSL
Sorghum02g028960 GAPSPSGSS-IGENSSSEAGEDDGE---EEVESKLKDGDA------------LGCLDALEDSLPIKKGLSSFYSGKSKSFTSL
ZmMTD186T7R4     GAPSPSGSS-IGENSSSEAGGDDGE---EEVESKLKEGDV------------LGCLDALEDSLPIKKGLSSFYSGKSKSFTSL
AtKCL1           --PPPESSSSVGETSENEEDEDDAVS-----SSQGRWLN-------------SFSSSLEDSLPIKRGLSNHYIGKSKSFGNL
AtKCL2           EESSDSSSS-IGESSENEEEEEE-----DDAVSCQRGTLD------------SFSSSLEDSLPIKRGLSNHYVGKSKSFGNL
AtKCP            DYSSDSSS--IGTPGDSEEDEEESENENDDVSSKELGLRG------------LASMSSLEDSLPSKRGLSNHYKGKSKSFGNL
BnKCP1           DYSSESSSS-IGTPGDSEEEDEESE---EDNDEEELGL--------------ASLRSLEDSLPSK-GLSSHYKGKSKSFGNL

                                       NLS          KID
OsSKIN2          AEAAA--KAAAKE-IAKPENPFNKRRR-VLAAWS-RRRASCSSLAT-TYLPPLLAPDHAVVEEEDEEDD--SDAE-------
ZmKCP            AEAAA--AAAARE-IAKPENPFNKRRR-VLQAWS-RRRASCSALAAA-YLPPLLAPDHAVVEEDDEEGADDEEEE-----DE
OsSKIN1          AEATASPAAAANE-LAKPENPFNKRRR-ILATWS--RRASCSSLATATYLPPLLAPDHAVAEGDEGEEEDDDSDD-----DE
ZmMTD1           AEATSTVAAAAKE-LAKPENPFNKRRR-ILANWS--RRASCSSLATAAYLPPLLGPDHAVAEGDEGEEDDSDSDD-----VE
Sorghum02g028960 AEATS-TVAAAKELLAKPENPFNKRRR-ILANWS--RRASCSSLATATYLPPLLGPDHAVAEGDEGEEDDSDDD------VE
ZmMTD186T7R4     AEAT--AAAAVKEVLAKPEKPFNKRRC-ILANWS--RRASCSSLATATYLPPLLGPDHAVAEGDEGEEDDSDDD------VE
AtKCL1           MEASN------TNDLVKVESPLNKRRRLLIANKL-RRRSSLSSFSIYTKINPNSMPLLALQESDNEDHKLNDDDD-----DD
AtKCL2           MEAASK-----AKDLEKVENPFNKRRRLVIANKLRRRGRSMSASNFYSWQNPNSMPLLALQEPNEEDHHIHNDDYE----DD
AtKCP            GEIGS------VKEVAKQENPLNKRRRLQICNKLARK-------SFYSWQNPKSMPLLPVNEDEDDDDEDDDEEDLKSGFDE
BnKCP1           GEIGS------VKEVPKQENPLNKKRRLQIYNKLARK-------SFYSWQNPKSMPLLPVHEDNDDEEGDDGD---------

OsSKIN2          --QCSGSGGGNR--RREPTFP--PPRLSLHAQKSSLTPRSS-N-PASSFRSPRSFSLSDLQNA----GSYN---
ZmKCP            DEEHGGGGGGLRGTRKPPTFP-S-PRLSVHVAAAGQMARNG------SFRSPRSFSMTDLHSAA---GYE----
OsSKIN1          RQHRGK-NGGRR-ESAAPPLPLPPPRLTLHTQMGGMVRRNG------TFRSPRSLSLSDLQNS-G--GSC----
ZmMTD1           CSQLPPPHRGKN-VRDARALP-P-LRL----GGAGMRRRNGPNGGLGSFRSPRSFSLSDLHSSAE--GE-----
Sorghum02g028960 YSQLP--HRGKN-VRDAPALPLPPTRL----GGVGMHRRNG----LGSFRSPRSFSLSDLHNSSSTDGSD----
ZmMTD1B6T7R4     YSQVP--HRGKN-VRDAPALPLPLPRL----GGVGSMQRNG----LGSFRSPRSFSLSDLRNSSA-DGSD----
AtKCL1           DSSSDDETSKLK---------------EKRMKMTNHRDF------MVPQTKSCFSLTSFQD-DD--DR-----
AtKCL2           D-GDGDDHRKI-----------------MMMMKNKKE------LMAQTRSCFCLSSLQEEDDGDGDDDEDE
AtKCP            NKSSSDEEGVK----------------KVVVRKGSFKN------RAYKSRSCFALSDLIEEEDDDDDQ----
BnKCP1           ---LSDEERGG----------------DVLARRPSFKN------RALKSMSCFALSDLQEEEEEEEDE----
                                              KID
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B

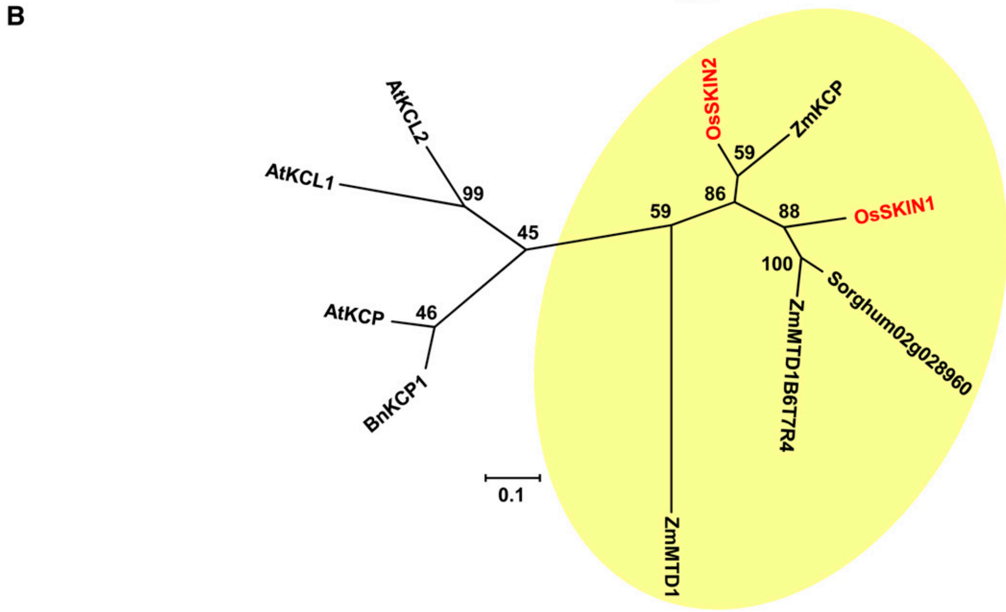

Figure 1. A Newly Identified Family of KSD-Containing Regulatory Proteins.

encoding a previously uncharacterized protein was identified, and the protein was designated as SnRK1A-interacting negative regulator 1 (SKIN1). Bioinformatics analysis of the rice genome also identified a SKIN1 homolog that we designated as SKIN2. The interaction between SKIN proteins fused to the Gal4 binding domain (GBD-SKIN) and GAD-SnRK1A was analyzed using the yeast two-hybrid assay. Both SKIN1 and SKIN2 interacted with SnRK1A in yeast (Supplemental Figure 1).

Amino acid sequences of the two SKINs share 59% identity and 69% similarity (Supplemental Figure 2). Bioinformatics analysis identified a highly conserved GKSKSF domain (KSD) present in SKIN1 and SKIN2 as well as in several other related proteins from various plant species (Figure 1A; Supplemental Figure 2). Additional conserved domains in these proteins include a putative nuclear localization signal (NLS) and protein kinase A–inducible domain (KID)–like sequences (Figure 1A). Among these related proteins, only the KID-containing protein from *Brassica napus* (KCP1) has been characterized. Bn-KCP1 is a nucleus-localized protein that interacts with a histone deacetylase in *Arabidopsis* (HDA19) via its C-terminal phosphorylated KID, and Ser-188 within the KID is necessary for the interaction with HDA19 and activation of downstream genes in response to cold stress and inomycin treatment (Gao et al., 2003). Amino acid sequences of SKINs share 40% identity and 54% similarity with Bn-KCP1. Phylogenetic analysis of amino acid sequences indicated that all KSD-containing proteins could be classified into monocot and dicot clusters (Figure 1B; Supplemental Data Set 1).

The N-Terminal Region of SKIN Interacts with the KD of SnRK1A

To map the functional domain of SKINs that interact with SnRK1A, five truncated versions of SKIN1 were fused with GBD and analyzed with the yeast two-hybrid assay (Supplemental Figure 3A). SKIN1 was truncated to contain amino acids 1 to 83, which were predicted to be a putative coiled-coiled domain by a bioinformatics program (SOSUI, http://bp.nuap.nagoya-u.ac.jp/sosui/coil/submit.html), and amino acids 1 to 159, which terminates at the N terminus of the KID. All truncated SKIN1 cDNAs lacking amino acids 1 to 83 did not interact with SnRK1A in yeast, whereas amino acids 1 to 83 alone did (Supplemental Figure 3B), indicating that SKIN1(1-83) is necessary and sufficient for interaction with SnRK1A in yeast.

To map the domain in SnRK1A that interacts with SKIN in the yeast two-hybrid assay, SnRK1A(1-279) containing the KD, SnRK1A(1-331) containing the KD and the autoinhibitory domain (AID), and SnRK1A(280-503) containing the RD (Lu et al., 2007) were fused with GAD. Only the full-length SnRK1A and SnRK1A (1-331) interacted with SKIN1 and SKIN2 (Supplemental Figure 3C), indicating that the KD and AID are necessary and sufficient for interaction with SKINs in yeast.

To further demonstrate the physical interaction of SKIN and SnRK1A in planta, a rice embryo two-hybrid assay was employed. Truncated SKIN1 and SKIN2 fused to GBD and expressed under the control of the *Ubi* promoter served as effectors, and five tandem repeats of the upstream activation sequence (UAS) fused upstream of the cauliflower mosaic virus 35S (*CaMV35S*) minimal promoter (-46 bp upstream of the transcription start site)–luciferase (*Luc*) cDNA (*5xUAS:Luc*) served as a reporter (Figure 2A). Luciferase activity was enhanced by coexpression of SnRK1A with each of the SKIN1 truncated versions except SKIN1(84-259) and SKIN2(86-261) lacking N-terminal regions (Figure 2B). The functional domain in SnRK1A that interacts with SKIN was also demonstrated in planta. The full length and KD of SnRK1A interacted with both SKIN1 and SKIN2 (Figure 2C), which is different from the result of yeast two-hybrid studies in which both KD and AID were required for interaction with SKIN1 and SKIN2 (Supplemental Figure 3C). These data confirmed the physical interaction between SKINs and SnRK1A in rice cells and that the N-terminal amino acids 1 to 83 and 1 to 85 of SKIN1 and SKIN2, respectively, interact with the KD of SnRK1A.

The Highly Conserved KSD Is Necessary for SKINs to Antagonize the Function of SnRK1A

The role of SKIN in the regulation of SnRK1A function was first investigated by gain- and loss-of-function analyses using a rice embryo transient expression assay. *SnRK1A* and *SKIN* cDNAs and a *SKIN* RNA interference (Ri) construct expressed under the control of the *Ubi* promoter served as effectors, and α*Amy3* SRC fused to the *CaMV35S* minimal promoter and *Luc* cDNA (*SRC-35Smp:Luc*) was the reporter (Figure 3A). Overexpression of SnRK1A enhanced, whereas *SKIN*s repressed and *SKIN*(Ri) derepressed, the α*Amy3* SRC promoter under +S and –S conditions (Figure 3B). Co-overexpression of *SKIN* with *SnRK1A* repressed the α*Amy3* SRC promoter to a level similar to overexpression of *SKIN* alone, while co-overexpression of *SKIN*(Ri) with *SnRK1A* significantly enhanced the α*Amy3* SRC promoter under +S and –S conditions (Figure 3B). These results indicate that SKINs act antagonistically to the SnRK1A-activation of α*Amy3* expression.

The accumulation of endogenous SnRK1A in nontransfected rice embryos was increased under sugar starvation (Figure 3C, lanes 1 and 2; Supplemental Figure 4A), as reported previously (Lu et al., 2007). Transient overexpression of SKINs alone or with SnRK1A did not alter the level of SnRK1A accumulation, except

Figure 1. (continued).

(A) Sequence comparison among KSD-containing proteins in plants. Identical amino acids are shown as white letters on a black background, and similar amino acids are indicated as black letters on a gray background. Boxes indicate KSD, putative NLS, and KID. Asterisks denote conserved domains in monocots.

(B) Phylogenetic analysis of KSD-containing proteins in plants. The scale value of 0.1 indicates 0.1 amino acid substitutions per site. The colored area denotes the monocot specific gene cluster.

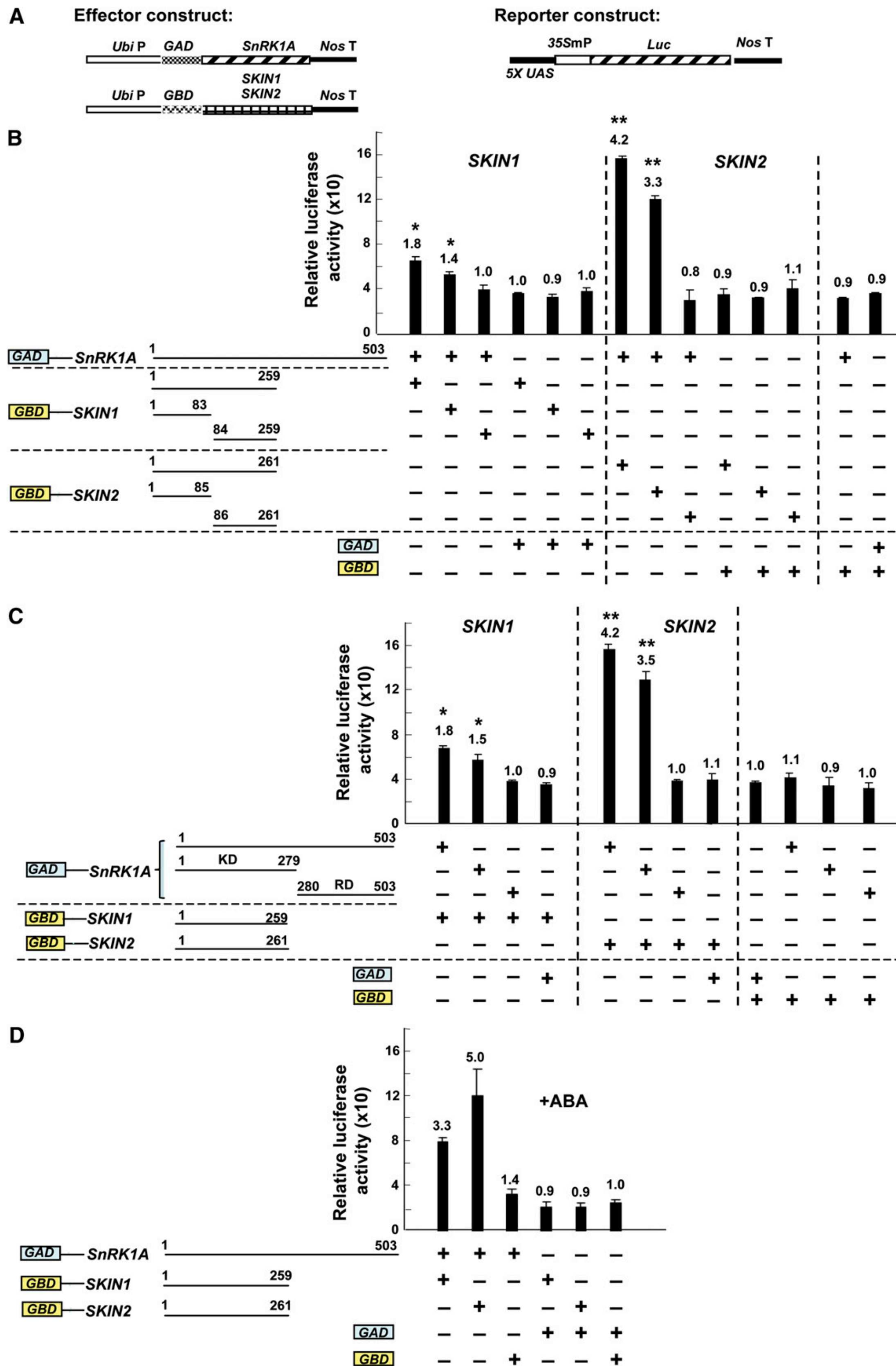

Figure 2. The N Terminus of SKIN Interacts with the KD of SnRK1A.

in that the recombinant SnRK1A increased the level of total SnRK1A (Figure 3C, lanes 5 to 12; Supplemental Figure 4A), indicating that SKINs antagonize the activity instead of affecting the protein accumulation of SnRK1A.

To further understand the mechanism of SKIN antagonism of SnRK1A function, the functional domain in SKIN that antagonizes SnRK1A activity was investigated. Wild-type and truncated versions of *SKIN1* expressed under the control of *Ubi* promoter were used as effectors and *SRC-35Smp:Luc* as the reporter (Figure 3A). SKIN1(1-83) and SKIN1(160-259) did not antagonize the function of SnRK1A (Figure 3D), indicating that the region within amino acids 84 to 159 of SKIN1 might be responsible for antagonism of SnRK1A function. This notion was further confirmed by the loss of inhibitory effect of SKIN1 with internal deletion of amino acids 84 to 159 (Figure 3D).

Because the highly conserved KSD is found within amino acids 84 to 159 of SKIN1 (Supplemental Figure 2), the KSD was deleted from SKIN1 or replaced with six Ala. Both mutated versions of SKIN1 lost their inhibitory effects on α-*Amy3* SRC promoter (Figure 3E). It is interesting to note that SKINs missing amino acid 84 to 159 or the KSD domain actually enhanced the function of SnRK1A under both +S and –S conditions (Figures 3D and 3E), suggesting that these truncated versions of SKIN might function as dominant-negative regulators of the endogenous SKIN. Nevertheless, these studies demonstrated that SKINs are negative regulators of SnRK1A, and the KSD in SKINs is necessary for SKINs to play a role in this repression.

SKINs Repress the SnRK1A-Dependent Sugar and Nutrient Starvation Signaling Pathway

The role of SKINs in the regulation of the SnRK1A-dependent sugar starvation signaling pathway was further explored in transgenic rice carrying constructs *Ubi:SKIN* and *Ubi:SKIN (Ri)*. In 2-d-old transgenic rice seedlings, the accumulation of endogenous *SKIN* mRNAs in the wild type was upregulated under –S conditions and decreased in the *SKIN*-silencing (SKIN-Ri) line under both +S and –S conditions, while the accumulation of recombinant *SKIN* increased significantly in the SKIN-overexpressing (SKIN-Ox) line under +S and –S conditions (Figure 4A, panel 1). The expression of hallmarks of the SnRK1A-dependent sugar starvation signaling pathway, including *MYBS1*, α*Amy3*, and α*Amy8*, were all induced in the wild type under –S conditions and reduced significantly in the SKIN-Ox line under both +S and –S conditions (Figure 4A, panels 2 to 4).

Previously, we showed that the expression of hydrolases and transporters for mobilization of various nutrients stored in the endosperm is coordinately turned on by any nutrient starvation signals at the onset of germination (Hong et al., 2012). To determine whether the SnRK1A-dependent pathway also regulates these genes, we selected four representative genes responsible for carbon, nitrogen, and phosphate nutrient mobilization for further analysis. These included the genes for sugar transporter (*ST*), GDSL-motif lipase (*Lip1*), Cys protease (*EP3A*), and phosphatase-like protein (*Phospho1*). The transcription of these four genes is normally low but activated by nutrient starvation (Hong et al., 2012). We found that the accumulation of mRNAs of the four genes was also activated under –S conditions and suppressed in the SKIN-Ox line (Figure 4A, panels 5 to 8). The accumulation of all tested genes was slightly increased in SKIN-Ri lines under +S but not under –S conditions, likely due to the functional redundancy of SKIN1 and SKIN2 under the experimental conditions. The expression of a rice ubiquitin gene, *UbiQ5*, used as a control was unaltered in SKIN-Ox and SKIN-Ri lines (Figure 4A, panel 9).

The accumulation of endogenous SnRK1A was slightly higher under –S conditions, and the pattern was unaltered by overexpression of SKINs in transgenic rice, except that the recombinant SnRK1A slightly increased the level of total SnRK1A (Figure 4B; Supplemental Figure 4B), indicating that the suppression of the SnRK1A-dependent signaling pathway was not due to the reduction of SnRK1A protein accumulation.

SKINs Repress Seedling Growth by Inhibiting Starch and Nutrient Mobilization from the Endosperm

Previously, we showed that germination and seedling growth are retarded in SnRK1A knockout (*snf1a*) and knockdown (SnRK1-Ri) mutants (Lu et al., 2007). Since SKINs repress the SnRK1A-dependent nutrient starvation signaling pathway in transgenic rice (Figure 4), the physiological function of SKINs in plant growth was further investigated. SKIN-Ox and SKIN-Ri transgenic lines were grown under a light/dark cycle or continuous dark conditions for 6 d. The growth of shoots and roots under the light/dark cycle was reduced in SKIN-Ox lines but enhanced

Figure 2. (continued).

For the *GAL4-UAS* two-hybrid assay, rice embryos were cotransfected with effector and reporter plasmids, incubated in –S medium for 24 h, and assayed for luciferase activity. For the relative values given above the bars, the luciferase activity in rice embryos bombarded with effectors *Ubi:GAD*, *Ubi:GBD*, and reporter *5XUAS-35S mp:Luc* was set to 1, and other values were calculated relative to this value. Error bars indicate the SE for three replicate experiments. Significance levels with the *t* test: $^{*}P < 0.1$ and $^{**}P < 0.05$. The *y* axis indicates the relative luciferase activity with different constructs.

(A) Plasmid constructs.

(B) Rice embryos were cotransfected with effectors *Ubi:GAD-SnRK1A* and *Ubi:GBD-SKIN* (wild-type or truncated) and reporter *5XUAS-35S mp:Luc*.

(C) Rice embryos were cotransfected with effectors *Ubi:GAD-SnRK1A* (wild-type, KD, or RD), *Ubi:GBD-SKIN*, and reporter *5XUAS-35S mp:Luc*.

(D) Rice embryos were cotransfected with effectors *Ubi:GAD-SnRK1A* and *Ubi:GBD-SKIN* and reporter *5XUAS-35S mp:Luc* and incubated in –S medium containing 1 μM ABA.

[See online article for color version of this figure.]

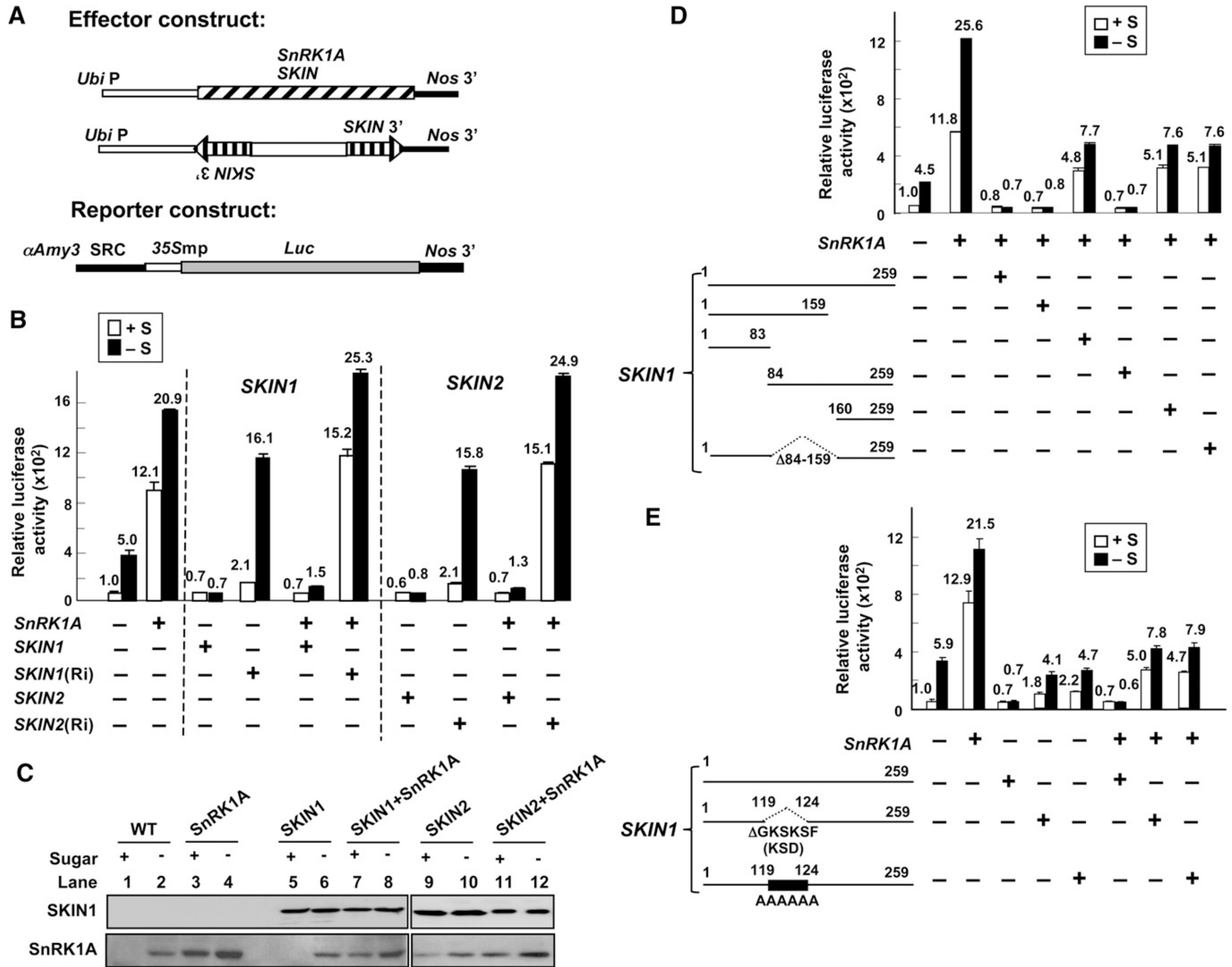

Figure 3. The Highly Conserved KSD Is Essential for SKINs to Antagonize the Function of SnRK1A.

Rice embryos were transfected with plasmids, incubated in +S or –S medium for 24 h, and assayed for luciferase activity. For the relative values given above the bars, the luciferase activity in rice embryos bombarded with the *SRC-35S* mp-*Luc* construct only and in +S medium was set to 1, and other values were calculated relative to this value. Error bars indicate the SE for three replicate experiments.

(A) Plasmid constructs.

(B) Rice embryos were cotransfected with effector *Ubi:SnRK1A*, *Ubi:SKIN1*, or *Ubi:SKIN(Ri)* alone and reporter *SRC-35Smp:Luc* or cotransfected with effectors *Ubi:SnRK1A* and *Ubi:SKIN* or *Ubi:SKIN(Ri)* and reporter *SRC-35Smp:Luc*.

(C) Total cellular proteins were extracted from rice embryos transfected with *Ubi:SnRK1A*, *Ubi:SKIN*, or *Ubi:SnRK1A* and *Ubi:SKIN* by particle bombardment and subjected to immunoblot analysis using antibodies against SnRK1A and the HA tag fused to at the N terminus of SKINs. Protein loading control by the Ponceau S staining is shown in Supplemental Figure 4A.

(D) Rice embryos were cotransfected with effectors *Ubi:SnRK1A* and *Ubi:SKIN1* (wild-type or truncated) and reporter *SRC-35Smp:Luc*.

(E) Rice embryos were cotransfected with effectors *Ubi:SnRK1A* and *Ubi:SKIN* (wild-type, KSD deleted, or KSD replaced with 6 Ala) and reporter *SRC-35Smp:Luc*.

in SKIN1-Ri lines compared with the wild type, and the difference was more evident under continuous darkness (Figures 5A and 5B, panel 1). No difference in shoot and root growth was detected regardless of the growth conditions if SKIN-Ox and SKIN-Ri lines were provided with 3% (88 mM) Suc (Figures 5A and 5B, panel 2), which indicates that Suc could recover the growth of SKIN-Ox lines.

To test whether the inhibition of seedling growth by overexpression of SKINs resulted from reduced expression of α-amylase, which generates the high-demand carbon source from hydrolysis of seed starch, the expression of *αAmy3* was examined. The expression of *αAmy3* was induced in 3-d-old seedlings in the wild type under continuous darkness, but the induction was reduced in SKIN-Ox lines and enhanced in SKIN-Ri lines under all growth conditions (Figure 5C, panel 1). Nitrogen is also essential for seedling growth. The expression of *EP3A*, which digests seed storage proteins to amino acids rich in nitrogen, was

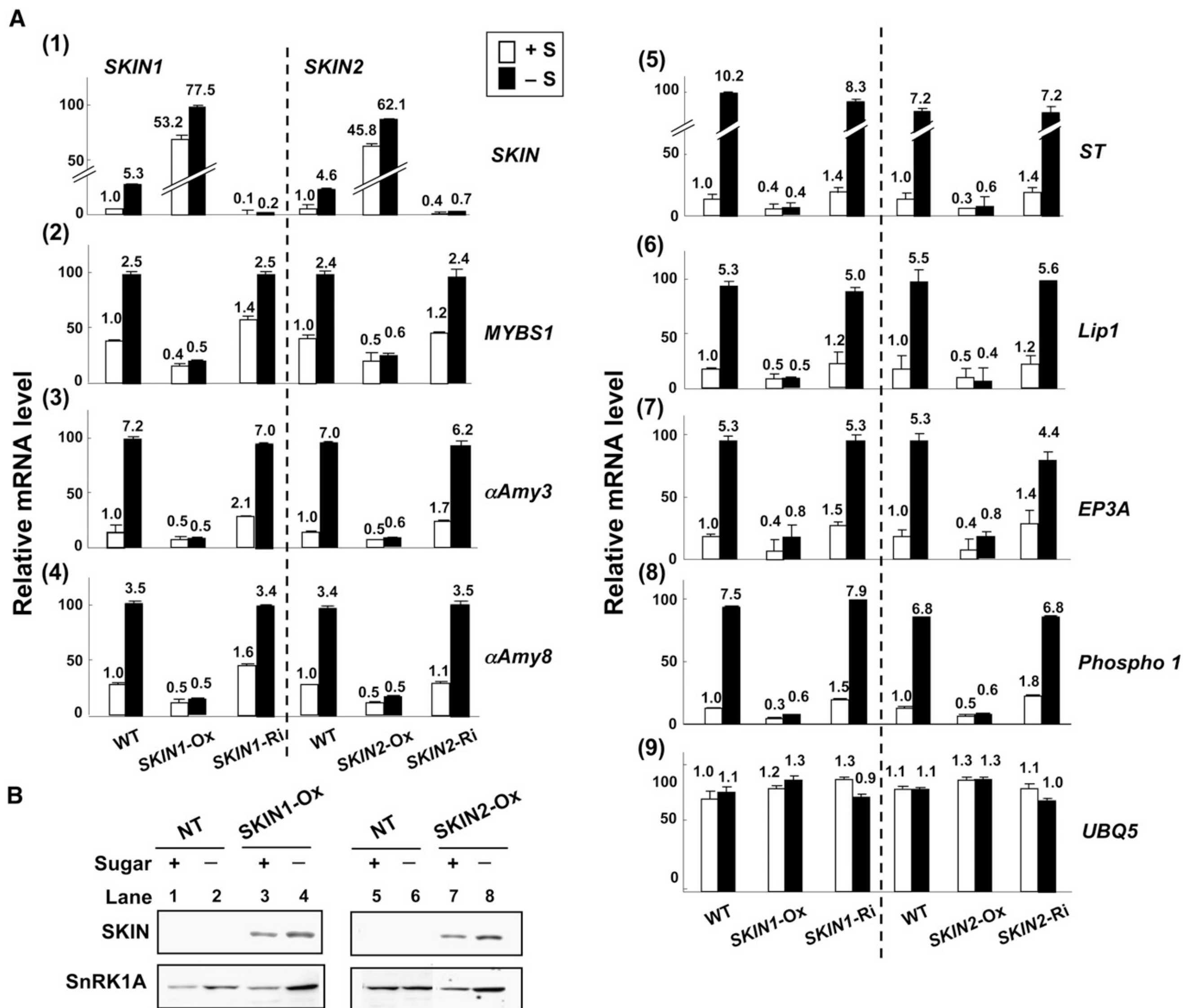

Figure 4. SKIN Suppresses the SnRK1A-Dependent Sugar and Nutrient Starvation Signaling Pathway.

(A) Two-day-old seedlings from the wild type and transgenic lines SKIN1-Ox (O3), SKIN1-Ri (R3), SKIN2-Ox (O2), and SKIN2-Ri (R1) were grown under +S or –S conditions with a 14-h-light/10-h-dark cycle for 18 h. Total RNA was purified from cells and subjected to quantitative RT-PCR analysis using primers specific for indicated genes, and mRNA levels were normalized against the level of *Act1* mRNA. The highest mRNA level in the *y* axis was set to 100 for normalization. Then, the lowest mRNA level of the wild type was set to 1, and the values for the other samples were calculated relative to this value. Error bars indicate the SE for three replicate experiments.

(B) Total proteins were extracted from 2-d-old seedlings of SKIN-Ox transgenic lines and subjected to immunoblot analysis using antibodies against SnRK1A and the HA tag fused to the N terminus (NT) of SKINs. Protein loading control by the Ponceau S staining is shown in Supplemental Figure 4B.

regulated similarly to α*Amy3* by SKINs, except that its expression was not enhanced in SKIN-Ri lines under continuous darkness (Figure 5C, panel 2).

SKINs Repress the Production of Sugars Necessary for Seedling Growth under Hypoxia

Previously, we showed that SnRK1A acts as an important regulator for germination and seedling growth in rice under hypoxic conditions (Lee et al., 2009). Consequently, the role of SKINs in regulating the hypoxic stress response was also investigated. In air, shoot elongation of SKIN-Ox lines was slightly slower than the wild type (Figure 6A; Supplemental Figure 5A), but under water, shoot elongation was severely arrested (Figure 6B; Supplemental Figure 5B). Under water, the retarded shoot elongation was significantly recovered by Suc (Figure 6C; Supplemental Figure 5C). The growth of SKIN-Ri lines was similar to the wild type. These results further confirm that SKINs

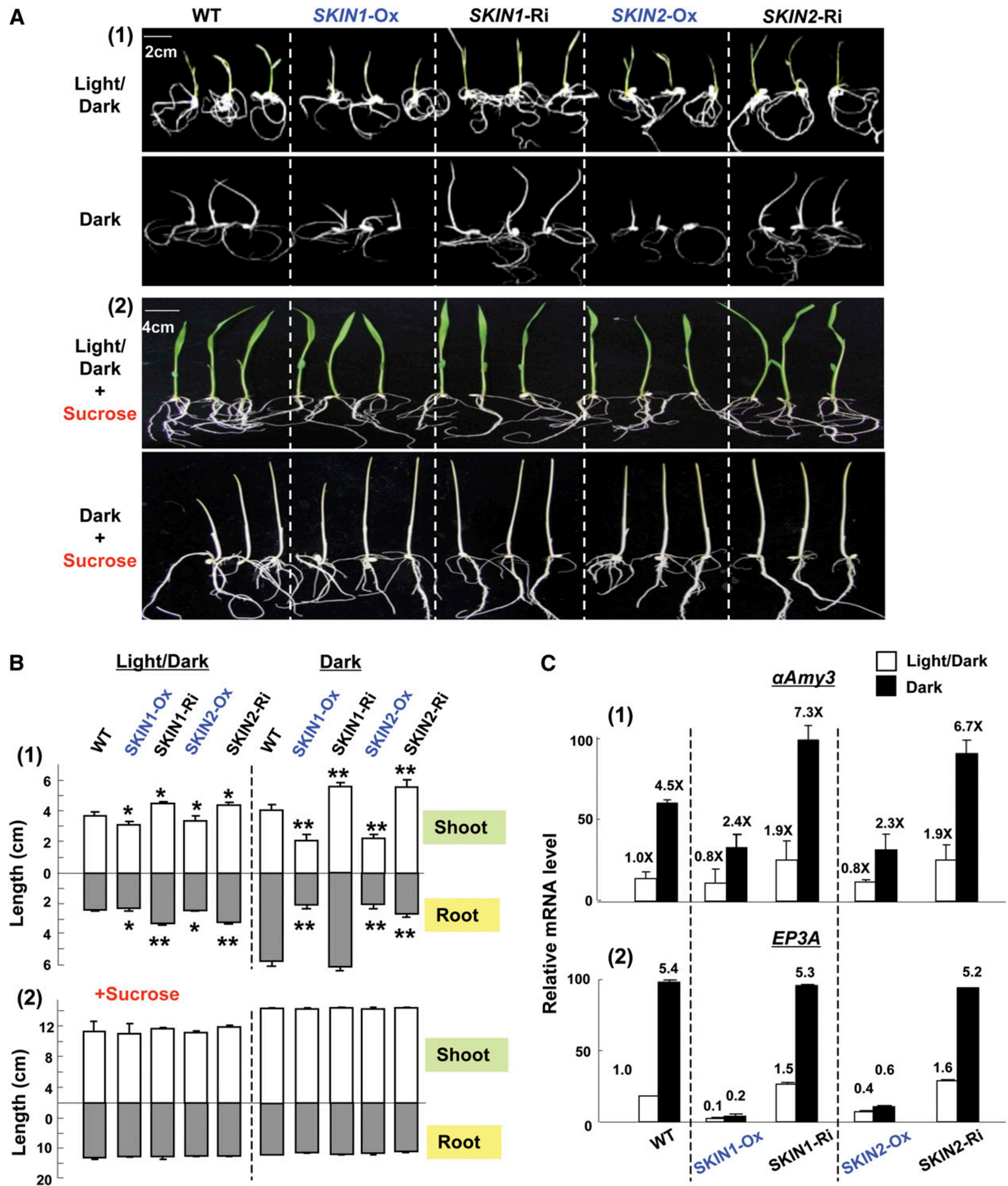

Figure 5. SKINs Repress Seedling Growth by Inhibiting Nutrient Mobilization in the Endosperm.

Transgenic lines SKIN1-Ox(O3), SKIN1-Ri(R3), SKIN2-Ox(O2), and SKIN2-Ri(R1) were used in the following experiments.

(A) Seeds were germinated and grown in water at 28°C under a 14-h-light/10-h-dark cycle or continuous darkness without (panel 1) or with (panel 2) 3% (88 mM) Suc for 6 d.

(B) Lengths of shoots and roots of seedlings in **(A)** were quantified. Panels 1 and 2, without and with Suc, respectively.

(C) Seedlings were grown under a 14-h-light/10-h-dark cycle or continuous darkness for 3 d. Total RNA was extracted and subjected to quantitative (real-time) RT-PCR analysis using primers specific for *aAmy3* (panel 1) and *EP3A* (panel 2).

Error bars represent SE (n = 12) at significance levels with the t test: *P < 0.1 and **P < 0.05 in **(B)** and **(C)**.

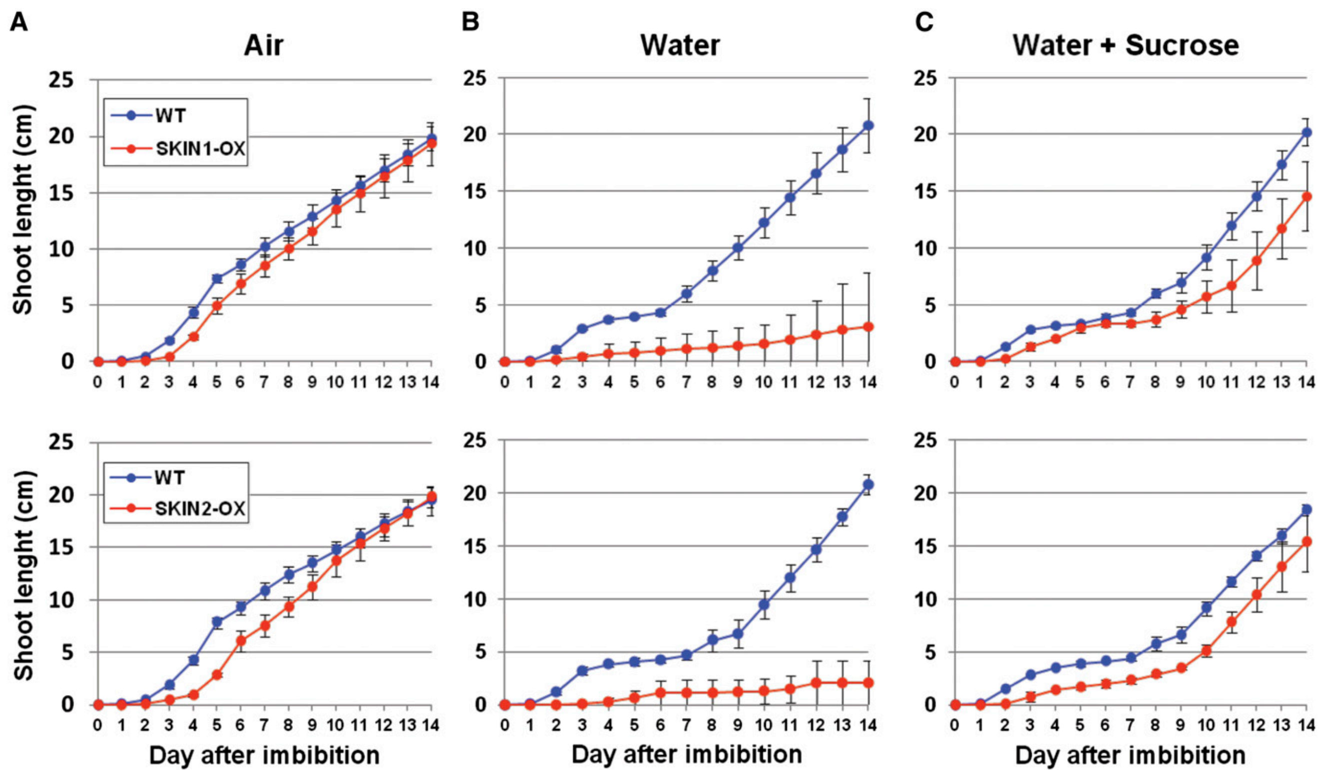

Figure 6. SKINs Suppress Sugar Production Necessary for Seedling Growth under Hypoxia.

Rice seeds were germinated in air or in water with or without 90 mM Suc at 28°C under a 14-h-light/10-h-dark cycle for various lengths of time. Shoot length of seedlings was measured daily. Error bars indicate the SE of shoot length (n = 10).

(A) In air.

(B) In water.

(C) In water with 88 mM Suc. For data using more SKIN-Ox and SKIN-Ri lines, see Supplemental Figure 5.

suppress the SnRK1A-dependent pathway, leading to impaired sugar production from starch hydrolysis in seeds during the postgermination seedling growth under hypoxia.

SKINs and SnRK1A Interact Primarily in the Cytoplasm

We next determined the subcellular localizations of SKIN and SnRK1A. As SKINs interact with the KD of SnRK1A, the full length, KD, and RD of SnRK1A were fused to the green fluorescent protein (GFP) and expressed under the control of the *Ubi* promoter in a barley aleurone cell transient expression system (Hong et al., 2012). As shown in Figure 7 and Supplemental Figure 6, SnRK1A-GFP and SnRK1A-KD-GFP were largely localized in the cytoplasm and with minor signal in the nucleus, whereas SnRK1A-RD-GFP was mainly in the nucleus. SKIN1-GFP was predominantly localized in the nucleus and with a minor amount in the cytoplasm. Coexpression of SnRK1A-GFP with SKIN1 excluded all SnRK1A-GFP from the nucleus. Coexpression of SKIN1-GFP with SnRK1A or SnRK1A-KD sequestered all SKIN1-GFP in the cytoplasm, whereas coexpression with SnRK1A-RD maintained the nuclear localization of SKIN1-GFP. These studies demonstrate that SKIN1 interacts with SnRK1A through SnRK1A-KD, which is consistent with result using the plant two-hybrid assay (Figure 2C), and the interaction retained SKINs and SnRK1A in the cytoplasm.

SKINs Antagonize the Function of SnRK1A in Both the Cytoplasm and Nucleus

Since SnRK1A and SKINs are present in both the cytoplasm and nucleus (Figure 7), we determined whether SKINs could antagonize the function of SnRK1A in both the nucleus and cytoplasm. Constructs were prepared for the expression of SKIN proteins lacking the putative NLS (SKIN∆NLS) and fused to GFP (Figure 8A). Whereas SKIN-GFP was mainly localized in the nucleus, SKIN∆NLS-GFP was exclusively localized in the cytoplasm under both +S and –S conditions (Figure 8B; Supplemental Figure 7), which indicates that the predicted NLS was functional. Coexpression of SKIN-GFP with or without the NLS with SnRK1A repressed the α*Amy3* SRC promoter to a level similar to overexpression of SKIN-GFP alone (Figure 8C). This indicates that SKIN in the cytoplasm could still trap SnRK1A in the cytoplasm and prevent the upregulation of *MYBS1* expression that is needed for α*Amy3* SRC activity.

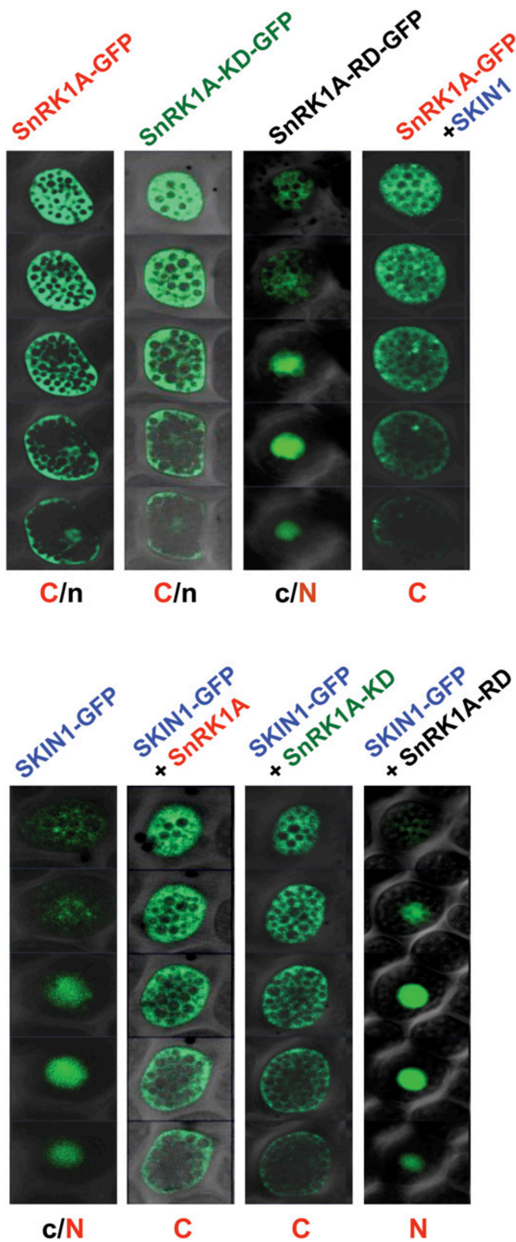

Figure 7. SKIN1 and SnRK1A Interact Primarily in the Cytoplasm.

Barley aleurone was transfected with plasmid constructs and incubated in –S medium for 24 h. Thirty optical sections of 0.9- to 1.1-μm thickness were prepared for each cell, and only five regularly spaced sections (sections 3, 9, 15, 21, and 27) are shown here. C and N indicate stronger GFP signals, and c and n indicate weaker GFP signals in the cytoplasm and nucleus, respectively. For more section images of each cell, see Supplemental Figure 6.

The Expression of *SKIN* Is Induced by Various Abiotic Stresses and ABA, and SKINs Promote ABA Sensitivity

Expression of both *SKIN*s could be detected in all tissues in seedlings, mature plants, flowers, and immature panicles and was particularly highly induced in the first leaf of seedlings and at day 4 after flowering (Supplemental Figure 8). We also determined whether the expression of *SKIN*s is regulated by abiotic stresses. Rice seedlings were subjected to drought (exposure to dry air), salt (200 mM NaCl), cold (4°C), and hypoxia treatments. The accumulation of *SKIN1* and *SKIN2* mRNAs was induced up to 79- and 66-fold, respectively, at 4 h after drought stress, 2.3- and 1.7 fold, respectively, 6 h after salt stress, 4.6-fold for both *SKIN1* and *SKIN2* 48 h after cold stress, 4.2- and 1.7-fold, respectively, 24 h after ABA, and 3.5- and 5.1-fold, respectively, 48 h after hypoxia treatment (Figure 9A).

To determine whether SKINs are important for ABA response/signaling, SKIN-Ox and SKIN-Ri lines were germinated in water containing various concentrations of ABA. The degree of inhibition of growth of wild-type and all transgenic lines increased with ABA concentrations from 1 to 10 μM; however, the growth of SKIN-Ri lines was less inhibited, and that of SKIN-Ox lines was more severely inhibited, by 1 and 5 μM ABA compared with the wild type (Figure 9B; Supplemental Figure 9). These results demonstrate that SKINs promote ABA sensitivity.

ABA Restricts SKINs, SnRK1A, and MYBS1 to the Cytoplasm under Sugar Starvation

The above studies showed that SKINs are exclusively localized in the nucleus in +S medium but levels are increased in the cytoplasm in –S medium, and they could antagonize the function of SnRK1A in both the nucleus and cytoplasm (Figures 7 and 8). Since the expression of SKINs is induced by various abiotic stresses and ABA, it is essential to determine whether SKINs are shuttling between the nucleus and cytoplasm in a stress-dependent manner. ABA and sorbitol, the latter used to mimic osmotic stress, not only by themselves suppressed, but also antagonized, the SnRK1A-mediated activation of α*Amy3* SRC promoter in both rice embryos and barley aleurone (Supplemental Figure 10). ABA also enhanced the interaction between SnRK1A and SKINs in rice embryos (Figure 2D). Consequently, ABA was used as a stress signal inducer. SKINs, SnRK1A, and MYBS1 fused to GFP were transiently expressed in barley aleurone incubated in +S or –S medium with or without ABA. SKIN-GFP and SnRK1A-GFP were exclusively localized in the nucleus and cytoplasm, respectively, in +S medium with or without ABA (Figure 10A; Supplemental Figure 11A, panels 1 to 3). SKIN-GFP became detectable in the cytoplasm and a considerable amount of SnRK1A was in the nucleus in –S medium without ABA; however, both SKIN-GFP and SnRK1A-GFP became exclusively localized in the cytoplasm in –S medium containing ABA (Figure 10A; Supplemental Figure 11A, panels 5 to 7). Quantitative analyses revealed that, in the absence of ABA, the percentage of SnRK1A-GFP localized in the nucleus was 19.7 and 64.0% in +S and –S medium, respectively, indicating that sugar starvation promotes the nuclear localization of SnRK1A (Table 1). In –S medium, the percentage of SnRK1A-GFP localized in the nucleus was reduced from 64.0% in the absence of ABA to 8.0% in the presence of ABA, indicating that ABA inhibits the nuclear localization of SnRK1A (Table 1).

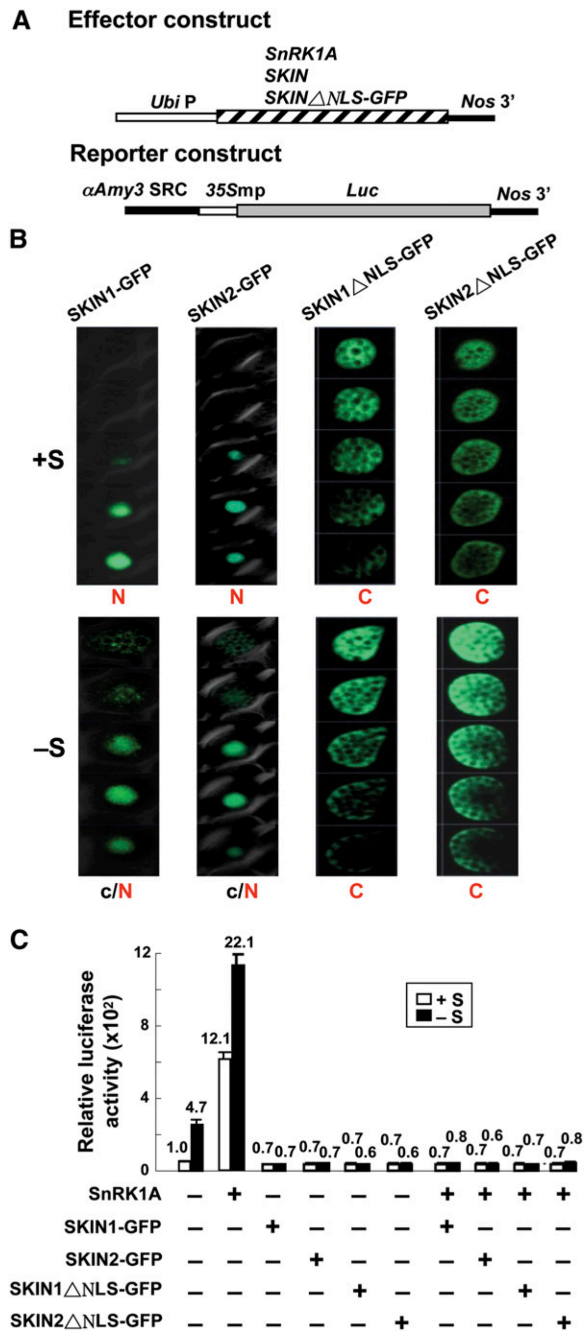

Figure 8. SKINs Could Antagonize the Function of SnRK1A in Both the Cytoplasm and Nucleus.

(A) Plasmid constructs.

(B) Barley aleurone cells were bombarded with *Ubi:SKIN-GFP* or *Ubi:SKIN△NLS-GFP.* Cells were incubated in +S or −S medium for 24 h. Thirty optical sections of 0.9- to 1.1-μm thickness were prepared for each cell, and only five regularly spaced sections (sections 3, 9, 15, 21, and 27) are shown here. C and N indicate stronger GFP signals, and c and n indicate weaker GFP signals in the cytoplasm and nucleus, respectively. For more section images of each cell, see Supplemental Figure 7.

(C) Rice embryos were cotransfected with SnRK1A and *Ubi:SKIN-GFP* or *Ubi:SKIN△NLS-GFP*, incubated in +S or −S medium for 24 h, and assayed for luciferase activity. For the relative values given above the bars, the luciferase activity in rice embryos bombarded with the *SRC-35S mp-Luc* construct only and in +S medium was set to 1, and other values were calculated relative to this value. Error bars indicate the SE for three replicate experiments.

MYBS1-GFP was mostly localized in the cytoplasm in +S medium and exclusively in the nucleus in –S medium without ABA, which is consistent with our previous study (Hong et al., 2012); however, MYBS1-GFP became exclusively localized in the cytoplasm in –S medium containing ABA (Figure 10A; Supplemental Figure 11A, panels 4 and 8). MYBS1 has been shown to be activated transcriptionally by SnRK1A (Lu et al., 2007). Here, we found that the nuclear import of MYBS1 was also promoted by overexpression of SnRK1A in +S medium and inhibited by silencing of SnRK1A in –S medium (Figure 10B; Supplemental Figure 11B, panels 2 and 3, respectively), indicating that SnRK1A is necessary and sufficient for promoting the nuclear localization of MYBS1. These studies also indicate that the nuclear localization of SnRK1A and MYBS1 is suppressed by ABA in –S medium.

To determine whether the exclusive cytoplasmic localization of SnRK1A-GFP and MYBS1-GFP resulted from the cytoplasmic interaction between SKIN and SnRK1A in –S medium containing ABA, SnRK1A-GFP was transiently coexpressed with SKIN(Ri) in barley aleurone. SnRK1A-GFP was highly accumulated in the nucleus in the presence of SKIN(Ri) in –S medium regardless of the presence or absence of ABA (Figure 10C; Supplemental Figure 11C). Transgenic rice overexpressing SKIN(Ri) was also transfected with SnRK1A-GFP and MYBS1-GFP. Similarly, SnRK1A-GFP and MYBS1-GFP became highly accumulated in the nucleus in –S medium despite the presence of ABA (Figure 10D; Supplemental Figure 11D). These studies indicate that ABA promotes the cytoplasmic interaction between SKINs and SnRK1A as well as reducing the nuclear localization of SnRK1A and MYBS1.

DISCUSSION

SKINs Are Regulators Interacting with and Antagonizing the Function of SnRK1A

SKINs physically interact with SnRK1A in yeast and plant cells (Figure 2; Supplemental Figure 1). Only a few proteins interacting with SnRK1 have been identified in plants. For example, the PLEIOTROPIC REGULATORY LOCUS1 WD protein, which interacts with the two *Arabidopsis* SnRK1s (AKIN10 and AKIN11) in yeast, negatively regulates the activity of these two SnRK1s and downstream Glc-regulated genes in *Arabidopsis* (Bhalerao et al., 1999). A barley protein, SnRK1-INTERACTING PROTEIN1, interacts with a seed-specific SnRK1 in vitro (Slocombe et al., 2002). Two proteins, Snf1-RELATED KINASE INTERACTOR1 (SKI1) and SKI2, from the moss *Physcomitrella patens* interact with SnRK1 and inhibit its activity in yeast (Thelander et al., 2007). However, these proteins do not share sequence similarity with SKINs.

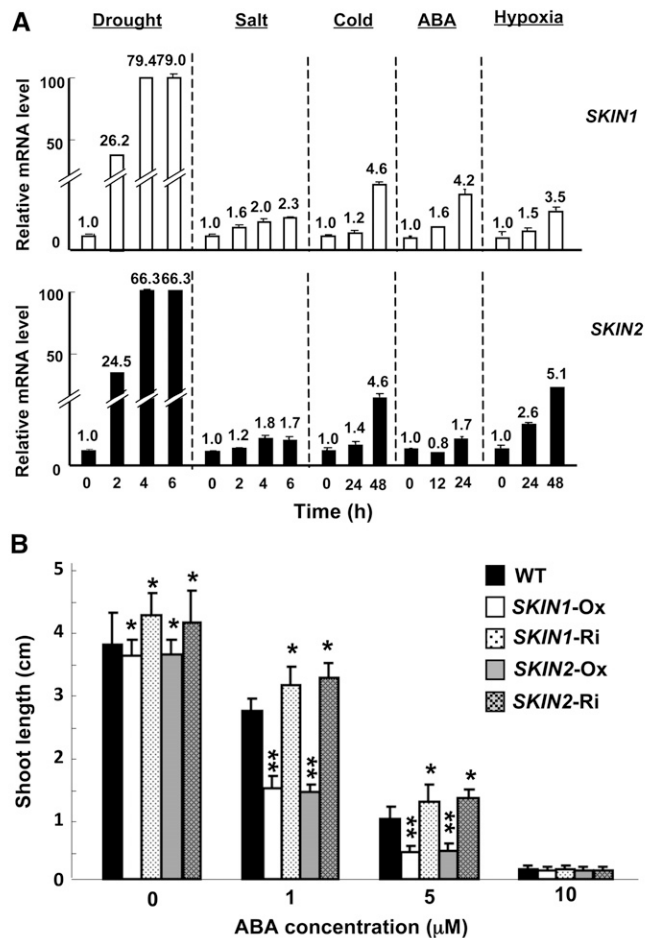

Figure 9. The Expression of *SKIN* Is Induced by Various Abiotic Stresses and ABA, and SKINs Promote ABA Sensitivity.

(A) Total RNA was purified from leaves of 2-week-old rice seedlings that had been air dried, treated with 200 mM salt, incubated at 4°C, or treated with 1 μM ABA, or from embryo tissues at the base of seedlings grown underwater (hypoxia), for various lengths of time. RNAs were subjected to quantitative RT-PCR analysis using primers specific for *SKIN1* and *SKIN2*. The highest mRNA level in the *y* axis was set to 100 for normalization. For the relative values given above the bars, the mRNA level at the zero time point for each treatment was assigned a value of 1, and mRNA levels of other samples were calculated relative to this value. Error bars indicate the SE for three replicate experiments.

(B) Seeds of transgenic lines SKIN1-Ox(O3), SKIN1-Ri(R3), SKIN2-Ox (O2), and SKIN2-Ri(R1) were germinated and grown in water containing various concentrations of ABA at 28°C under a 14-h-light/10-h-dark cycle for 6 d. Lengths of shoots were measured. Error bars represent SE (n = 8) at significance levels with the t test: *P < 0.1 and **P < 0.05. For photos of treated seedlings, see Supplemental Figure 9.

The KSD in SKINs is highly conserved in all SKIN homologs from monocots and dicots and along with a conserved C-terminal NLS represent the most distinct signature of the SKIN closely related family members identified in five plant species (Figure 1A). A few additional conserved domains are prominent in this protein family from monocots, suggesting distinct structural and/or functional features may exist between monocots and dicots. The function of the KSD was not elucidated for any member of the SKIN-related family previously; here, we showed that the KSD was necessary for antagonism of SnRK1A function (Figure 3D). The N-terminal amino acids 1 to 83 and 1 to 85 of SKIN1 and SKIN2, respectively, interacted with the SnRK1A-KD in yeast and plant cells (Figure 2; Supplemental Figure 3); however, the KSD does not reside within these regions (Figure 3D). It remains unclear how the SKIN-KSD interferes with SnRK1A function. A few domains are highly conserved in the N terminus of SKINs, and some of them are also monocot specific. The core domain in SKINs that interacts with the SnRK1A-KD remains to be better defined.

The only member of this protein family to have been functionally studied is *B. napus* KCP1, which is proposed to be a transcription factor that interacts with the histone deacetylase HDA19 and activates cold-inducible genes in *Arabidopsis* (Gao et al., 2003). The KID in Bn-KCP1 is essential for interaction with HDA19 and shares some functional similarities with the KID in the mammalian cAMP-responsive element binding protein family (Gao et al., 2003). The typical KID composed of RRXS (where X means any amino acid) (Gonzalez et al., 1991) is conserved in both SKIN1 and SKIN2 (as RRAS); however, its relative position in the entire protein amino acid sequence is quite distinct from that in Bn-KCP1 (Figure 1). Whether KID plays a function in the rice SKINs also remains to be determined.

Similar structural, functional, and regulatory interactions among subunits in the SnRK1 complex observed in yeast also exist in plants (Lu et al., 2007; Polge and Thomas, 2007; Halford and Hey, 2009). In yeast, Snf1 is in the cytoplasm in Glc-containing medium but largely translocated into the nucleus with the assistance of Gal83 upon Glc starvation (Vincent et al., 2001), and Snf1-RD is responsible for the interaction with Gal83 (Jiang and Carlson, 1997). The detection of SnRK1A-RD in the nucleus in –S medium (Figure 7) could be due to its lack of interactions with other cytoplasmic factors or efficient interactions with the rice Gal83 homolog. The high amount of cytoplasmic localization of SnRK1A-GFP was probably due to trapping by other cytoplasmic factors through the SnRK1A-KD or insufficient amounts of endogenous Gal83 homolog for conuclear import (Figure 7). Nevertheless, the accumulation of SnRK1A in the nucleus was increased significantly in cells in –S medium compared with in +S medium (Table 1).

The nuclear localization of Snf1 and SnRK1 has been shown to be essential for their protein kinase activities in yeast cells and *Arabidopsis* leaf mesophyll protoplasts, respectively (Vincent et al., 2001; Cho et al., 2012). It is unclear whether the nuclear localization of SnRK1A is essential for regulating the nutrient starvation signaling pathway. Previously, we showed that the expression of SnRK1A is induced by sugar starvation (Lu et al., 2007); therefore, the level of SnRK1A in the nucleus may be increased in –S medium. SKINs with or without NLSs maintained their antagonist activities (Figure 8C), indicating that the antagonism of SKINs against SnRK1A is independent of its cellular localization. Without ABA, SnRK1A is absent in the nucleus under +S conditions (Figure 10A, panel 3) but present in both the nucleus and cytoplasm under –S conditions (Figure 10A, panel 7). Although SnRK1A significantly enhanced α*Amy3* SRC promoter activity, the SRC activity was suppressed by SKINs to the

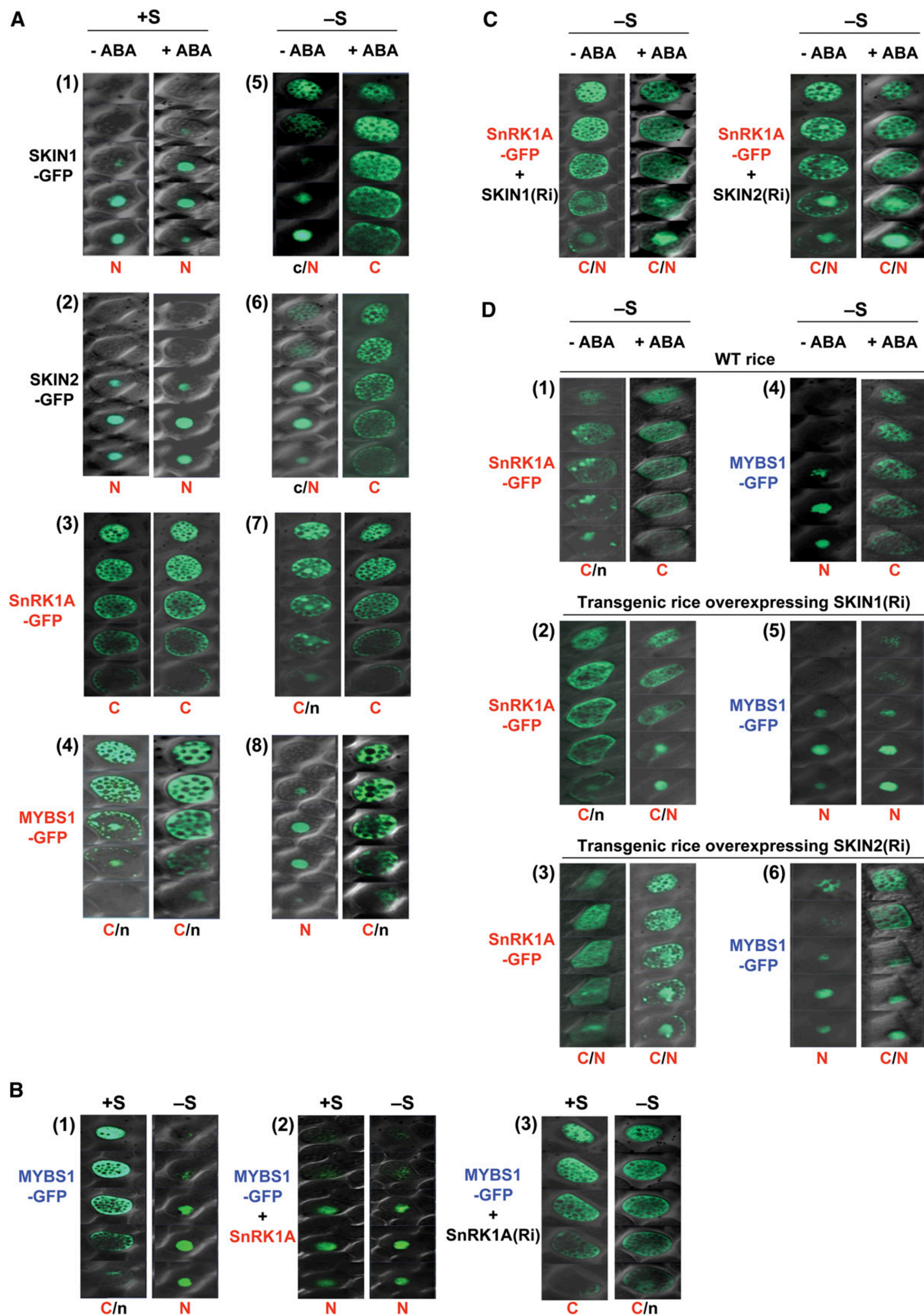

Figure 10. ABA Restricts SKINs, SnRK1A, and MYBS1 to the Cytoplasm under Sugar Starvation.

Barley aleurone was cotransfected with indicated plasmid constructs and incubated in +S or –S medium with ABA (+ABA) or without ABA (−ABA) for 48 h. Thirty optical sections of 0.9- to 1.1-μm thickness were prepared for each cell, and only five regularly spaced sections (sections 3, 9, 15, 21, and 27) are

background levels under –S conditions (Figures 3B and 8C). Consequently, the endogenous SnRK1A might be antagonized by SKINs in both the nucleus and cytoplasm.

The SnRK1A-Dependent Nutrient Starvation Signaling Pathway Plays a Key Role Regulating Source-Sink Communication

SnRK1 has been shown to regulate similar physiological activities in moss and land plants in terms of adaptation to limited energy. The double knockout mutant of two SnRK1 genes, *SNF1a* and *SNF1b*, of *P. patens* has impaired capability to mobilize starch reserves in response to darkness and can be kept alive only by feeding with Glc or providing constant light (Thelander et al., 2004). This mutant is unable to grow in a normal day (16 h)/night (8 h) cycle, presumably due to an inability to conduct normal carbohydrate metabolism under darkness (Thelander et al., 2004). Overexpression of two *Arabidopsis* SnRK1s, KIN10 and KIN11, increases primary root growth under low light with limited energy, while the double *kin10 kin11* knockdown mutant, generated by virus-induced gene silencing, impairs starch mobilization from leaves at night and, thus, seedling growth (Baena-González et al., 2007). Although SnRK1 has been proposed to regulate carbon partitioning between source and sink tissues in plants (Roitsch, 1999), the molecular and cellular mechanisms of its functions in source-sink communication are not well understood due to the inherent growth defects of *snrk1*-null mutants in land plants.

In rice, the *SnRK1* family has two members, *SnRK1A/OSK1* and *SnRK1B/OSK24*, with amino acid sequences sharing 74% identity (Takano et al., 1998; Lu et al., 2007). Our previous studies demonstrated that *SnRK1A*, but not *SnRK1B*, mediating the sugar starvation signaling cascade in growing seedlings (Lu et al., 2007). *SnRK1A* is thought to play a broader role in sugar regulation than *SnRK1B*, as *SnRK1A* is uniformly expressed in various growing tissues (including young roots and shoots, flowers, and immature seeds) (Takano et al., 1998). *SnRK1A* functions upstream of *MYBS1* and α*Amy3* SRC and plays a key role in regulating seed germination and seedling growth in rice (Lu et al., 2007). Expression of both *SKIN*s could be detected in all tissues in seedlings, mature plants, flowers, and immature panicles (Supplemental Figure 8). These studies indicate that *SnRK1A* and *SKIN*s are both expressed in germinating seeds and growing seedlings.

We showed that SKINs are sufficient and necessary for antagonism of SnRK1A function (Figure 3B). Furthermore, in transgenic rice, the source-sink communication regulating nutrient mobilization in the endosperm during early seedling growth stages is found to act through the SnRK1A-dependent nutrient starvation signaling pathway. The expression of SKINs is induced by sugar starvation, similar to components in the sugar starvation signaling pathway (Figure 4, panel 1). The accumulation of mRNA for MYBS1 and a variety of hydrolases was suppressed in SKIN-Ox lines under +S and –S conditions and only slightly increased in SKIN-Ri lines under +S but not under –S condition. SKIN1 and SKIN2 may have redundant functions, which would lead to insignificant responses in terms of enhancing endogenous gene expression in single-*SKIN* silenced lines under –S conditions.

Seedling shoot and root growth were inhibited in SKIN-Ox plants but promoted in SKIN-Ri plants, and these effects were more evident in the dark, conditions that mimic sugar starvation, than in the light/dark cycle that produce sugars through photosynthesis (Figures 5A and 5B). The delay and promotion of seedling growth were accompanied by the decrease and increase of α*Amy3* expression in SKIN-Ox and SKIN-Ri plants, respectively (Figure 5C). Moreover, growth of SKIN-Ox seedlings could be recovered by the application of exogenous sugars. Similar negative effects of SKIN overexpression on seedling growth under hypoxia were also observed (Figure 6). These studies indicate that SnRK1A-dependent sugar-demand signaling is necessary and sufficient for promoting sugar supply from the endosperm/aleurone (source), where hydrolases are produced for nutrient mobilization (Figures 4 and 5), to the germinated embryo/growing seedling (sink), where nutrients are used, and allows plants to grow under darkness or hypoxia. The expression of *EP3A* was regulated by SKINs similarly to α*Amy3* in seedlings (Figure 5C), indicating that although required at lower amounts, other nutrients likely are also coordinately produced through by the SnRK1A-regulated pathway.

Differential Cellular Localization of Key Factors Regulates Source-Sink Communication under Abiotic Stresses

Plants are frequently exposed to environmental stresses, such as water deficit, flooding, extreme temperatures, and high salinity, that inhibit photosynthesis, influence carbohydrate partitioning, constrain growth, and thus cause substantial yield loss. Several lines of evidences suggest that ABA might be a key signaling molecule regulating the SnRK1A-dependent sugar starvation signaling pathway via SKINs under abiotic stresses. First, the expression of *SKIN*s was induced by various abiotic stresses and ABA (Figure 9A). Second, ABA antagonizes the

Figure 10. (continued).

shown here. C and N indicate stronger GFP signals, and c and n indicate weaker GFP signals in the cytoplasm and nucleus, respectively. For more section images of each cell, see Supplemental Figure 11.

(A) Barley aleurone was transfected with *Ubi:SKIN1-GFP*, *Ubi:SKIN2-GFP*, *Ubi:SnRK1A-GFP*, or *Ubi:MYBS1-GF*P alone.

(B) Barley aleurone was transfected with *Ubi:MYBS1-GFP* alone (panel 1) or cotransfected with *Ubi:MYBS1-GFP* and *Ubi:SnRK1A* (panel 2) or with *Ubi:MYBS1-GFP* and *Ubi:SnRK1A(Ri)* (panel 3).

(C) Barley aleurone was cotransfected with *Ubi:SnRK1A-GFP* and *Ubi:SKIN(Ri)*.

(D) Wild-type rice or transgenic rice overexpression *Ubi:SKIN(Ri)* was transfected with *Ubi:SnRK1A-GFP* (panels 1 to 3) or *Ubi:MYBS1-GFP* (panels 4 to 6).

Table 1. ABA Inhibits the Nuclear Localization of SnRK1A

Location of SnRK1A-GFP	No. of Cells in Different Locations (% of Total)			
	+S		−S	
	−ABA	+ABA	−ABA	+ABA
Cytoplasm and nucleus	13 (19.7%)	6 (15.8%)	71 (64.0%)	7 (8.0%)
Cytoplasm	53 (80.3%)	32 (84.2%)	40 (36.0%)	81 (92.0%)
Total cell number	66	38	111	88

Barley aleurone was transfected with *Ubi:SnRK1A-GFP* and incubated in +S or –S medium with ABA (+ABA) or without ABA (−ABA) for 48 h. Percentages indicate the number of cells with GFP distribution in the indicated category divided by the total number of cells examined.

function of SnRK1A similarly to SKINs (Figure 10). Third, ABA promotes the interaction between SnR1A and SKINs (Figure 2D). Fourth, overexpression of SKINs promotes the ABA-mediated inhibition of seedling growth (Figure 9B). The notion is further supported by the discovery that sugar starvation promotes, whereas ABA inhibits, the nuclear localization of SnRK1A (Table 1). Interestingly, SKINs were relocalized from the nucleus to the cytoplasm, accompanied by the exclusion of SnRK1A and MYBS1 from the nucleus under –S conditions in the presence of ABA (Figure 10A, panels 5 to 8). The exclusion of SnRK1A from the nucleus resulted from its interaction with SKINs in the cytoplasm, as the accumulation of SnRK1A in the nucleus was significantly enhanced by silencing of *SKIN*s in barley aleurone cells transiently overexpressing SKIN(Ri) (cf. Figure 10C with Figure 10A, panel 7) and in transgenic rice aleurone cells stably overexpressing SKIN(Ri) (Figure 10D, compare panels 2 and 3 with panel 1) under –S conditions with ABA treatment.

SnRK1 has been shown to regulate enzyme activity in the cytoplasm directly as well as acting as a regulator of gene expression (Halford and Hey, 2009). SnRK1A seems to regulate the sugar starvation signaling pathway through various mechanisms. Previously, we showed that SnRK1A activates *MYBS1* promoter activity and likely also phosphorylates MYBS1 directly (Lu et al., 2007). Additionally, the nuclear import of MYBS1 was inhibited by sugars and promoted by sugar starvation (Figure 10B, panel 1) as has been reported previously (Hong et al., 2012). Here, we further show that SnRK1A is necessary and sufficient for promoting the nuclear import of MYBS1 under +S and –S conditions, respectively (Figure 10B, panels 2 and 3). However, as significant amounts of SnRK1A are localized in the cytoplasm compared with the nucleus, it is unclear how MYBS1 is regulated by SnRK1A in the cytoplasm or nucleus. The recovery of nuclear localization of SnRK1A by *SKIN* silencing also recovered the nuclear enrichment of MYBS1 in transgenic rice under –S conditions with ABA treatment (Figure 10D, compare panels 5 and 6 with panel 4), indicating that the nuclear localization of SnRK1A and MYBS1 are tightly linked and suppressed by SKINs. It is conceivable that SKIN in the cytoplasm prevents the nuclear localization of SnRK1A and MYBS1, rendering them ineffective in upregulating α*Amy3* SRC activity.

In summary, as illustrated in Figure 11, the sink strength serves as a driving force and SnRK1A plays a central regulatory role in source-sink communication. Differential cellular localization appears to be a key factor in this regulatory process. It has been demonstrated previously that the crucial GA regulator MYBGA facilitates the function and nuclear import of MYBS1 (Chen et al., 2006; Hong et al., 2012). Here, we further showed that sugar and nutrient demands, which are important signals from the sink tissue (germinating embryo and seedling), trigger the conuclear localization of two starvation signaling factors

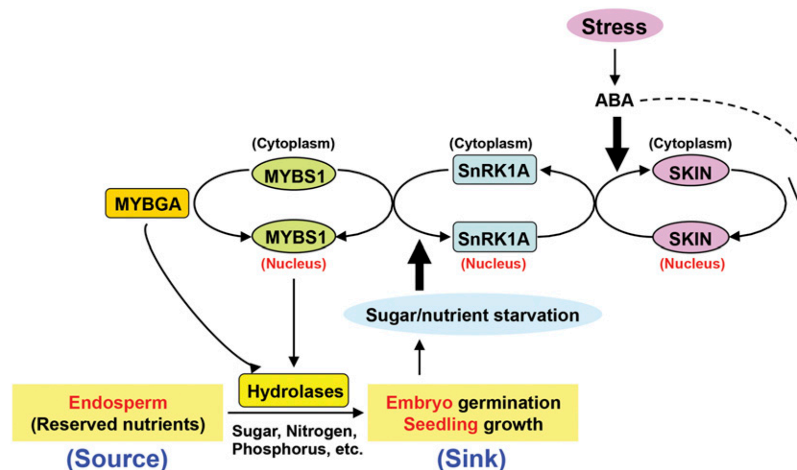

Figure 11. SnRK1A Plays a Central Role Regulating the Source-Sink Communication for Nutrient Mobilization in Cereal Seedlings, and Differential Cellular Localization of Key Factors Regulates the Process under Abiotic Stress.

Sugar starvation signals from sink tissues (germinating embryo and seedling) in demand of nutrients trigger the conuclear localization of SnRK1A and MYBS1, leading to the induction of hydrolases necessary for the mobilization of nutrients in the source tissue (endosperm). Stress and ABA facilitate the cytoplasmic localization of SKIN, which binds to SnRK1A and prevents SnRK1A and MYBS1 from entering the nucleus. More details are described in the text.

(i.e., SnRK1A and MYBS1), leading to the induction of α-amylase and other hydrolases necessary for the mobilization of nutrients in the source tissue (endosperm). Furthermore, stress and ABA not only induce the synthesis of SKIN but also facilitate its exit from the nucleus to the cytoplasm or prevent its import from the cytoplasm to the nucleus. The cytoplasmic SKIN in turn binds to SnRK1A and prevents SnRK1A and MYBS1 from entering the nucleus, eventually leading to the suppression of hydrolase production. However, since SnRK1A is highly accumulated in the cytoplasm even under sugar starvation, and SnRK1 protein kinase has substrates in the cytoplasm (Halford and Hey, 2009), the possibility that SnRK1A may also regulate the sugar starvation signaling pathway in the cytoplasm could not be ruled out. It is noted that SKIN is localized in the nucleus in the absence of ABA or stress, but the functional relevance of this is unknown.

The current global climate changes tend to shift weather to more extreme perturbations (e.g., high and low temperatures, flooding, and water scarcity), which aggravate a world crop productivity that has already plateaued (Lucas, 2013). As the world population rises rapidly, development of crops that are more tolerant of various abiotic stresses while maintaining yield potentials remains an important and challenging task. In plants, SnRK1s regulate many aspects of growth and development during vegetative and reproductive stages (Polge and Thomas, 2007). To alleviate the negative effect of SKIN overexpression on plant growth, understanding the mode of action of SKINs on the restriction of plant growth temporally and spatially under abiotic stresses may facilitate the improvement of cereals with enhanced tolerance of abiotic stresses without yield penalty.

METHODS

Plant Materials

Rice (*Oryza sativa* cv *Tainung* 67) and barley (*Hordeum vulgare* cv *Himalaya*) were used in this study. Embryo calli were induced in the Murashige and Skoog (MS) medium containing 3% Suc and 10 mM 2,4-D for 5 d. For hydroponic culture of rice seedlings, seeds were sterilized with 1.5% NaOCl plus Tween 20 for 1 h, washed extensively with distilled water, and germinated in a Petri dish with wetted filter papers at 28°C under a 14-h-light/10-h-dark condition unless otherwise indicated. The SnRK1A Knock-down transgenic rice was generated previously (Lu et al., 2007).

Our previous studies showed that sugar regulation of MYBS1 function in barley aleurone (Lu et al., 2002), SnRK1A regulation of MYBS1 function using rice embryos (Lu et al., 2007), CIPK15 regulation of SnRK1A expression using rice suspension cells (Lee et al., 2009), and regulation of MYBS1 and MYBGA interaction and nucleocytoplasmic shuttling of MYBS1 using rice and barley aleurone (Hong et al., 2012) are all consistent regardless of different systems being used. For transient expression assay of luciferase activity, aleurone/embryos are preferred compared with rice endosperms due to easier manipulation for large-scale sample preparation, particle bombardment, and protein extraction. For cellular localization of GFP fused to target protein, barley aleurone is preferred as the rice aleurone has a single layer of cells and is fragile, while the barley aleurone has three to four layers and is relatively stronger and easier to manipulate under the microscope. Additionally, barley or rice aleurone cells have relatively much larger nuclei but smaller vacuoles compared with onion epidermal cells, which facilitate the study of nuclear import of proteins.

Plasmids

Plasmid p3Luc.18 contains α*Amy3* SRC (−186 to −82 upstream of the transcription start site) fused to the *CaMV35S* minimal promoter-*Adh1* intron-luciferase cDNA (*Luc*) fusion gene (Lu et al., 1998). Plasmid pUG contains β-glucuronidase cDNA (*GUS*) fused between the *Ubi* promoter and *Nos* terminator (Christensen and Quail, 1996). Plasmid pUbi-SnRK1A-Nos contains *SnRK1A* full-length cDNA between a *Ubi* promoter and a *Nos* terminator (Lu et al., 2007). Plasmid pUbi-SnRK1A-KD-Nos contains a cDNA encoding the KD of SnRK1A between the *Ubi* promoter and *Nos* terminator (Lu et al., 2007). Plasmid pUbi-SnRK1A-RD-Nos contains a cDNA encoding the RD of SnRK1A between the *Ubi* promoter and a *Nos* terminator (Lu et al., 2007). Plasmid p5xUAS-35SminiP-Luc-Nos contains five tandem repeats of *UAS* fused to the upstream of *CaMV35S* minimal promoter-*Adh1* intron-*Luc* fusion gene (Lu et al., 1998). pAHC contains the Luc cDNA between the *Ubi* promoter and the *Nos* terminator (Bruce et al., 1989).

Yeast Two-Hybrid Assay

For cloning of SnRK1A-interacting proteins, a yeast (*Saccharomyces cerevisiae*) two-hybrid cDNA library was constructed by fusion of cDNAs, which were derived from poly(A) mRNAs isolated from rice suspension cells starved of Suc for 8 h, with the GAD DNA in the phagemid vector pAD-GAL4-2.1. Approximately 10^6 transformants were subjected to two-hybrid selection on a synthetic complete medium lacking Leu, Trp, and His but containing 15 mM 3-amino-1,2,4-triazole (3-AT). The expression of the *HIS3* reporter gene allowed colonies to grow on the selective medium, and putative positive transformants were tested for the induction of other reporter genes, such as *lacZ*. Positive colonies were assessed by retransformation into yeast, and cDNA inserts were identified by DNA sequencing analysis.

For studying the interaction between SnRK1A and SKIN, the Yeast-marker Transformation System 2 was used as described by the manufacturer (Clontech). The two-hybrid assay was performed in yeast (*S. cerevisiae*) strains AH109 and Y187 (Clontech) that contain reporter genes *HIS3* and *lacZ* under the control of a *GAL4*-responsive element (Chien et al., 1991). Colonies were grown on selective medium and tested for β-galactosidase activity by a colony-lift filter assay method (Breeden and Nasmyth, 1985).

Phylogenetic Analysis

The SKIN family members were identified by BLAST search of The Institute for Genomic Research database (http://www.tigr.org/tdb/e2k1/osa1/irgsp.shtml), the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST/), and the Rice Genome Annotation (RiceGAAS) database (http://ricegaas.dna.affrc.go.jp) with the full-length SKIN1 and conserved KSD domain. Deduced amino acid sequences of SKINs were aligned with the ClustalW2 (version2.0.8) and AlignX (Vector NTI, version 9.0.1; Invitrogen) programs (Supplemental Data Sets 1 and 2). The unrooted phylogenetic tree was constructed using the MEGA 3.1 phylogenetic analysis program (Supplemental Data Set 3). Evolutionary relationships were deduced using the neighbor-joining algorithm. Bootstrapping was performed using the MEGA3.1 program with 1000 replicates. The Knowledge-Based Oryza Molecular Biological Encyclopedia database (http://cdna01.dna.affrc.go.jp/cDNA) was used for the cDNA search.

Plasmid Construction

The Gateway gene cloning system (Invitrogen) was used to generate all constructs. First, destination vectors that could be used in all experiments were generated. For constructs used in the rice embryo transient

expression assays, plasmid pAHC18 was digested with *Bam*HI to remove the *luciferase* cDNA insert, followed by the addition of a double-HA tag, generating pAHC18-2HA. pAHC18-2HA was linearized with *Eco*RV, and a *ccdB* DNA fragment flanked by *attR1* and *attR2* was inserted between the *Ubi* promoter and *Nos* terminator, generating the destination vector pUbi-2HA-ccdB-Nos. For constructs used in the rice stable transformation, pUbi-2HA-ccdB-Nos was linearized with *Hin*dIII and inserted into the binary vector pSMY1H (Ho et al., 2000), which had been linearized with the same restriction enzyme, generating the destination vector pSMY1H-pUbi-2HA-DEST-Nos.

For constructs used in the yeast two-hybrid assay, pAS2-1 containing the *ADH1* promoter fused to the Gal4 binding domain DNA (*ADH1-GAD*) and pGAD424 containing the *ADH1* promoter fused to Gal4 activation domain DNA (*ADH1-GBD*) were linearized with *Sma*I, and the *ccdB* DNA fragment flanked by *attR1* and *attR2* sties was inserted downstream of *ADH1-GAD* or *ADH1-GBD*, generating destination vectors *GAD-ccdB* and *GBD-ccdB*. The coding sequences of *SKIN1*, *SKIN2*, and *SnRK1A* (wild type or truncated) were synthesized by PCR and inserted between the *attL1* and *attL2* sites in pENTR/Directional TOPO cloning kits (Invitrogen), generating pENTR-SKIN and pENTR-SnRK1A. Various genes fused to the C termini of *GAD* and *GBD* were driven by the *ADH1* promoter through the GATEWAY lambda recombination system (LR Clonase II enzyme mix kit; Invitrogen).

For the SKIN RNA interference construct, two 307- and 245-bp fragments derived from the 3′ untranslated region of *SKIN1* and *SKIN2* cDNA were synthesized by PCR and fused in antisense and sense orientations flanking the 750-bp *GFP* cDNA. The *SKIN* RNA interference fragments were inserted into pENTR/D-TOPO, generating pENTR-SKIN (Ri). pENTR-SKIN(Ri) was recombined with pSMYY1H-Ubi-ccdB-GFP-ccdB-Nos through the Gateway lambda recombination system, generating pSMY1H-SKIN(Ri).

For protein cellular localization, the full-length *SKIN* cDNA was inserted into pENTR/D-TOPO, generating the entry vector *pENTR-SKIN*. *SKIN* in *pENTR-SKIN* was then inserted downstream of pUbi-2HA-GFP in pSMY1H-pUbi-2HA-GFP-DEST-Nos through the Gateway lambda recombination system, generating pSMY1H-Ubi-2HA-GFP-SKIN-Nos.

For construction of SKIN without NLS (SKINΔNLS), *SKIN* cDNA lacking DNA encoding the NLS (KRRR) was inserted into pENTR/D-TOPO, generating the entry vector *pENTR-SKINΔNLS*. *SKINΔNLS* in *pENTR-SKINΔNLS* was then inserted downstream of pUbi-2HA in pUbi-2HA-DEST-GFP-Nos through the Gateway lambda recombination system, generating pUbi-2HA-SKINΔNLS-GFP-Nos, and inserted downstream of pUbi-GFP in pUbi-GFP-DEST-Nos, generating pUbi-GFP-SKINΔNLS-Nos.

Rice Transformation

Plasmids pSMY1H-pUbi-2HA-SKIN and pSMY1H-SKIN-Ri were introduced into *Agrobacterium tumefaciens* strain EHA105, and rice transformation was performed as described (Ho et al., 2000).

Rice Embryo and Barley Aleurone Transient Expression Assays

Rice embryos were prepared for particle bombardment as described (Chen et al., 2006). The rice embryos were bombarded with reporter, effectors, and internal control at a ratio of 4:2:1 for single effector or 4:2:2:1 for two effectors. The internal control (*Ubi*:*GUS*) was used to normalize the reporter enzyme activity because different transformation efficiency might occur in each independent experiment. Bombarded rice embryos were divided into two halves, with half being incubated in MS liquid medium containing 100 mM Glc, and the other half grown in MS liquid containing 100 mM mannitol, for 24 h. Total proteins were extracted for embryos with a cell lysis buffer (0.1 M K-phosphate, pH 7.8, 1 mM EDTA, 10% glycerol, 1% triton X-100, and 7 mM β-mercaptoethanol). GUS assay buffer (0.1 M Na-phosphate, 20 mM EDTA, 0.2% sarcosine, 0.2% Triton X-100, and 20 mM β-mercaptoethanol) was used for GUS activity assay. The activity assay of GUS and luciferase were described elsewhere (Lu et al., 1998). All bombardments were repeated at least three times.

The barley aleurone/endosperm transient expression assays were performed as described (Hong et al., 2012). Each independent experiment consisted of three replicates, with six endosperms for each treatment, and was repeated three times with similar results. Luciferase and GUS activity assays were performed as described (Hong et al., 2012). Error bars indicate the standard error for three replicate experiments.

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from leaves of rice seedlings with the Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega). Five to 10 μg of RNA was used for cDNA preparation using reverse transcriptase (Applied Biosystems), and cDNA was then diluted to 10 ng/μL for storage. Five microliters of cDNA was mixed with primers and the 2× Power SYBR Green PCR Master Mix reagent (Roche) and applied to an ABI 7500 Real-Time PCR system (Applied Biosystems). The quantitative variation between different samples was evaluated by the Δ-Δ cycle threshold method, and the amplification of 18S rRNA was used as an internal control to normalize all data.

Antibodies and Immunoblot Analysis

The anti-SnRK1 polyclonal antibodies were produced against synthetic peptides (5′-RKWALGLQSRAHPRE-3′, amino acid residues 385 to 399) derived from SnRK1A. Mouse monoclonal antibody against HA tag was purchased from Sigma-Aldrich. The immunoblot analysis with the anti-SnRK1A primary antibody diluted at 1:2000 was performed as described (Lu et al., 2007). Horseradish peroxidase–conjugated antibody against rabbit immunoglobulin G (Amersham Biosciences) was used as a secondary antibody. Protein signals were detected by chemiluminescence with ECL (Amersham Bioscience). Ponceau S staining of proteins was used for a loading control.

Seed Germination in Air or under Water

The experiment was performed as described (Lee et al., 2009). For germination in air, seeds were placed on 3M filter papers wetted with water in a 50-mL centrifuge tube that contained half-strength MS agar medium. For germination under water, seeds were placed in a 50-mL centrifuge tube, autoclaved water was carefully poured into the tube to avoid any air bubbles, and tubes were sealed with lids.

Confocal Microscopy for Detection of GFP

Detection of cellular localization of SKIN-GFP, SnRK1A-GFP, and MYBS1-GFP fusion proteins were performed as described (Hong et al., 2012). Embryo-less barley and rice seeds were sterilized with 1% NaOCl for 30 min and incubated in a buffer containing 20 mM $CaCl_2$ and 20 mM sodium succinate, pH 5.0, for 4 d. Aleurone layers were isolated by scratching away starch in the endosperm with a razor blade. Four aleurone layers were arranged in a 10-cm dish for bombardment. Aleurone layers expressing GFP were examined with a Zeiss confocal microscope (LSM510META) using a 488-nm laser line for excitation and a 515- to 560-nm long-pass filter for emission.

Primers

All primers used for the cloning of plasmid constructs are listed in Supplemental Tables 1 and 2. Primers used for quantitative RT-PCR are listed in Supplemental Table 3.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *SKIN1* (AK060116), *SKIN2* (AK072516), *SnRK1A* (AB101655.1), *MYBS1* (AY151042.1), *aAmy3/RAmy3D* (M59351.1), *aAmy8/RAmy3E* (M59352.1), *EP3A* encoding Cys protease (AF099203), *Lip1* encoding GDSL-motif lipase (AK070261), *Phospho1* encoding phosphatase-like (AK061237), *ST* encoding sugar transporter family protein (AK069132), Zm-*MTD1* (ACG28615.1), Zm-*KCP* (ZAA48125.1), Sorghum02g028960 (XP_002462609.1), At-*KCP* (NC_003076.8), At-*KCL1* (NC_003075), At-*KCL2* (NC_003071), Bn-*KCP1* (AY211985), and Zm-MTD186T7R4 (EU961029).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. SKIN1 and SKIN2 Interact with SnRK1A in Yeast.

Supplemental Figure 2. Amino Acid Sequence Alignment between SKIN1 and SKIN2.

Supplemental Figure 3. The N-Terminal Amino Acids 1 to 83 of SKIN1 Interact with the Kinase and Autoinhibitory Domains of SnRK1A in Yeast.

Supplemental Figure 4. Ponceau S Staining of Nitrocellulose Membrane to Visualize the Protein Loading in Immunoblot Analysis.

Supplemental Figure 5. SKINs Suppress Sugar Production Necessary for Underwater Seedling Growth.

Supplemental Figure 6. SKIN1 and SnRK1A Interact Primarily in the Cytoplasm.

Supplemental Figure 7. SKINs without NLSs Are Localized in the Cytoplasm.

Supplemental Figure 8. *SKIN* Is Expressed in Most Rice Tissues.

Supplemental Figure 9. Growth of Transgenic Rice Overexpressing SKIN Is More Sensitive to ABA Inhibition.

Supplemental Figure 10. ABA and Sorbitol Suppress the Function of SnRK1A in Activation of the α*Amy3* SRC Promoter.

Supplemental Figure 11. ABA Restricts SKINs, SnRK1A, and MYBS1 to the Cytoplasm under Sugar Starvation.

Supplemental Table 1. Primer List.

Supplemental Table 2. Primer Pairs Used for Plasmid Construction.

Supplemental Table 3. Primer List for Quantitative RT-PCR Analysis.

Supplemental Data Set 1. Text File of Alignments Used to Generate the Phylogenetic Tree Shown in Figure 1B.

Supplemental Data Set 2. Text File of Alignments Used to Generate the Phylogenetic Tree Shown in Figure 1B.

Supplemental Data Set 3. Text File of Alignments Used to Generate the Phylogenetic Tree Shown in Figure 1B.

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AUTHOR CONTRIBUTIONS

C.-R.L. and S.-M.Y. designed the research. C.-R.L., K.-W.L., C.-Y.C., Y.-F.H., J.-L.C., C.-A.L., K.-T.C., and T.-H.D.H. performed the research. C.-R.L., T.-H.D.H., and S.-M.Y. wrote the article.

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