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The hybrid Four-CBS-Domain KINβγ-subunit functions as the canonical γ subunit of the plant energy sensor SnRK1

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Summary

The AMPK/SNF1/SnRK1 protein kinases are a family of ancient and highly conserved eukaryotic energy sensors that function as hetero-trimeric complexes. These typically comprise catalytic α and regulatory β and γ subunits, the latter functioning as the energy sensing modules of animal AMPK through adenosine nucleotide binding. The ability to accurately monitor and adapt to changing environmental conditions and energy supply is essential for optimal plant growth and survival, but mechanistic insight in the plant SnRK1 function is still limited. In addition to a family of γ-like proteins, plants also encode a hybrid βγ protein, that combines the four-CBS-domain (FCD) structure in γ subunits with a glycogen binding domain (GBD), typically found in β-subunits. We used integrated functional analyses by ectopic SnRK1 complex reconstitution, yeast mutant complementation, in-depth phylogenetic reconstruction, and a seedling starvation assay to show that only the hybrid KINβγ protein, that recruited the GBD around the emergence of the green, chloroplast-containing plants, acts as the canonical γ-subunit required for hetero-trimeric complex formation. Mutagenesis and truncation analysis further show that complex interaction in plant cells and γ subunit function in yeast depend on both a highly conserved FCD and a pre-CBS domain, but not the GBD. In addition to novel insight in canonical AMPK/SNF/SnRK1 γ subunit function, regulation and evolution, we provide a new classification of plant *FCD* genes as a convenient and reliable tool to predict regulatory partners for the SnRK1 energy sensor and novel *FCD* gene functions.

Keywords

energy signaling; SnRK1; SNF1; AMPK; γ-subunit; *Arabidopsis thaliana*; *Saccharomyces cerevisiae*

Introduction

All living organisms require a continuous input of energy to maintain their thermodynamically unlikely level of organization and activity. Early in evolution, eukaryotic

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cells developed a sophisticated energy sensing protein kinase complex to monitor metabolic status and maintain energy homeostasis both during normal growth and development and in stress conditions. The animal AMP-activated kinase (AMPK), yeast SNF1 (Sucrose non-fermenting1) and plant SnRK1 (SNF1-related kinase 1) kinases act as conserved fuel gauges that share both function and a characteristic heterotrimeric structure (Baena-González et al, 2007; Baena-Gonzalez & Sheen, 2008; Ghillebert et al, 2011; Hardie et al, 2012; Hedbacker & Carlson, 2008; Polge & Thomas, 2007)(Figure 1A). Upon environmental stress and energy limitation, these kinases generally down-regulate ATP consuming biosynthetic processes, while stimulating energy generating catabolic reactions through gene expression and post-transcriptional regulation. While several triggers of SnRK1 signaling and a large number of conserved target genes have been identified, SnRK1 complexes also show particular features (Polge & Thomas, 2007) and important mechanistic links are still missing. Plants produce their own energy-rich organic molecules by solar energy-driven photosynthesis as sessile organism, therefore depending heavily on the ability to accurately monitor and adapt to changing environmental conditions for optimal growth and survival.

The catalytic AMPK/SNF1/SnRK1 α-subunits typically function in hetero-trimeric complexes and, in addition to a highly conserved Ser/Thr kinase domain, contain a large C-terminal domain for interaction with regulatory β and γ subunits (Figure 1A). The β subunits, which exhibit a scaffolding function and contribute to substrate binding and complex localization, are characterized by an internal kinase-interacting sequence (or KIS domain) and a C-terminal γ-subunit interacting ASC (association with SNF1 complex) domain (Jiang & Carlson, 1997). Overlapping with the KIS domain, all β-subunits also harbor a glycogen-binding domain (GBD) (Hudson et al, 2003; Polekhina et al, 2003). The γ-subunit, finally, consists of a divergent N-terminus, a recently identified pre-CBS domain (Viana et al, 2007) and a highly conserved domain with four cystathionine β-synthase (CBS) motifs (Lang et al, 2000). Tandem pairs of these CBS motifs make up the two adenosine nucleotide (and S-adenosylmethionine, SAM/AdeMet) binding sites (Bateman domains) that function as the energy sensing modules of AMPK (Kemp, 2004; Scott et al, 2004). CBS motif pairs are also found in other types of proteins, including SAM-activated cystathionine β-synthase (hence the name), CLC voltage-dependent chloride channels and IMP (inosine-5'-monophosphate) dehydrogenase and, like for the γ-subunit, mutations in their conserved nucleotide binding residues have been associated with a variety of hereditary diseases in man (Ignoul & Eggermont, 2005; Scott et al, 2004).

The AMPK/SNF1/SnRK1 kinases are rigorously controlled by a phospho-switch; phosphorylation of the α-subunit T-loop is a prerequisite for activity. The AMP:ATP ratio, a sensitive indicator of cellular energy supply, was established as the major regulator of AMPK activity by binding to the γ-subunit (Carling et al, 1989), but while there is a clear correlation between cellular adenine nucleotide levels and its activation state, yeast SNF1 is not directly activated by AMP (Wilson et al, 1996). Similarly, the plant SnRK1 kinases are not directly activated by AMP (Sugden et al, 1999). However, both in yeast and mammals, AMP is believed to stabilize the active form of the complex by triggering a conformational change that makes it resistant to dephosphorylation; similar effects are reported for ADP (Oakhill et al, 2011; Rubenstein et al, 2008; Sanders et al, 2007; Xiao et al, 2011).

Plants typically encode several isoforms of each subunit with environmentally controlled and developmental stage or tissue-specific expression patterns (Bouly et al, 1999; Bradford et al, 2003; Buitink et al, 2003) and alternative splicing further increases the number of putative complexes (Gissot et al, 2006). Based on structural similarity with animal and yeast subunits, an extended family of plant γ-like subunits (comprising SNF4- and PV42-like proteins) can be discerned (Gissot et al, 2006; Robaglia et al, 2012). While the Arabidopsis KINγ (At3g48530) was not shown to complement the yeast *snf4* γ subunit mutant (Bouly et al, 1999), tomato (LeSNF4) and Medicago (MtSNF4b) γ-like subunits did (Bolingue et al, 2010; Bradford et al, 2003). In addition, plants have acquired a unique hybrid βγ-subunit, combining an N-terminal GBD with a 4CBS motif C-terminal γ part (Gissot et al, 2006; Lumbreras et al, 2001), and a truncated β-subunit (β3), lacking the N-terminal extension and GBD domain (Gissot et al, 2004). The βγ-subunit functionally complements a yeast γ-subunit (*snf4*) mutant, interacts with α- and β-subunits in yeast two-hybrid assays and assembles into plant-specific SnRK1 complexes (Bitrian et al, 2011; Gissot et al, 2006; Kleinow et al, 2000; Lopez-Paz et al, 2009). It was also suggested that the KINβγ subunit can form complexes without β-subunits or homo-dimers (Lopez-Paz et al, 2009). The truncated β3 protein is also functional in yeast cells and interacts with the catalytic α-subunits and the βγ-subunit in yeast two-hybrid assays (Gissot et al, 2004; Polge et al, 2008). Thus, different types of complexes, with KINγ or KINβγ subunits, respectively, have been proposed to be functional in plants (Lopez-Paz et al, 2009; Polge et al, 2008; Robaglia et al, 2012), but *in vivo* interaction studies with regulatory subunits are lacking. In addition, the diverse roles of plant 4CBS domain proteins in seed stress responses (SNF4-like) (Bolingue et al, 2010; Bradford et al, 2003; Rosnoblet et al, 2007) and reproductive development (PV42-like) (Fang et al, 2011) have also been linked to SnRK1 signaling.

To analyse whether plant SnRK1 γ-function is indeed exerted by different members of the CBS domain protein family and how more prototypical γ and plant-specific βγ hybrid proteins differ in function, we used a comprehensive approach with functional yeast complementation, leaf cell transient expression and whole plant assays. To our surprise, our data showed that in plants KINγ does not interact with β-subunits in vivo and that only the hybrid KINβγ protein acts as the canonical γ-subunit, required for hetero-trimeric complex formation with α- and β-subunits. Mutagenesis and truncation analyses showed that complex interaction and (heterologous) γ-subunit function depend on both a highly conserved CBS and a pre-CBS domain. However, this does not require the GBD, suggesting other, plant-specific functions for this domain. Phylogenetic reconstruction and functional analysis by yeast mutant complementation further indicated that in higher plants only the subclass of 4CBS domain (FCD) proteins that acquired a glycogen binding domain (GBD) has retained the canonical γ-subunit function. Based on the resolved phylogeny, we propose a new classification of plant *FCD* genes as a convenient and reliable tool to predict regulatory partners for the SnRK1 energy sensor or novel *FCD* gene functions. Finally, consistent with a unique role for the hybrid protein in SnRK1 signaling, Arabidopsis KINγ KO plants showed wild type starvation responses in a novel seedling assay, while transient knockdown of KINβγ affected SnRK1 target gene expression. Our findings have important implications for SnRK1 regulation, revealing plant-specific adaptations to a conserved eukaryotic mechanism.

Results

Specific KINβγ binding to the regulatory KINβ-subunits

Co-immunoprecipitation experiments in transiently transfected Arabidopsis leaf mesophyll protoplasts (Baena-Gonzalez et al., 2007) showed that the SnRK1 catalytic a-subunit, KIN10, was able to bind all three regulatory β-subunits, and that these interactions depended on the kinase-interacting sequence (KIS) (Jiang & Carlson, 1997) (Figure 1B). Deletion (KINβ2 w/o ASC) or truncation (KINβ2–242) of the ASC (association with SNF1 complex) domain, abolishing normal binding between regulatory β- and γ-subunits (Jiang & Carlson, 1997), eliminated the interaction between KINβ2 and KIN10, indicating the requirement of a second regulatory subunit for SnRK1 complex formation (Figure 1B). Since Arabidopsis was thought to have two regulatory γ-subunits, KINγ (At3g48530) and KINβγ (At1g09020), we tested both. Surprisingly, no binding was found between the KINγ and KINβ regulatory subunits, but strong interactions could be observed between the β-regulatory subunits and the hybrid KINβγ protein (Figure 1C), suggesting that only the latter contributes to SnRK1 complex formation. Furthermore, KINβγ was able to directly bind to KIN10, albeit not tightly (Figure 1B), while deletion or truncation of the ASC domain of the β2-regulatory subunit abolished binding between KINβγ and KINβ2 (Figure 1D), confirming the potential role of KINβγ in SnRK1 complex formation. All interactions were confirmed by pull-down in both directions.

The hybrid KINβγ uniquely confers canonical γ-subunit functionality

Heterologous yeast mutant complementation is well established for determining SnRK1/AMPK functionality (Gissot et al, 2004; Gissot et al, 2006; Lumbreras et al, 2001; Polge et al, 2008). Deletion of the yeast γ-subunit gene, *SNF4*, does not affect growth on fermentable carbon sources like glucose, but leads to severe growth defects on media with non-fermentable carbon sources (Neigeborn & Carlson, 1984) (Figure 2A). Transformation of the *snf4Δ* strain with Arabidopsis KINγ could not restore growth on glycerol/ethanol medium, while growth on glucose was not affected (Fig 2A). In contrast, expression of yeast Snf4 and Arabidopsis KINβγ both restored growth of the yeast *snf4Δ* strain on non-fermentable carbon sources, suggesting an important and conserved role for KINβγ in SnRK1 functioning (Figure 2A). All proteins tested were efficiently expressed in yeast (Figure 2B). As KINβγ is a hybrid protein of a regulatory γ-subunit and the GBD domain present in regulatory β-subunits (Lumbreras et al, 2001), and hence is sometimes classified as regulatory β-subunit (Robaglia et al, 2012), we also expressed it in the yeast triple β-subunit deletion strain (*sip1Δ sip2Δ gal83Δ*). No complementation could be observed, confirming that KINβγ does not have β-functionality (Figure S1).

Dual requirement for yeast snf4Δ complementation

In order to better understand structural requirements for yeast *snf4Δ* complementation and therefore γ-subunit functionality, we systematically generated and tested different truncation and fusion proteins. Interestingly, deletion of the GBD domain (KINβγ 151–487) did not seem to affect complementation (Figure 3A). Additional truncation of the pre-CBS domain (KINβγ171–487 and KINβγ Δ151–170), however, compromised the ability of *snf4Δ* complementation (Viana et al, 2007) (Figure 3A). Expression of the pre-CBS domain

together with the GBD domain (KINβγ 1–170) was not sufficient for growth on non-fermentable carbon sources (Fig 3A). Also, when the pre-CBS domain of the KINβγ was fused to the FCD part of KINβγ, no growth was observed, suggesting that in addition to a functional pre-CBS domain, a functionally conserved FCD is required for yeast *snf4Δ* complementation (Figure 3A). All truncated and fused KINβγ proteins were efficiently expressed in yeast (Figure 3B).

These modified proteins were then also transiently expressed in leaf cell protoplasts together with KINβ2 (Figure 3C). Deletion of the GBD domain did not affect interaction of KINβγ with KINβ2. In contrast, removal of the pre-CBS domain alone severely compromised binding (Figure 3C) (Viana et al, 2007), suggesting the necessity of a functional pre-CBS sequence for correct complex formation. Interestingly, an interaction between the KINβ2 and the KINβγ pre-CBS-KINγ FCD fusion protein could be observed (Figure 3C), suggesting that a structurally similar four CBS motif region is sufficient for normal binding (cfr. further).

A large gene family of plant FCD-containing proteins

To identify the true SNF4/AMPK orthologs in land plants, we performed phylogenetic analyses with *SNF4/AMPK*-like *FCD* genes from fungi and animals and *FCD* genes from land plants. These include orthologs of *KINβγ, KINγ* and *PV42* (Fang et al, 2011) and inosine-5-monophosphate dehydrogenase (IMDH) related genes. Our results show that *KINβγ*-like genes are present in all Viridiplantae and form a supported monophyletic clade (98 Bootstrap support, BS; 1.00 Bayesian posterior probability, BPP). Interestingly, this Viridiplantae-specific *KINβγ* gene clade is positioned within a highly supported larger monophyletic clade consisting of yeast and animal *SNF4/AMPKγ*-like genes and Amoebozoa, Heterokontophyta and Rhodophyta *SNF4* homologs (92 BS, 1.00 BPP) (Figure 4). However, all other plant genes that encode FCD proteins are positioned outside of this clade, including genes that were previously reported as γ-type subunits, like *KINγ*, *LeSNF4* and *PV42* (Figure 4). Our phylogeny therefore indicates that *KINβγ*-like genes are in fact the true orthologs of γ-subunit genes from fungi and animals. Furthermore, the position of *KINβγ*-like genes with an additional GBD-encoding domain within a larger group of γ-type subunits that lack this domain, suggests that the recruitment of the GBD-domain to an ancestral γ-type subunit is a derived feature for all Viridiplantae (Figure 4).

To avoid future miscommunication about the different FCD proteins in land plants, we propose a classification based on their evolutionary relationship. Our phylogenetic inferences indicate that *SNF4-, AMPKγ-* and *KINβγ*-like genes form one strongly supported monophyletic family, which we will call type Ia *FCD* genes. The other four CBS motif-containing genes in land plants that are structurally similar, not yet functionally characterized and lacking the characteristic GBD-domain of *KINβγ*-like genes then belong to the FCD-Ib and FCD-Ic families, respectively. Finally, *IMDH*-like gene encode proteins containing an additional Phox and Bem1p (PB1) domain and are clearly distinguishable from the FCD-I genes, which is why we classify them as *FCD* type II genes (FCD-II) (Figure 4). A more detailed tree of all available FCD-Ia protein sequences can be found in Supporting Figure S2. As we were unable to unambiguously position animal and fungal

FCD genes other than *SNF4/AMPKγ*-like genes in relationship to FCD-Ib, c and FCD-II, these were left out. Future phylogenetic reconstruction, focusing solely on families FCD-Ib, c and FCD-II, could help to identify true animal and fungal orthologs.

Non-hybrid plant FCD proteins lack the canonical γ-subunit functionality

To confirm that higher plant γ-subunit function is restricted to the FCD-Ia family of our phylogeny, a representative gene from each class was cloned and transformed into the yeast *snf4Δ* strain. None could complement the yeast *snf4* growth deficiency on glycerol/EtOH (Figure 5A) despite efficient expression of all proteins (Figure 5B), confirming the dual requirement for *snf4Δ* complementation. To pinpoint which amino acids in the FCD structure are important for γ-subunit functionality, we first aligned the FCD-Ia proteins from maize, rice, Medicago, tomato, soybean and Arabidopsis with the yeast SNF4 and the AMPKγ proteins to find the highly conserved residues (Figures S3 and S4). Seventeen evolutionarily highly conserved amino acids were identified. Next, we aligned all tested non-γ FCD proteins with the FCD-Ia family. Under stringent conditions, only six of the original seventeen amino acids were retained as conserved in the FCD-Ia proteins and diverged in non-γ FCD proteins (Figures S5 and S6) (Figure 5). The first four amino acids are found in the first CBS domain, while the last two are located in the third CBS domain. Modeling the KINβγ and SNF4 protein based on the resolved AMPKγ1 structure (Xiao et al, 2007) shows that these three proteins might have a very similar overall structure (Figure 5C). When the six amino acids are highlighted on the putative KINβγ structure, most turn out to be positioned at the protein surface and cluster together (Figure 5D). The structural differences between the KINγ and AMPKγ1 are obvious outside the CBS domains (Figure S7).

Although tomato *LeSNF4* was reported to complement the yeast *snf4*Δ growth defect on sucrose (Bradford et al, 2003), it classifies in FCD family Ic. To confirm the predictive value of our phylogeny, we expressed both *LeSNF4* and *LeKINβγ2*, a tomato class Ia member, in the yeast *snf4D* deletion strain. As expected, only LeKINβγ2 could complement the growth deficiency on glycerol/EtOH, suggesting that also in tomato the βγ-like FCD-Ia family proteins are the canonical SnRK1 γ-subunits (Figure 6A). All proteins were efficiently expressed (Figure 6B).

KINγ is not directly involved in SnRK1 signaling

After screening of several potential KINγ knockout mutants, a SALK T-DNA insertion line (SALK_074554.52.55) was characterized as a complete null mutant (Figure 7A, Figure S8). Pull-down experiments in protoplasts showed that KIN10 could still efficiently bind to KINβ2 in this mutant background (Figure 7B). To confirm the hypothesis that KINγ is not directly involved in SnRK1 function, we studied the responses of SnRK1 target genes (Baena-González et al, 2007) in the *kinγ* knockout background. WT and mutant protoplasts were transfected with *SEN1* promoter-luciferase reporter and KIN10 (SnRK1α) effector constructs. Basal levels and induction of promoter activity was similar in WT and mutant background, suggesting that *kinγ* knockout does not affect SnRK1 responses (Figure 7C). More KIN10 target gene responses were analyzed by qRT-PCR in a scaled-up experiment

(Figure S9). Phenotypic characterization also revealed no obvious differences between *kinγ* knockout and WT plants (Figure S8).

In order to study fast SnRK1 responses in intact plants, we also developed a new starvation assay. WT seedlings were grown in six well plates under continuous light in 0.5xMS medium supplemented with 50 mM glucose. After 5 days, the medium was replaced with 0.5xMS without sugars and samples were taken after 0, 30, 60 and 120 minutes. Under these conditions, all SnRK1 target genes tested were responsive to the sugar starvation and showed specific response patterns (Figure 7D). *SEN1* expression was induced 1 hour after sugar removal, while *SEN5*, *DIN10* and *MYB75* were already activated after 30 min, demonstrating the feasibility to study fast starvation responses with this assay. SnRK1 target gene responses were not significantly altered in *kinγ* knockout plants (Figure 7D), confirming the protoplast data *in planta.*

We were unable to isolate homozygous KINβγ KO plants, consistent with the *kin10 kin11* double mutant lethality (Baena-González et al, 2007) and suggesting non-redundant vital functions during plant development. To assess KINβγ involvement in SnRK1 signaling, we used a transient RNAi approach in protoplasts and found that reduced KINβγ expression correlated well with reduced basal target gene expression (Figure 7E).

Discussion

Key to AMPK/SNF1/SnRK1 function and regulation is the hetero-trimeric protein complex of the catalytic α with regulatory β- and γ-subunits, but composition of the plant energy sensor complex could be very diverse and has not been fully characterized. Our co-IP results show that the hybrid KINβγ protein (At1g09020, FCD-Ia), and not KINγ (At3g48530, FCD-Ib), strongly interacts with all 3 KINβ-subunits in Arabidopsis leaf cells (Figure 1). In a yeast *snf4* γ-subunit mutant complementation assay, only the hybrid protein confers the canonical γ-subunit functionality (Figure 2) (Kleinow et al, 2000). Previous Y2H analyses, however, showed interaction of the KINγ subunit with KINβ1 and KINβ2 (Bouly et al, 1999), but not KINβ3 (Gissot et al, 2004), although no yeast mutant complementation could be shown (Bouly et al, 1999; Lumbreras et al, 2001). Consistent with the yeast complementation data (Figure 2), our comprehensive and high-resolution phylogenetic reconstruction (with extended data sets and useful out-group for appropriate rooting) puts the KINγ (At3g48530) protein in a clade that evolutionarily significantly diverged from animal and yeast AMPK/SNF1 γ-subunits, that cluster in a monophyletic clade together with KINβγ. This was not correctly interpreted in earlier, lower resolution analyses (Gissot et al, 2006) and thus suggests that the observed Y2H interactions between KINγ and KINβ1/2 might not be physiologically relevant. We analyzed *kinγ* T-DNA KO plants in a protoplast transient expression experiment and a novel starvation assay (Figure 7, Figure S9). This assay enables the efficient assessment of fast SnRK1 signaling with intact seedlings, showing responses as early as 30 min after sugar deprivation in wild type plants. Similar responses were found in *kinγ* T-DNA KO plants, again indicating that KINγ does not act directly in the SnRK1 complex and signaling pathway. Homozygous KINβγ KO plants could not be isolated, consistent with the *kin10,11* double (VIGS) mutant phenotypes (Baena-González et al, 2007) and its vital functions during plant development, but transient

RNAi in mesophyll protoplasts clearly suggests an important role for KINβγ in the SnRK1 respons (Figure 7E). Future detailed insight in its exact functions will come from transgenic induced silencing, *in vitro* complex reconstitution and directed mutagenesis.

In addition to the expected heterogeneity based on the different subunit isoforms, differential transcriptional regulation and alternative splicing, plant-specific heterotrimeric complexes of KINβγ with a catalytic α- and any of the 3 β-subunits have been proposed to exist alongside KINγ-containing complexes including KINβ1 or KINβ2, but not KINβ3 (Gissot et al, 2006; Lumbreras et al, 2001). Hetero-dimeric α-βγ complexes have also been proposed to exist (Lumbreras et al, 2001) and maize KINβγ not only assembles into SnRK1 complexes, but was also found to specifically homo-dimerize through the GBD (Lopez-Paz et al, 2009), suggesting complex-independent functions as well. In the latter study, increased interaction of KINα and KINβγ upon co-expression of a β-subunit supports the tendency to form stable hetero-trimeric complexes (Lopez-Paz et al, 2009). Our analyses now indicate that such complexes need to include KINβγ and that KINγ does not assemble in hetero-trimeric plant SnRK1 complexes (Figures 1, 2, 7). Truncations of the KINβ2 subunit, disrupting subunit interaction, further demonstrate that the βγ-subunit is absolutely required for hetero-trimeric SnRK1 complex formation with α- and β-subunits (Figure 1). In addition to a complete ASC truncation (Jiang & Carlson, 1997) we also tested a more limited truncation (KINβ2–242) of the ASC, avoiding dramatic structural changes.

Subsequently, we used mutagenesis and truncation of known domains to identify the exact structural requirements of the KINβγ subunits for canonical γ-subunit functionality, (Figure 3). While deletion of the GBD does not affect functionality in yeast, additional removal or specific deletion of the pre-CBS sequence results in loss of growth complementation (Figure 3A) and significant loss of binding to the KINβ2 subunit (Figure 3C), indicating its requirement for both activity and binding. This conserved 20–25 aa sequence immediately preceding the FCD was identified in AMPKγ and found to be required for b-subunit but not a-subunit interaction (Viana et al, 2007). Our analyses suggest that this function is conserved in the canonical plant γ-subunits but the pre-CBS domain alone is not sufficient and deletion of the FCD results in complete loss of binding and activity (Figure 3A,B). Interestingly, fusion of the diverged KINγ FCD to the KINβγ pre-CBS domain still confers sufficient structural similarity for efficient KINβ binding (Figure 3C, Figure S7A), but not for γ-subunit functionality. Molecular modeling based on the AMPKγ1 subunit confirms conservation of the overall FCD structure in KINγ (Figure 5C, Figure S7A). However, besides this conserved structure, the FCD clearly requires additional features for the canonical γ-subunit functionality in yeast (Figure 3A). Since in the animal system the γ-subunits serve as energy sensing modules by binding of nucleotides to CBS pairs (Bateman domains) in the FCD, we considered the possible involvement of altered or deficient nucleotide binding in the lack of *snf4* complementation by KINγ. We used docking of AMP in the crystallized structure of AMPKγ1 (17) and in optimized homology models of KINβγ and KINγ (Figure 5C, D and Figure S7A) using Glide in Schrödinger Suite 2011 (Friesner et al, 2004; Friesner et al, 2006)(Figure S7). Changes in Glide scores upon *in silico* mutation of binding pocket amino acids in binding sites AMP1 and AMP2 of AMPKγ1 confirmed the validity of this approach. Interestingly, Glide scores for AMP binding in KINβγ and KINγ AMP1 and AMP2 sites were considerably higher and comparable to

those for mutated AMPKγ1 sites. Moreover, *in silico* mutation of putative binding pocket amino acids in KINβγ did not significantly alter values (Figure S7B). Alignment of KINβγ FCD's from five plant species with AMPKγ's and yeast SNF4 revealed 17 conserved amino acids, most of them in the CBS domains; stringent alignment with non-γ FCD proteins retained six (Figure S3–S6). Four of these (L179, K182, P197, G208) are located in the first CBS domain, two (S376, Y391) in the third CBS domain. Interestingly, *in silico* mutation of these conserved amino acids also does not significantly alter Glide scores for KINβγ or for AMPKγ1, where the score for the AMP2 site even decreases further (Figure S7). These results suggest that the difference between KINβγ and KINγ in yeast mutant complementation is not likely due to deficient AMP binding in KINγ and that AMP binding is probably not the major regulatory mechanism in the canonical plant γ-subunit function in controlling SnRK1 activity. Consistently, AMP was shown not to be a direct activator of SNF1 and SnRK1, although AMP can inhibit SnRK1 T-loop dephosphorylation and thus inactivation at physiological concentrations (Adams et al, 2004; Momcilovic & Carlson, 2011; Sugden et al, 1999). This also suggests that other metabolites might be sensed by or allosterically regulate the SnRK1 complex to signal metabolic status. Interestingly, plant SnRK1 activity is inhibited by sugar phosphates, like glucose-6-P and trehalose-6-P (Ramon et al, 2008; Toroser et al, 2000; Zhang et al, 2009) providing a direct link between metabolic status and SnRK1 activity. However, direct targets and mechanisms have not been identified yet. Clustering of the six highly conserved amino acids in proteins with canonical γ-subunit function at the surface in two distinct regions (Figure 5D) also suggests their involvement in protein interaction or interaction with regulatory molecules, a mechanism that might also be functional in yeast (and possibly animals).

Sequence analysis also reveals the presence of an extended family of γ-subunit-like FCD proteins in plants. Based on homology and yeast *snf4* mutant complementation, several have been implicated in SnRK1 signaling (Bolingue et al, 2010; Bradford et al, 2003; Fang et al, 2011; Rosnoblet et al, 2007). The phylogenies we generated of the extended family of FCD proteins now identified a distinct *KINβγ* family within a highly supported larger monophyletic clade consisting of yeast and animal *SNF4/AMPKγ*-like genes, encoding canonical functional γ-subunit proteins (Fig 4). All other plant *FCD* genes are positioned outside of this clade and sometimes show very (e.g. flower or seed) specific expression profiles (Figure S10). Furthermore, functional analysis by yeast mutant complementation of the Arabidopsis FCD genes *At1g69800*, *At1g15330 (PV42a)* and *At3g52950*, each belonging to a different sub-clade, indicated that in higher plants only the FCD proteins that acquired a GBD and pre-CBS domain have retained the canonical γ-subunit function in SnRK1. Based on these phylogenetic and functional analyses, we now propose a classification of plant FCD genes into four subfamilies, FCD-Ia being the major monophyletic family comprising *SNF4-, AMPKγ- and KINβγ*-like genes (Figure S2). The other structurally similar but poorly characterized land plant FCD genes, that lack the characteristic GBD-domain sequence, make up families FCD-Ib (including *At3g48530/KINγ* and *At1g69800*) and FCD-Ic (*At1g15330/AtPV42a*), respectively. Finally, *IMDH*-like genes encoding proteins with an additional Phox and Bem1p (PB1) domain are classified as FCD-II (Figure 4). This classification can serve as a resource and tool to predict function when plant *FCD* genes are picked up in mutant, functional, genomic or proteomic screens.

For this purpose, a more detailed tree of FCD-Ia plant genes is also provided (Figure S2). Inconsistent with our classification, however, the seed specific tomato *LeSNF4* (*Solyc06g068160*, clustering in family FCD-Ic) was reported to complement a yeast *snf4* mutant (Bradford et al, 2003). To resolve this, we cloned and tested this gene and a tomato hybrid βγ gene (*LeKINbg2/Solyc01g099280*, clustering in family FCD-Ia) in our more stringent *snf4* complementation assay on glycerol/ethanol (instead of semi-fermentable sucrose). This assay showed efficient growth complementation by *LeKINβγ2*, but not *LeSNF4* (Figure 6), confirming the accuracy and usefulness of our phylogenetic study and classification. Interestingly, our analysis also provides insight in the evolutionary origin of the hybrid KINβγ proteins. Recruitment of the GBD, possibly acting as a sensor of energy reserves in the form of glycogen in animals (McBride et al, 2009), coincides with the appearance of the chloroplastidal Viridiplantae (Figure 4) and hence rewiring of an ancenstrally cytosolic storage polysaccharide synthesis to chloroplastic starch metabolism (Ball et al, 2011). This must have created the need for mechanisms controlling carbon and energy homeostasis through retrograde (plastid to nucleus) signaling, possibly via starch or starch breakdown product binding proteins. Interestingly, the PTPKIS1/SEX4 (STARCH EXCESS4) phosphoglucan phosphatase, that was reported to interact with the SnRK1 catalytic α-subunit KIN11 through a KIS domain (Fordham-Skelton et al, 2002) and is required for starch breakdown (Niittyla et al., 2006; Kotting et al, 2009), contains a carbohydrate binding domain with homology to the GBD, that effectively binds starch (and glycogen) and interacts with the phosphatase domain to form a single continuous active site pocket (Vander Kooi et al., 2010). Two related chloroplastic proteins, LSF1 (Like Sex Four1) and LSF2 (Like Sex Four2, lacking the carbohydrate binding domain), were similarly found to be involved in starch turnover (Comparot-Moss et al., 2010; Santelia et al., 2011). A Bayesian phylogenetic tree of the carbohydrate binding domains of β-subunits, KINβγ, SEX4 and LSF1 homologs based on the sampling and phylogenetic reconstructions of Janeček et al. (2011), suggests that the LSF1 and KINβγ modules have a common ancestor (Figure S11). This may imply that the KINβγ GBD could still bind starch, starch breakdown product or analogous carbohydrates. In any case, the GBD (or SBD) in the hybrid KINβγ proteins must have acquired plant-specific (not required for yeast mutant complementation; Figure 3) but essential regulatory functions, as only these hybrid plant proteins have retained conserved FCD and pre-CBS domains and hence the canonical γ-subunit function in SnRK1γ. The GBD of the hybrid KINβγ proteins also shows higher sequence similarity to the animal β-subunit protein KIS/GBD than to that in plant β-subunit proteins (Lumbreras et al, 2001)(Figure S11), suggesting that part of the original β-subunit GBD function might have been transferred to the plant KINβγ. The yeast β-subunit GBD, for example, was found to contribute to recruitment of a PP1 phosphatase, controlling SNF1 activity (Mangat et al, 2010). Truncation of the KIS/GBD domain in the plant KINγ3-type proteins, that still assemble in SnRK1 complexes, could be consistent with an ongoing evolution towards loss of GBD function in plant KINβ proteins. A major challenge thus will be the identification of the exact factors signaling metabolic status to SnRK1 complex formation and activity and the possible role of the KINβγ GBD/SBD in this process.

Experimental procedures

Plant growth and protoplast isolation

For leaf mesophyll protoplast isolation, Arabidopsis Columbia WT plants were grown in a 12h light/12h dark diurnal cycle with 70 μE light intensity for 4 weeks. Protoplast isolation was performed as described (Niu & Sheen, 2012; Yoo et al, 2007). The *kinγ* SALK_074554.52.55 T-DNA line was obtained from ABRC and homozygous plants were selected on full MS medium with 50mg/ml kanamycin. For Western blot and PCR confirmation, vapor-sterilized and stratified seedlings were grown in 1ml half strength MS medium with 0.5% sucrose in 6 well plates under continuous (65 μE) light for 7 days. For the starvation assay, 15 vapor-sterilized and stratified WT and *kinγ* knock-out seeds were germinated 1 ml half strength MS medium supplemented with 50mM glucose in 6-well plates. Plates were incubated under continuous light (65 μE) at 24°C for 5 days.

Plasmid construction

For the reporter construct, a 2.5 kb *SEN1* (At4g35770) promoter fragment was PCR amplified from Arabidopsis Columbia genomic DNA and inserted in front of the luciferase (LUC) gene in a pUC based expression vector (15). Full length *KIN10* (*At3g01090*), *KINβγ* (*At1g09020*), *KINγ* (*At3g48530*), *KINβ1* (*At5g21170*), *KINβ2* (*At4g16360*) and *KINβ3* (*At2g28060*) coding sequences (CDS) lacking the STOP codon were PCR-amplified from Arabidopsis Columbia cDNA and inserted (BamHI-StuI) in the HBT95 expression vector (Sheen, 1996) in frame with a double HA or FLAG tag. *KINβγ* and *KINγ* CDS and their truncated or mutated alleles were subcloned in the PYX212 vector for yeast complementation studies (cfr. further).

PCR was used for site-directed mutagenesis (SDM, including deletion) and truncation of KINβγ, KINγ and KINβ2 proteins. For SDM, primers were designed to extend 12–15 base pairs on either side of the modification. A typical 25 μl SDM PCR reaction contained: 2.5μl dNTPs (2.5mM), 2.5μl Pfu Turbo buffer 10x, 25 ng plasmid DNA, 10 ng primer A and B each, and 0.5μl Pfu Turbo enzyme (Stratagene). Half of the PCR reaction mixture was then subjected to 3 min at 95°C and 12–18 cycles (12 for point mutations, 16 for single amino acid changes, 18 for deletions or insertions) at 95°C (30s), 55°C (60s), 68°C (2 min/kb of plasmid). As a negative control, half of PCR the reaction mix was incubated at RT. *Dpn*I was then added to digest the methylated template DNA and 5μl was transformed in *E.coli.* Constructs were confirmed by sequencing. For cloning of the N-terminal 170 aa of KINβγ, reverse primer KINβγ/1–170 was used. For cloning of KINβγ fragment 171–487, forward primer KINβγ/171–487 was used. For the pre-CBS-KINγ fusion protein, the pre-CBS sequence was included in forward PCR primer KINγ/PRECBSβγ.

Two specific KINβγ RNAi constructs were made by PCR amplification of cDNA fragments −100 to +97 (relative to the ATG start codon; including a 5’ UTR sequence) and +1347 to +1531 (including a 3’ UTR sequence) and sense/antisense insertion in a pUC-based expression vector with an intron sequence for stem loop and efficient double stranded RNA formation.

qRT-PCR

For qRT-PCR quantification of gene expression in starved seedlings and KIN10 transfected protoplasts, RNA extraction was performed with Trizol (Invitrogen) according to manufacturer's instructions. 1µg of total RNA was used for reverse transcription (RT) with the Reverse Transcription System (Promega A3500, Madison, Wi, USA). qPCR was performed using the GoTaq® qPCR Master Mix kit (promega A6001) according to the manufacturer's instructions in a total volume of 10 µl with 5 µl FAST SYBR GREEN buffer, 0.2 µl of each primer (10 µM), 2.5 µl H_20, 0.1µl CXR and 2 µl cDNA (5ng/µl). The PCR program comprised an initial denaturation for 2 min at 95°C and amplification by 45 cycles of 3s at 95°C and 30s at 58°C in a StepOnePlus Real Time PCR system (Applied Biosystems). Expression levels were normalized to UBIQUITIN10 (UBQ10). All qRT-PCR experiments were performed 6 times and the graph values are means with standard deviation.

Luciferase and GUS assays

For luciferase activity measurement, protoplasts were lysed with 100µl lysis buffer (25mM Trip-phosphate pH 7.8, 2mM DTT, 2mM 1,2-diaminocylcohexane-N,N,N',N'-tetra-acetic acid, 10% glycerol, 1% Triton X-100). 20µl of the cell lysate was dispensed into a luminometer tube and mixed with 100µl luciferase assay reagent (Promega kit E1500). Luminescence was detected with a Berthold Lumat LB 9507 luminometer. b-glucuronidase activity from the UBQ-GUS control for transfection efficiency was measured with 10µl of cell lysate in 100ul 10mM MUG solution (4-methylumbelliferyl-β-D-glucuronide, Sigma M-9130). After 1h incubation at 37°C, the reaction was stopped with 900µl 0.2M Na_2CO_3, and fluorescense measured with a Hoefer DyNA Quant 200 fluorometer (Amersham Biosciences).

Protein expression

For co-immunoprecipitation experiments, around 400,000 leaf mesophyll protoplasts were co-transfected with 40µg of each (CsCl gradient purified) construct. After harvesting, cells were lysed with 200µl IP buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 5mM EDTA, 1% Triton X-100, 0.5mM DTT, 1 tablet complete protease inhibitor (Roche 04693159001)) and incubated for 3 hours with 30µl FLAG-conjugated agarose beads (Sigma A2220) (pre-washed 5 times with IP buffer) at 4°C under gentle rotation. 20µl lysate was not incubated with agarose beads and used as input control. After incubation, beads were washed 5 times with IP buffer. 40µl loading buffer (1x MOPS running buffer (50mM MOPS, 50mM Tris base, 0.1% SDS, 1mM EDTA), 16.22g urea, 11.5ml glycerol, 9.75ml 20% SDS)) was added to the agarose beads and samples were heated for 5 min at 95°C. 20µl of bead supernatant and 15µl of input lysate were loaded on a 10% SDS-PAGE gel and separated in a 1x MOPS running buffer at 60 Volts for 15 min and 160 Volts for 1h. After running, proteins were transferred to a PVDF membrane (Immobilon®-P, Millipore) with a semi-dry transfer system (Trans-Blot® SD, Bio-Rad) in 1x MOPS buffer with 10% methanol for 1h at 12 Volts. After incubation with 5% skim milk, the membrane was incubated with antibody in 1% milk for 2h (conjugated HA-antibody, conjugated FLAG antibody; Roche). The membrane was washed 5 times in TBST (50mM Tris, 150mM NaCl, 0,05% Tween 20),

incubated with Pierce SuperSignal® West Pico chemiluminescent substrate (Thermo Scientific, 34078) for 1 min and exposed to film for several minutes. To check protein expression in yeast, cells were grown to exponential phase on SD-ura medium and lysed with IP buffer and glass beads for 3 times 40 seconds at 4°C in a FastPrep FP120 Homogenizer (Thermo Savant). Protein concentrations were equalized after Bradford protein concentration measurements and 20µl was loaded on gel for Western blot analysis with conjugated HA-antibody (Roche). For KINγ protein determination in wild type and *kinγ* knock-out plants, seedlings were crushed in 200µl loading buffer and 20µl was loaded on gel. KINγ antibody was obtained from Agrisera (AS09 613).

Phylogenetic analyses

To study the inter-relationship between *SNF4/AMPKγ*-like genes in fungi and animalia and *KINβγ*-, *KINγ*-, *PV42*-, *IMDH*-like genes from land plants, homologues of these genes were identified through BLAST searches in the Phytozome (Goodstein et al, 2012), PLAZA (Proost et al., 2009) and Genbank (Benson et al, 2004) databases using *KINbg, KINγ*, *PV42a* and *At3g52950* sequences. FCD genes from bacteria were included to root the phylogenies and additional *SNF4/AMPKγ*-like genes from Amoebozoa, Heterokontophyte and Rhodophyta were included to improve resolution of the *SNF4/AMPKγ/KINβγ* monophyletic group.

Only the four CBS domains were used for the alignment and phylogenetic reconstruction, because some gene families contained additional domains apart from CBS domains. The CBS domains were detected using the simple modular architecture research tool or SMART (http://smart.embl-heidelberg.de/) (Schultz et al, 2000). The four concatenated CBS domain data matrix was then aligned using MAFFT v6 (Katoh & Toh, 2008) and manually refined in MacClade4 taken into consideration their amino acid translation (Maddison & Maddison, 2003). jModeltest was used to select the best model of evolution (Posada, 2008). Using the AiC criterion, the GTR+I+G model of substitution was selected. Phylogenetic trees were reconstructed using Maximum likelihood and Bayesian methods. Maximum likelihood reconstructions were performed using PhyML 3.0 (Guindon et al, 2010). Bootstrap values were estimated for 100 nonparametric bootstrap replicates. Bayesian analysis was carried out using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). Two independent runs with each 4 Markov Chain Monte Carlo chains were run for 15,000,000 generations and sampled every 1,000 generations. After convergence, we removed the first 5000 of the 15,000 sampled trees as burn-in. The remaining 10,000 were summarized as a majority-rule consensus tree with posterior probabilities at their respective nodes. Both trees were rooted using bacterial FCD genes.

The more articulated *SNF4/AMPKγ/KINβγ* phylogeny was reconstructed using the full-length genes from the Ia cluster from the first phylogeny together with additional *KINβγ* orthologs identified through BLAST searches from Phytozome, PLAZA and Genbank (Benson et al, 2004; Goodstein et al, 2012; Proost et al, 2009). The alignment, model selection and phylogenetic reconstructions were performed similarly to the above-mentioned reconstruction.

Based on the sampling and phylogenetic reconstructions of Janeček et al. (2011), carbohydrate binding domains of β-subunits, KINβγ, SNF4, AMPKγ, SEX4 and LSF1 homologs were obtained and aligned. The phylogenetic reconstruction was performed using MrBayes 3.2 (Ronquist & Huelsenbeck, 2003). Two independent runs for 3,000,000 generations with each 4 MCMC chains were sampled every 1,000 generations. The first 1,000 sampled trees were discarded as burn-in. The remaining ones were subsequently summarized as a majority-consensus tree.

Protein modeling and docking

Homology modeling of the 4 CBS domains is based on the crystal structure of the AMPKγ1 subunit of mammalian AMPK (2V8Q) (Xiao et al, 2007) and was done using MODELLER (Sali & Blundell, 1993). For evaluation of the models the internal DOPE energy scoring function was used. Figures were made using PyMOL. Optimal structures were imported in Maestro9.2 (Banks et al, 2005) for minimization, removing unfavorable steric contacts and improving the quality of the protein hydrogen bonding network without large rearrangements of heavy atoms. Docking of AMP was performed using Glide (Friesner et al, 2004; Friesner et al, 2006) in Schrödinger Suite 2011. Docking regions were defined by 8 Å cubic boxes centered on the ligand mass center. Subsequently, extra-precision (XP) docking and scoring were executed. The best scored poses were chosen as the optimal solution.

Alignments

Protein alignments were done on the biology workbench San Diego Supercomputer Center (http://workbench.sdsc.edu/) with the CLUSTAL W (Thompson et al, 1994). Multiple alignment was done with Gonnet Series protein weight matrix and gap open and extension penalties of respectively 10.00 and 0.20.

Yeast complementation

The yeast (*Saccharomyces cerevisiae*) MCY4024 (*MATα gal83Δ::TRP1 gal4 gal80 URA3::lexAop-lacZ ade2 his3 leu2 trp1)* (Wiatrowski et al, 2004) and MCY2634 (*MATa snf4–2 ura3 his3 leu2)* (Hubbard et al, 1994) strains were used for growth defect complementation assays. The different plant and yeast sequences were amplified from cDNA and cloned in a yeast multicopy pYX212 plasmid with an *HXT7* promoter and *URA3* marker, without stop codon and in frame with a C-terminal HA tag ((BamHI and SmaI restriction sites). Correct constructs were confirmed by sequencing. cDNA was synthesized from W303–1A WT yeast, Arabidopsis Columbia ecotype leaf and LA3021 tomato seed RNA. Cloning primers included BamHI and SmaI-compatible StuI restriction sites (Table S1). KINγ and KINβγ coding sequences were subcloned from the HBT95 expression vector. Yeast transformation was performed using a LiAc/SS carrier DNA/PEG transformation protocol (Gietz & Schiestl, 2007). For growth assays, cultures of the transformed strains were grown to exponential phase at 30°C on minimal medium without uracil (SD-ura) containing 2% glucose and drop-assays were performed on SD-ura with 2% glucose (control) or 2% glycerol - 3% ethanol. Several transformants were spotted at an OD_{600}1 and growth was analyzed after three days at 30°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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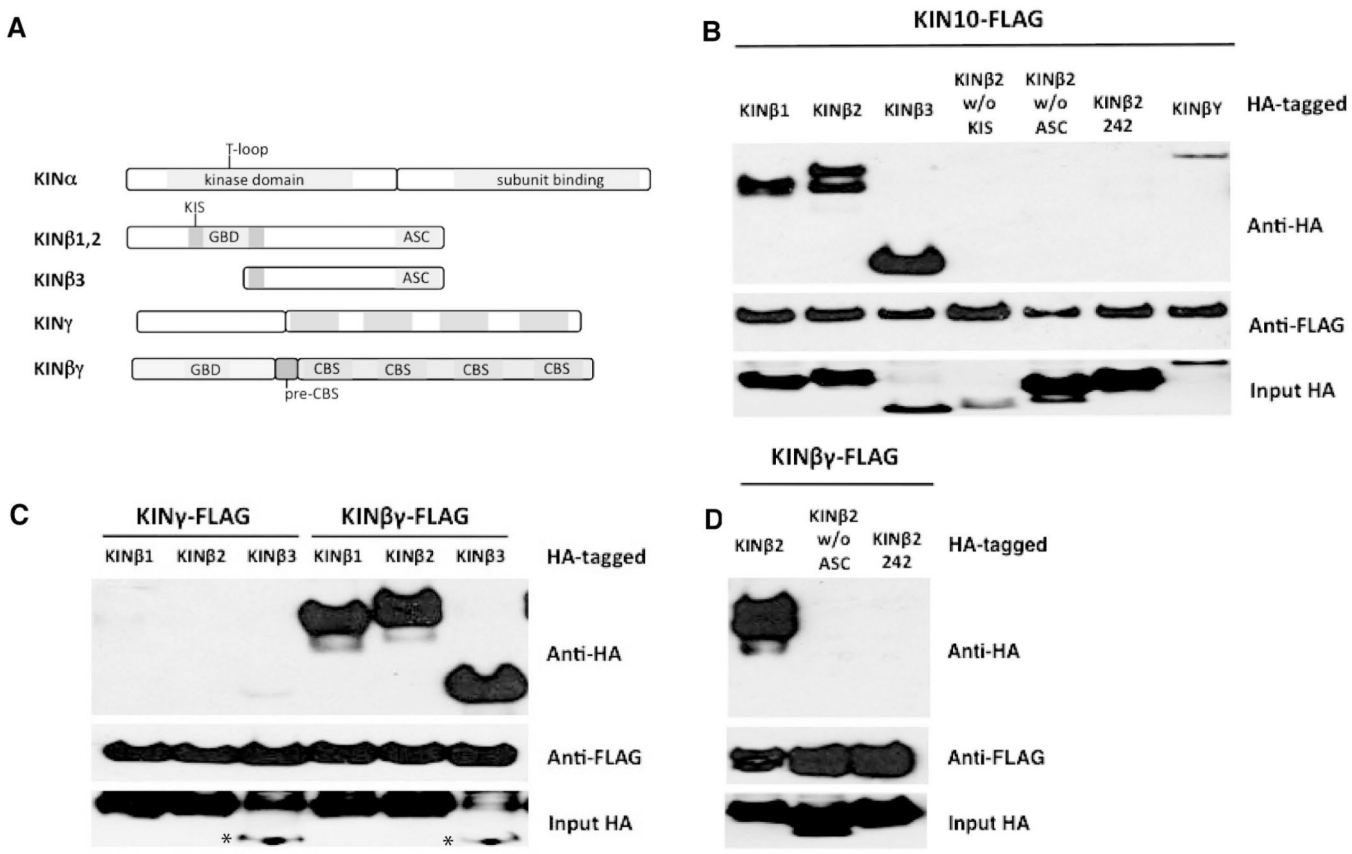

Figure 1.

Specific KINβγ binding to the regulatory KINβ-subunits (**A**) Structure and domain composition of the plant SnRK1 subunits. The GBD domain (light grey) in the β-subunits (KINβ1 and KINβ2) overlaps with the KIS domain (dark grey). (**B**) Co-immunoprecipitation of HA-tagged β-subunits with Flag-tagged KIN10 using FLAG-coupled beads. (**C**) Co-immunoprecipitation of HA-tagged β-subunits expressed in Arabidopsis mesophyll protoplasts with FLAG-tagged KINγ (At3g48530) or KINβγ expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled glutathione beads. (**D**) Co-immunoprecipitation of HA-tagged β-subunits with Flag-tagged KINβγ expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled glutathione beads. KIS: Kinase Interacting Sequence; GBD: Glycogen Binding Domain; ASC: Association with SNF1 Complex; CBS: cystathionine β-synthase domain; KINβ 242: partially truncated ASC (KINβ amino acids 1–242).

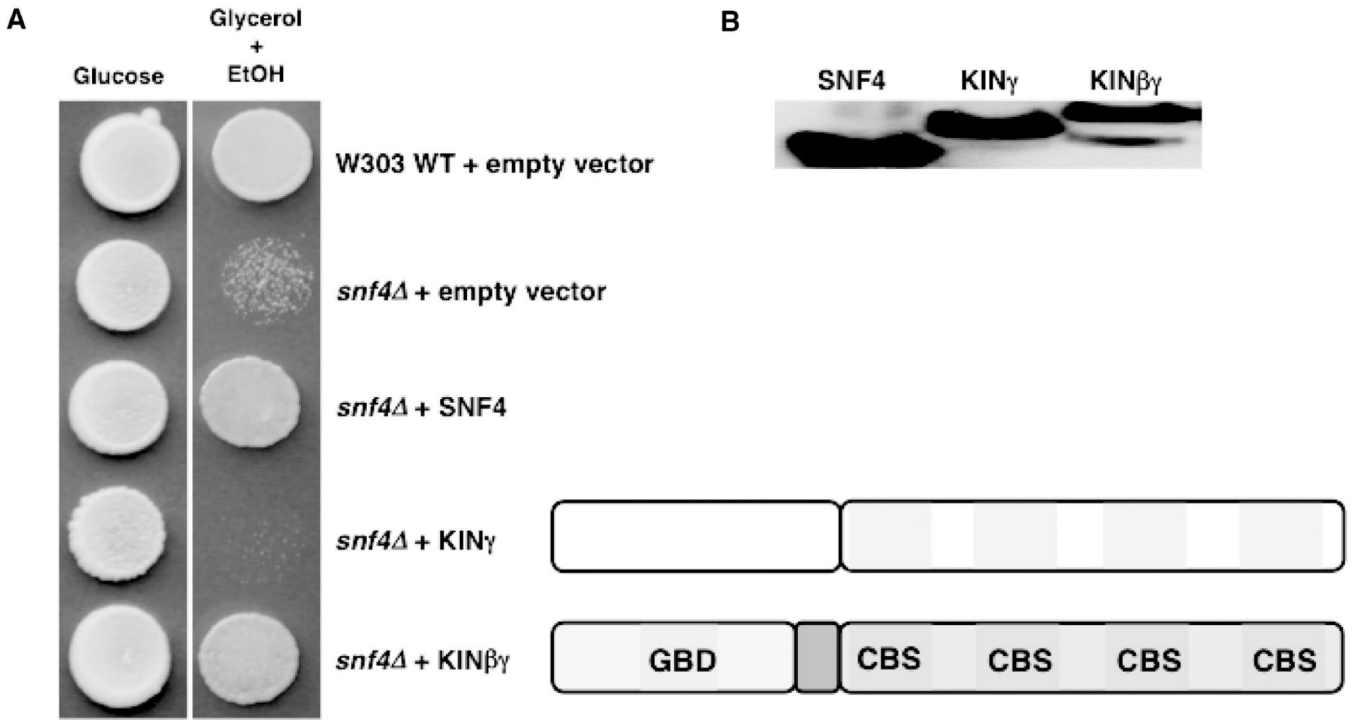

Figure 2.

Hybrid Arabidopsis KINβγ (At1g09020) but not KINγ (At3g48530), complements the yeast snf4 γ-subunit mutant growth defect on non-fermentable glycerol/ethanol medium. (**A**) Heterologous expression in yeast of Arabidopsis KINγ and KINβγ. As a positive control the yeast SNF4 gene was also expressed Cells growing exponentially in minimal medium (-uracil) with glucose as a carbon source were diluted to OD_{600} 1 and spotted on minimal medium (-uracil) plates with glucose or glycerol/ethanol as the only carbon source. Pictures were taken after 3 days. The overall structure and domain composition of KINγ and KINβγ are indicated. GBD: Glycogen Binding Domain; CBS: cystathionine β-synthase domain. After 3 days some background growth can be observed on glycerol/ethanol medium in the *snf4* mutant background. (**B**) Expression of the HA-tagged proteins in yeast was confirmed by Western blot analysis. Equal total amounts of solubilized protein were loaded.

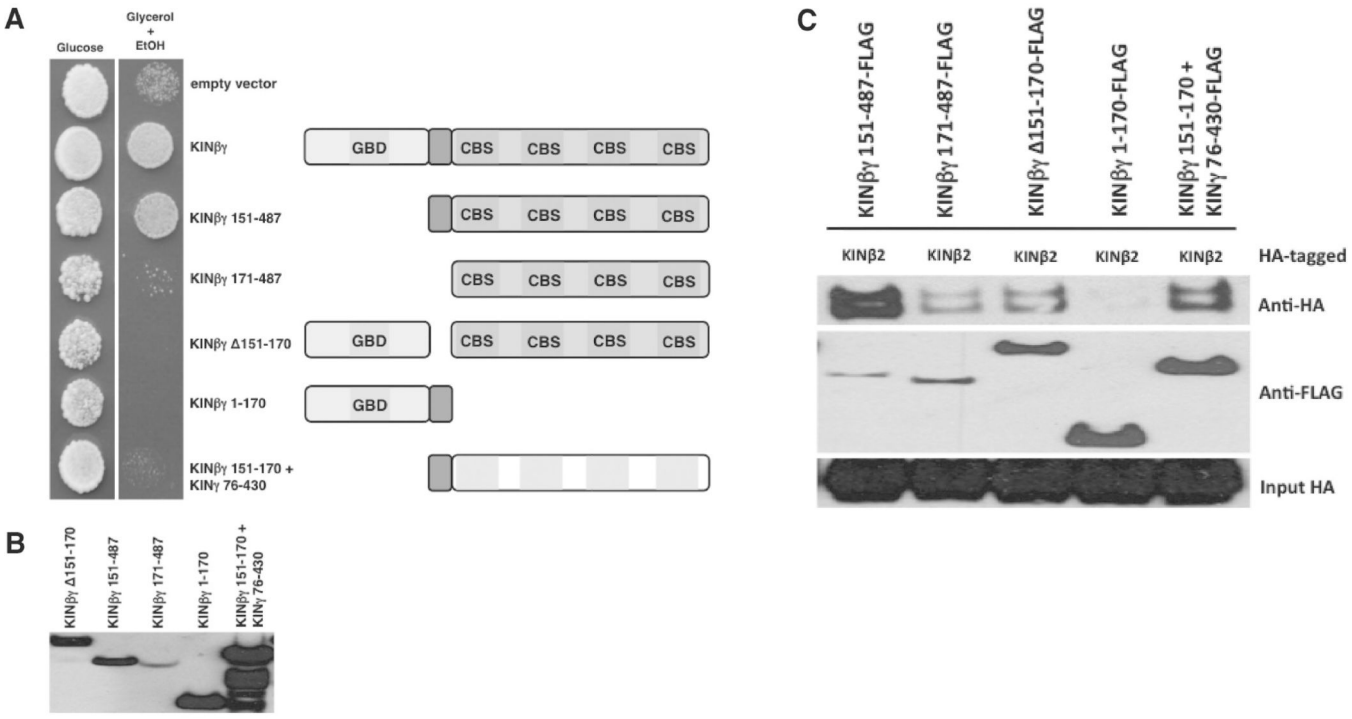

Figure 3.

A dual requirement for pre-CBS and 4 CBS domain (FCD) region for yeast snf4 mutant growth defect complementation on non-fermentable glycerol/ethanol medium. (**A**) Heterologous expression in yeast of the full length Arabidopsis KINβγ; KINβγ lacking the N-terminal part with the GBD (aa 151–487); in addition lacking the pre-CBS domain (dark grey)(aa 171–487); just lacking the pre-CBS domain (Δ151–170); lacking the FCD C-terminal part (aa 1–170); or the KINβγ pre-CBS domain fused to the KINγ FCD C-terminal part (KINβγ 151–170 + KINγ 76–430). Cells growing exponentially in minimal medium (-uracil) with glucose as a carbon source were diluted to OD_{600} 1 and spotted on minimal medium (-uracil) plates with glucose or glycerol/ethanol as the only carbon source. Pictures were taken after 3 days. CBS: cystathionine β-synthase domain. The overall structure and domain composition of KINγ and KINβγ are indicated. GBD: Glycogen Binding Domain; aa: amino acids. After 3 days, some background growth can be observed on glycerol/ethanol medium in the *snf4* mutant background. (**B**) Expression of the HA-tagged proteins in yeast was confirmed by Western blot analysis. Equal total amounts of solubilized protein were loaded. (**C**) Co-immunoprecipitation of HA-tagged β2-subunits with Flag-tagged full length and truncated KINβγ subunits expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled beads.

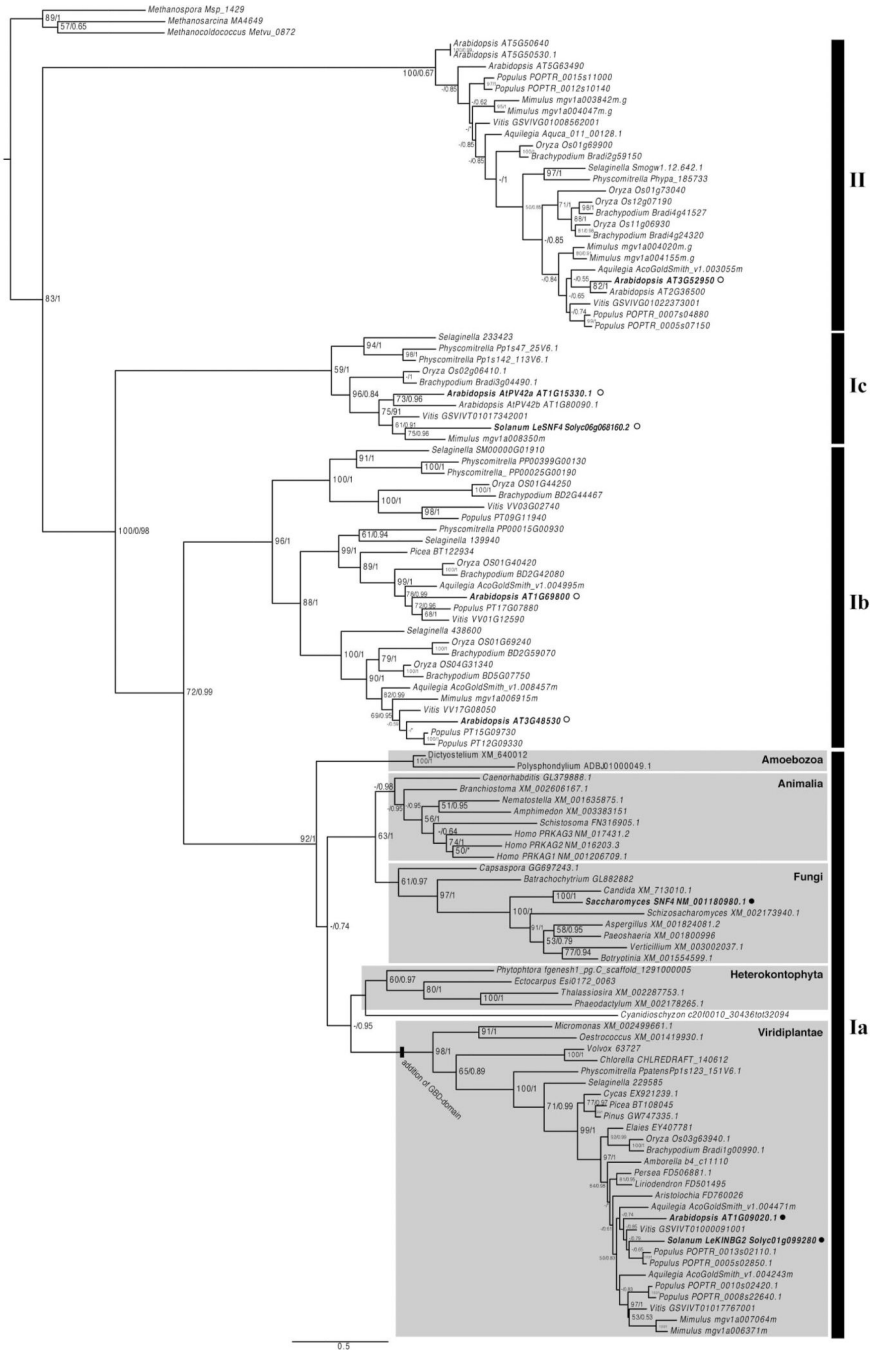

Figure 4.

Maximum likelihood phylogeny of the 4 CBS domain (FCD) of *SNF4*, *AMPKγ*, *KINβγ*, *KINγ*, *PV42* and *IMDH* genes. Numbers at the nodes represent ML bootstrap support values and Bayesian posterior probabilities. Genes in bold correspond to proteins that were assayed in this study. Filled circles indicate canonical γ-subunits functionality based on our yeast complementation experiments, while open circles suggest a lack of canonical γ-subunit functionality. Based on this phylogeny, a classification in families FCD-Ia, FCD-Ib, FCD-Ic and FCD-II is proposed.

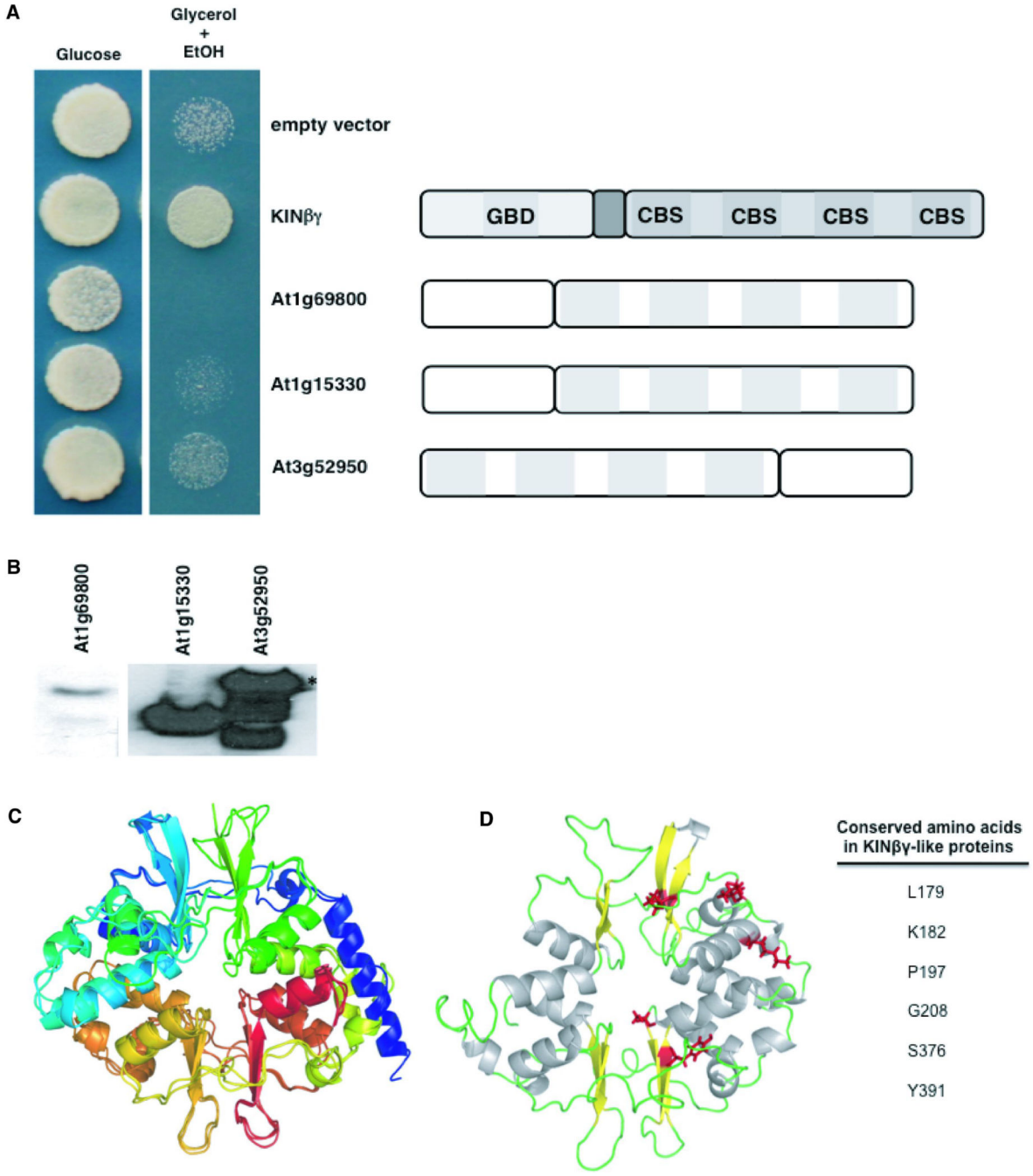

Figure 5.

Non-hybrid Arabidopsis Four-CBS-domain (FCD) proteins lack the canonical γ-subunit functionality. (**A**) Heterologous expression in the yeast *snf4* mutant of Arabidopsis KINβγ (FCD-Ia), At1g69800 (FCD-Ib), At1g15330 (FCD-Ic/AtPV42a) and At3g52950 (FCD-II). Cells growing exponentially in minimal medium (-uracil) with glucose as a carbon source were diluted to OD_{600} 1 and spotted on minimal medium (-uracil) plates with glucose or glycerol/ethanol as the only carbon source. Pictures were taken after 3 days. The overall structure and domain composition of KINγ and KINβγ are indicated. GBD: Glycogen

Binding Domain; CBS: cystathionine β-synthase domain. After 3 days, some background growth can be observed on glycerol/ethanol medium in the *snf4* mutant background. (**B**) Expression of the HA-tagged proteins in yeast was confirmed by Western blot analysis. Equal total amounts of solubilized protein were loaded, except for At1g69800 that consistently showed very low expression levels. (**C**) Overlap of the AMPKγ1 FCD structure and models of KINβγ and SNF4 revealing a similar overall organization. α-helices are indicated in silver, β-sheets in yellow, connecting loops in green. (**D**) Model of the KINβγ FCD structure based on the resolved structure of AMPKγ1 and position of the 6 highly conserved amino acids (highlighted in red) in KINβγ-like hybrid plant proteins and animal and fungal proteins with reported γ-subunit functionality.

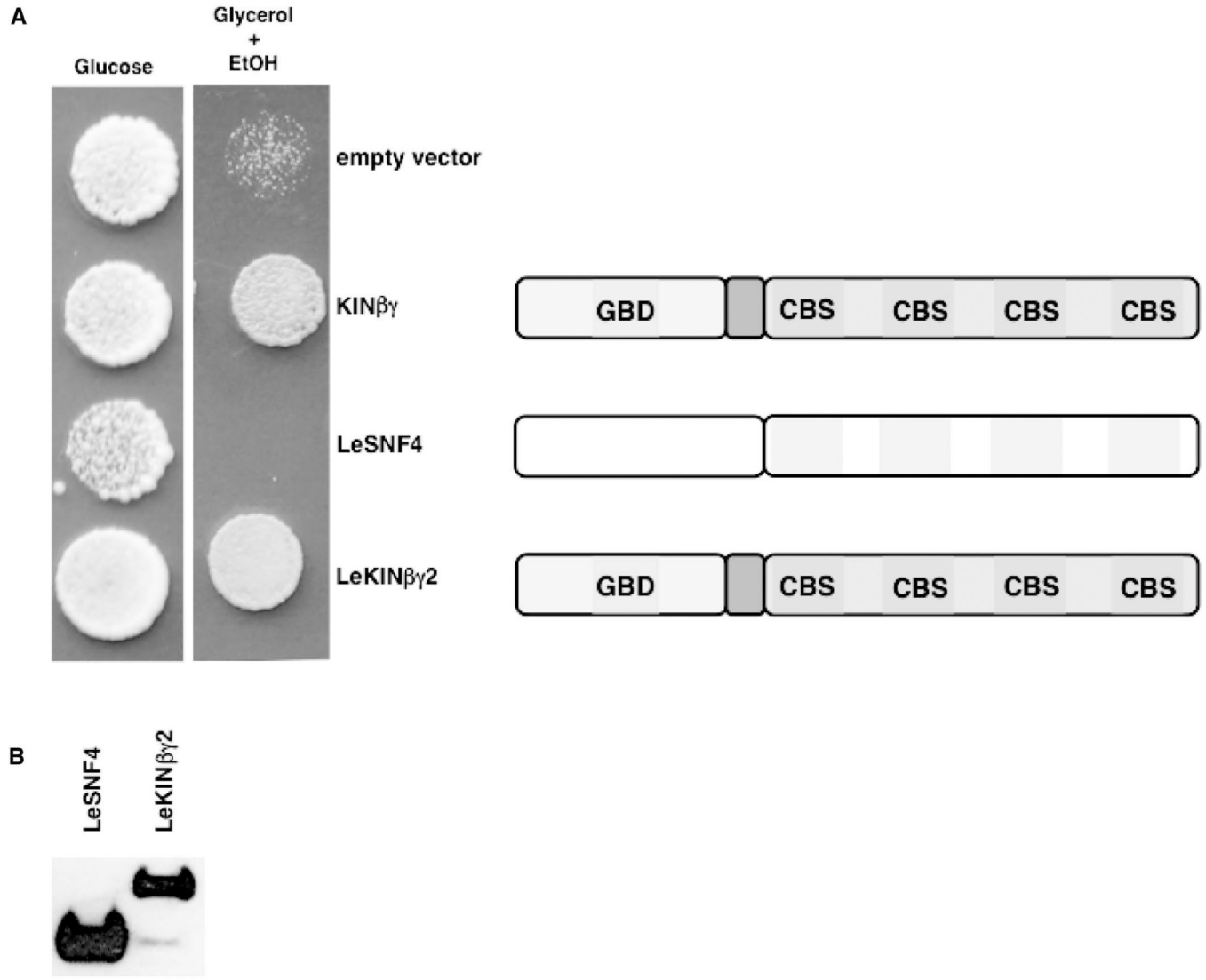

Figure 6.

Non-hybrid Tomato Four-CBS-domain (FCD) proteins lack the canonical γ-subunit functionality. (A) Heterologous expression in the yeast *snf4* mutant of Arabidopsis KINβγ (FCD-Ia) and tomato LeSNF4 (FCD-Ic) and LeKINβγ2 (FCD-Ia). Cells growing exponentially in minimal medium (-uracil) with glucose as a carbon source were diluted to OD_{600} 1 and spotted on minimal medium (-uracil) plates with glucose or glycerol/ethanol as the only carbon source. Pictures were taken after 3 days. The overall structure and domain composition are indicated. GBD: Glycogen Binding Domain; CBS: cystathionine β-synthase domain. After 3 days, some background growth can be observed on glycerol/ethanol medium in the *snf4* mutant background. (**B**) Expression of the HA-tagged proteins in yeast was confirmed by Western blot analysis. Equal total amounts of solubilized protein were loaded.

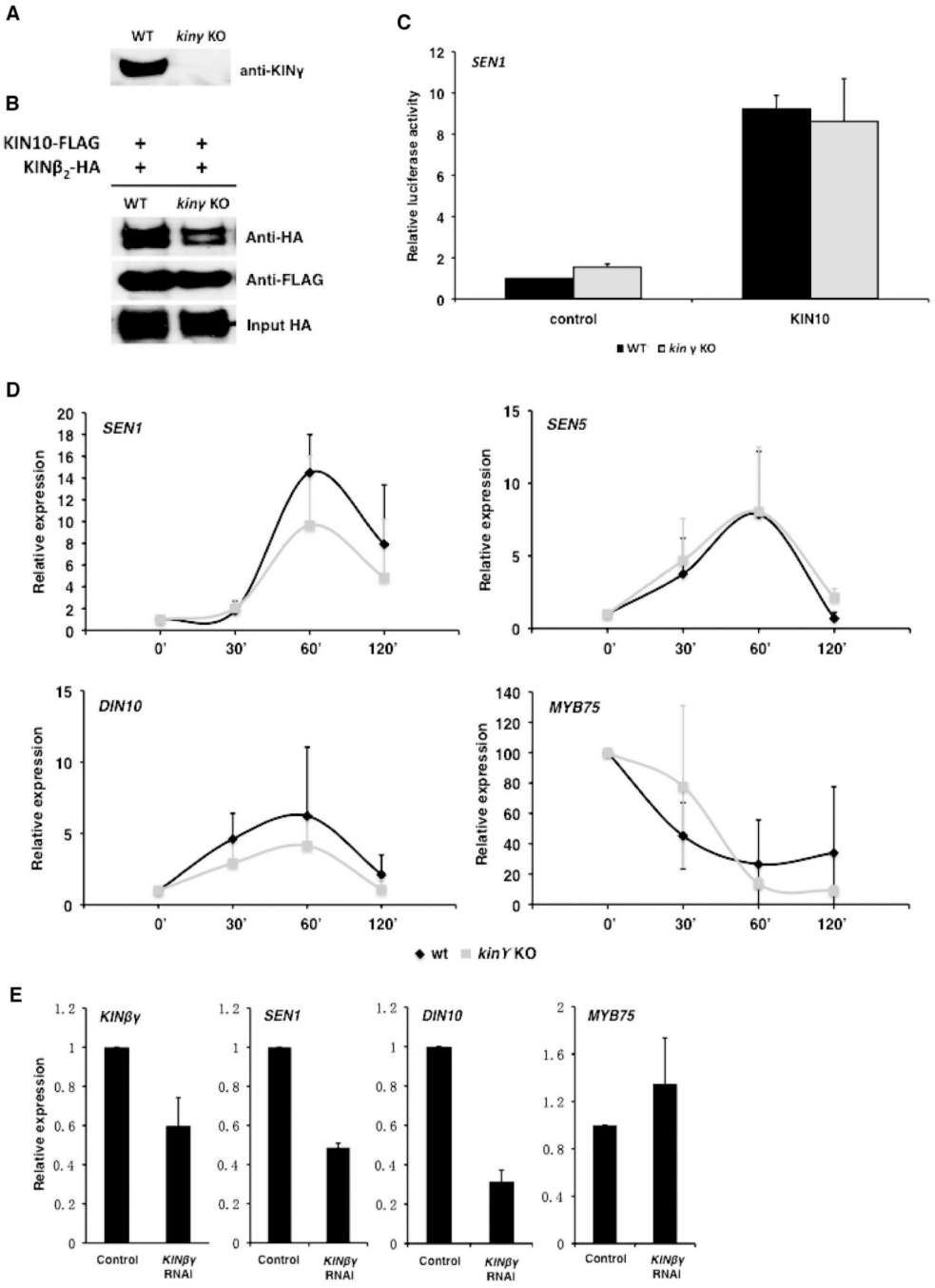

Figure 7.

KINγ is not directly involved in SnRK1 signaling. (**A**) T-DNA insertion line SALK_074554.52.55 is a complete *kinγ* null mutant as confirmed by Western blot analysis on wild type and mutant seedlings. (**B**) Efficient co-immunoprecipitation of the HA-tagged β2-subunit with Flag-tagged KIN10 expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled beads. (**C**) Wild type response of transient KIN10 over-expression in *kinγ* mutant mesophyll protoplasts using a *SEN1/DIN1*-Luciferase reporter construct. (**D**) Wild type SnRK1 target gene responses in the *kinγ* in a seedling sugar starvation assay. Relative

SEN1/DIN1, *SEN5*, *DIN10* (induced) and *MYB75* (repressed) target gene expression is assayed 0, 30, 60 and 120 minutes after removal of glucose from the growth medium using qRT-PCR. (**E**) Relative expression of *KINβγ* and of the SnRK1 target genes *SEN1*, *DIN10* and *MYB75* in control and transient *KINβγ* RNAi protoplasts.