the plant journal

SEB Society for Experimental Biology

The Plant Journal (2013) **75,** 11–25 doi: 10.1111/tpj.12192

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

Matthew Ramon[1], Philip Ruelens[1], Yi Li[1], Jen Sheen[2], Koen Geuten[1] and Filip Rolland[1,*]

[1]*KU Leuven Department of Biology, Laboratory of Molecular Plant Biology, Leuven B-3001, Belgium, and*

[2]*Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, MA 02114, USA*

Received 11 February 2013; revised 15 March 2013; accepted 26 March 2013; published online 1 April 2013.

*For correspondence (e-mail filip.rolland@bio.kuleuven.be).

SUMMARY

The AMPK/SNF1/SnRK1 protein kinases are a family of ancient and highly conserved eukaryotic energy sensors that function as heterotrimeric complexes. These typically comprise catalytic α subunits and regulatory β and γ subunits, the latter function as the energy-sensing modules of animal AMPK through adenosine nucleotide binding. The ability to monitor accurately 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-Cystathionine β-synthase (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 heterotrimeric 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 into 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 cells developed a sophisticated energy-sensing protein kinase complex to monitor metabolic status and maintain energy homeostasis during both normal growth and development and in stress conditions. Animal AMP-activated kinase (AMPK), yeast SNF1 (sucrose non-fermenting 1) kinase and plant SnRK1 (SNF1-related kinase 1) act as conserved fuel gauges that share both function and a characteristic heterotrimeric structure (Baena-González and Sheen, 2008; Baena-González *et al.*, 2007; Ghillebert *et al.*, 2011; Hardie *et al.*, 2012; Hedbacker and Carlson, 2008; Polge and Thomas, 2007; Figure 1(a)). 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 many conserved target genes have been identified, SnRK1 complexes also show particular features (Polge and Thomas, 2007) and important mechanistic links are still missing. Plants produce their own energy-rich organic molecules by solar energy-driven photosynthesis as sessile organisms, therefore they depend heavily on the ability to monitor accurately and adapt to changing environmental conditions for optimal growth and survival.

The catalytic AMPK/SNF1/SnRK1 α subunits typically function in heterotrimeric 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

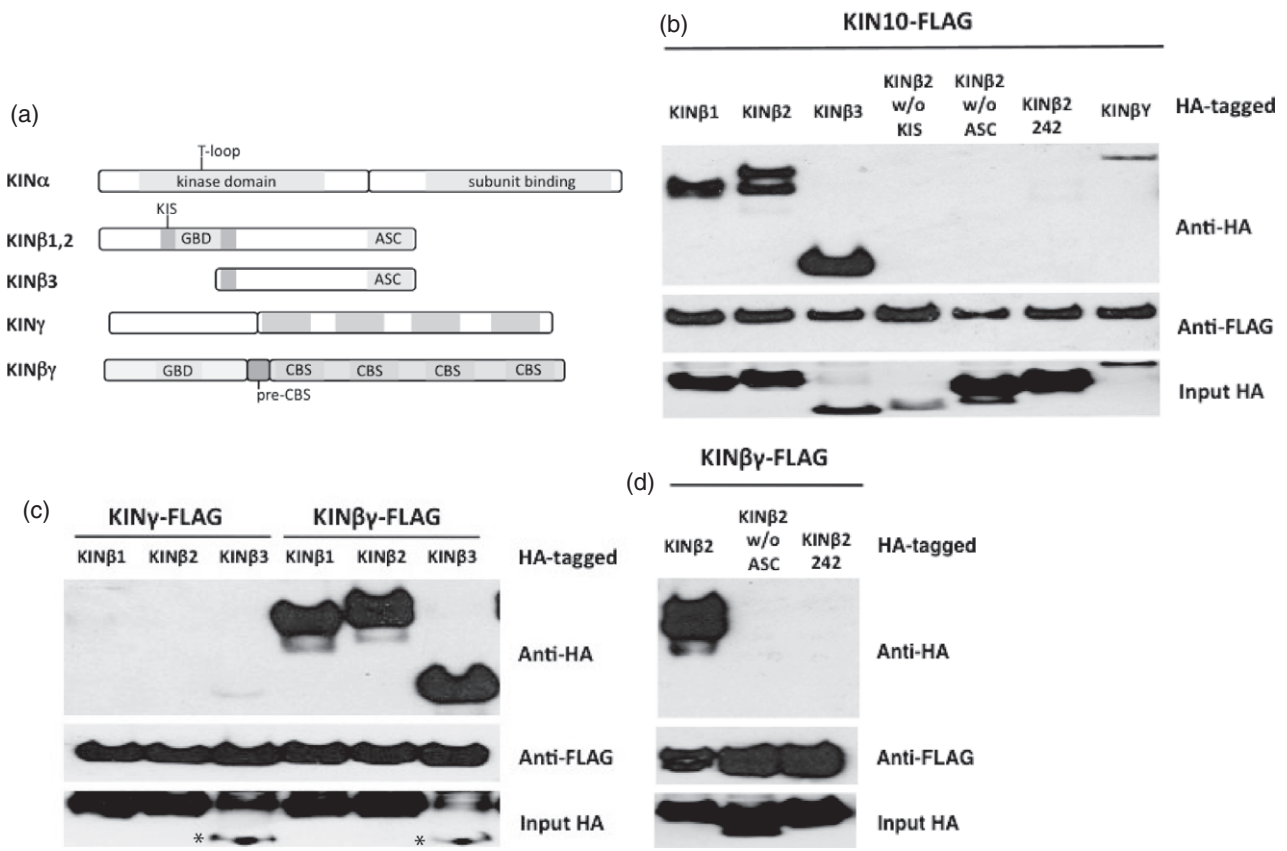

Figure 1. Specific KINβγ binding to the regulatory KINβ subunits.
(a) Structure and domain composition of the plant SnRK1 subunits. The GBD (light grey) in the β subunits (KINβ1 and KINβ2) overlaps with the kinase-interacting sequence (KIS) domain (dark grey).
(b) Co-immunoprecipitation of HA-tagged 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.
ASC, association with SNF1 complex; CBS, cystathionine β-synthase domain; GBD, glycogen-binding domain; HA, hemagglutinin; KINβ 242, partially truncated ASC (KINβ amino acids 1–242); KIS, kinase-interacting sequence.

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 and Carlson, 1997). Overlapping with the KIS domain, all β subunits also harbor a 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 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 CBS (hence the name), CLC voltage-dependent chloride channels and inosine-5′-monophosphate (IMP) dehydrogenase and, as for the γ subunit, mutations in their conserved nucleotide-binding residues have been associated with a variety of hereditary diseases in man (Scott *et al.*, 2004; Ignoul and Eggermont, 2005).

The AMPK/SNF1/SnRK1 kinases are controlled rigorously 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 activated directly 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 (Sanders *et al.*, 2007; Rubenstein *et al.*, 2008; Oakhill *et al.*, 2011; 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 SnRK1 regulatory subunit gamma (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 complement (Bradford *et al.*, 2003; Bolingue *et al.*, 2010). In addition, plants have acquired a unique hybrid βγ subunit, which combines an N-terminal GBD with a four CBS motif C-terminal γ part (Lumbreras *et al.*, 2001; Gissot *et al.*, 2006), and a truncated β subunit (β3), which lacks 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 (Kleinow *et al.*, 2000; Gissot *et al.*, 2006; Lopez-Paz *et al.*, 2009; Bitrian *et al.*, 2011). It was also suggested that the KINβγ subunit can form complexes without β subunits or homodimers (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 (Polge *et al.*, 2008; Lopez-Paz *et al.*, 2009; Robaglia *et al.*, 2012), but *in vivo* interaction studies with regulatory subunits are lacking. In addition, the diverse roles of plant four-CBS domain proteins in seed stress responses (SNF4-like) (Bradford *et al.*, 2003; Rosnoblet *et al.*, 2007; Bolingue *et al.*, 2010) 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. 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 heterotrimeric 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 four-CBS domain (FCD) proteins that acquired a 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-González *et al.*, 2007) showed that the SnRK1 catalytic α subunit, KIN10, was able to bind all three regulatory β subunits, and that these interactions depended on the KIS (Jiang and Carlson, 1997; Figure 1b). Deletion (KINβ2 w/o ASC) or truncation (KINβ2–242) of the ASC (association with SNF1 complex) domain, which abolished normal binding between regulatory β and γ subunits (Jiang and Carlson, 1997), eliminated the interaction between KINβ2 and KIN10, indica the requirement of a second regulatory subunit for SnRK1 complex formation (Figure 1b). As 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), a finding that suggested that only the latter contributes to SnRK1 complex formation. Furthermore, KINβγ was able to bind directly 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 (Lumbreras *et al.*, 2001; Gissot *et al.*, 2004, 2006; Polge *et al.*, 2008). Deletion of the yeast γ subunit gene, *SNF4*, does not affect growth on fermentable carbon sources such as glucose, but leads to severe growth defects on media with non-fermentable carbon sources (Neigeborn and Carlson, 1984)

(Figure 2a). Transformation of the *snf4*Δ strain with Arabidopsis KINγ could not restore growth on glycerol/ethanol medium, while growth on glucose was not affected (Figure 2a). In contrast, expression of yeast Snf4 and Arabidopsis KINβγ both restored growth of the yeast *snf4*Δ strain on non-fermentable carbon sources, a finding that suggested an important and conserved role for KINβγ in SnRK1 functioning (Figure 2a). All proteins tested were expressed efficiently 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 (*sip1*Δ *sip2*Δ *gal83*Δ). 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 *snf4*Δ complementation and therefore γ subunit functionality, we generated and tested systematically 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 *snf4*Δ 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 (Figure 3a). Also, no growth was observed when the pre-CBS domain of the KINβγ was fused to the FCD part of KINγ, a finding that suggested that, in addition to a functional pre-CBS domain, a functionally conserved FCD is required for yeast *snf4*Δ complementation (Figure 3a). All truncated and fused KINβγ proteins were expressed efficiently in yeast (Figure 3b).

These modified proteins were then also expressed transiently 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 compromised binding severely (Figure 3(c); Viana *et al.*, 2007), which suggested 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 genes 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 that consists of yeast and animal *SNF4/AMPKγ*-like genes and Amoebozoa, Heterokontophyta and Rhodophyta *SNF4* homologs (92 BS, 1.00 BPP; Figure 4). However, all

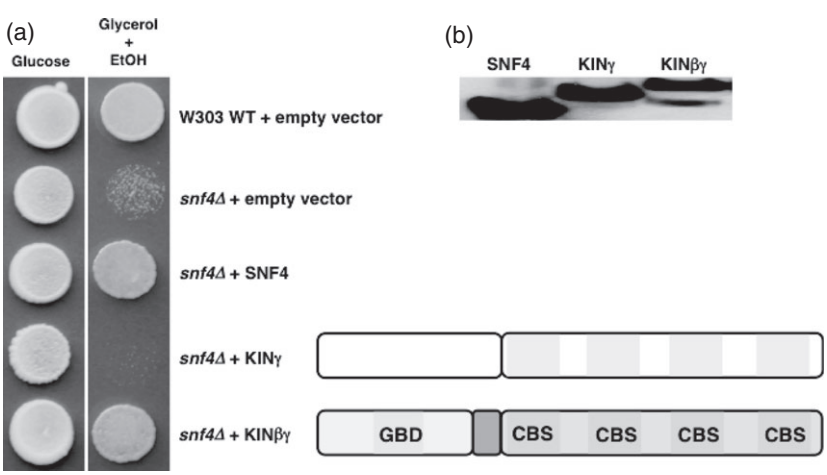

Figure 2. Hybrid Arabidopsis KINβγ (At1g09020) but not KINγ (At3g48530), complements the yeast snf4 γ subunit mutant growth defect on non-fermentable glycerol/ethanol medium.
(a) Heterologous expression in yeast of Arabidopsis KINγ and KINβγ Asa positive control the yeast SNF4 was also expressed. Cells 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. 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.
CBS, cystathionine β-synthase domain; GBD, glycogen-binding domain.

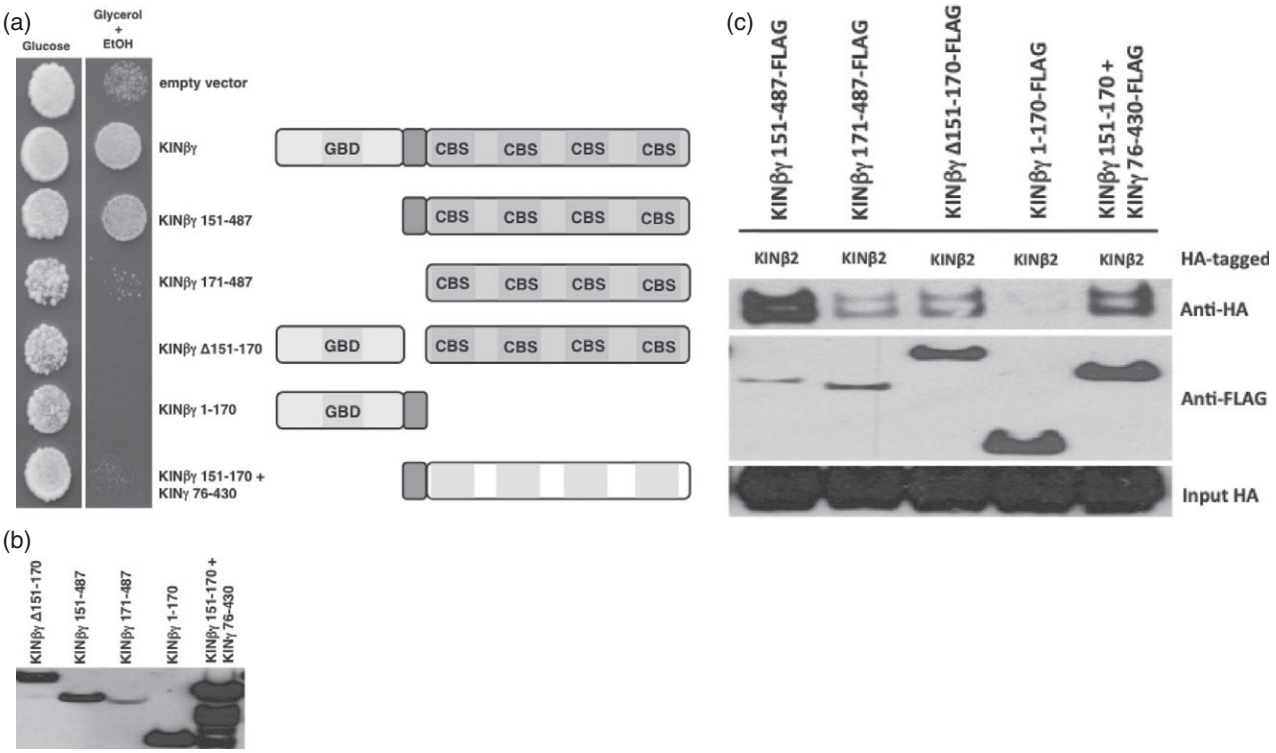

Figure 3. A dual requirement for the pre-CBS and Four-CBS-domain (FCD) region for yeast snf4 mutant growth defect complementation on non-fermentable glycerol/ethanol medium.

(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 gray) (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. The overall structure and domain composition of KINγ and KINβγ are indicated. 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 βγ subunits with FLAG-tagged full-length and truncated KINβγ subunits expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled beads.

aa, amino acids; CBS, cystathionine β-synthase domain; GBD, glycogen-binding domain.

other plant genes that encode FCD proteins are positioned outside this clade, including genes that have been reported previously to be γ-type subunits, such as *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 characterized functionally and lack 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 that contain an additional Phox and Bem1p (PB1) domain and are clearly distinguishable from the FCD-I genes, which is why we have classified 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 Figure S2. Animal and fungal FCD genes, other than the *SNF4/AMPKγ*-like genes, were left out, as we were unable to position these unambiguously in relationship to FCD-Ib, c and FCD-II. 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

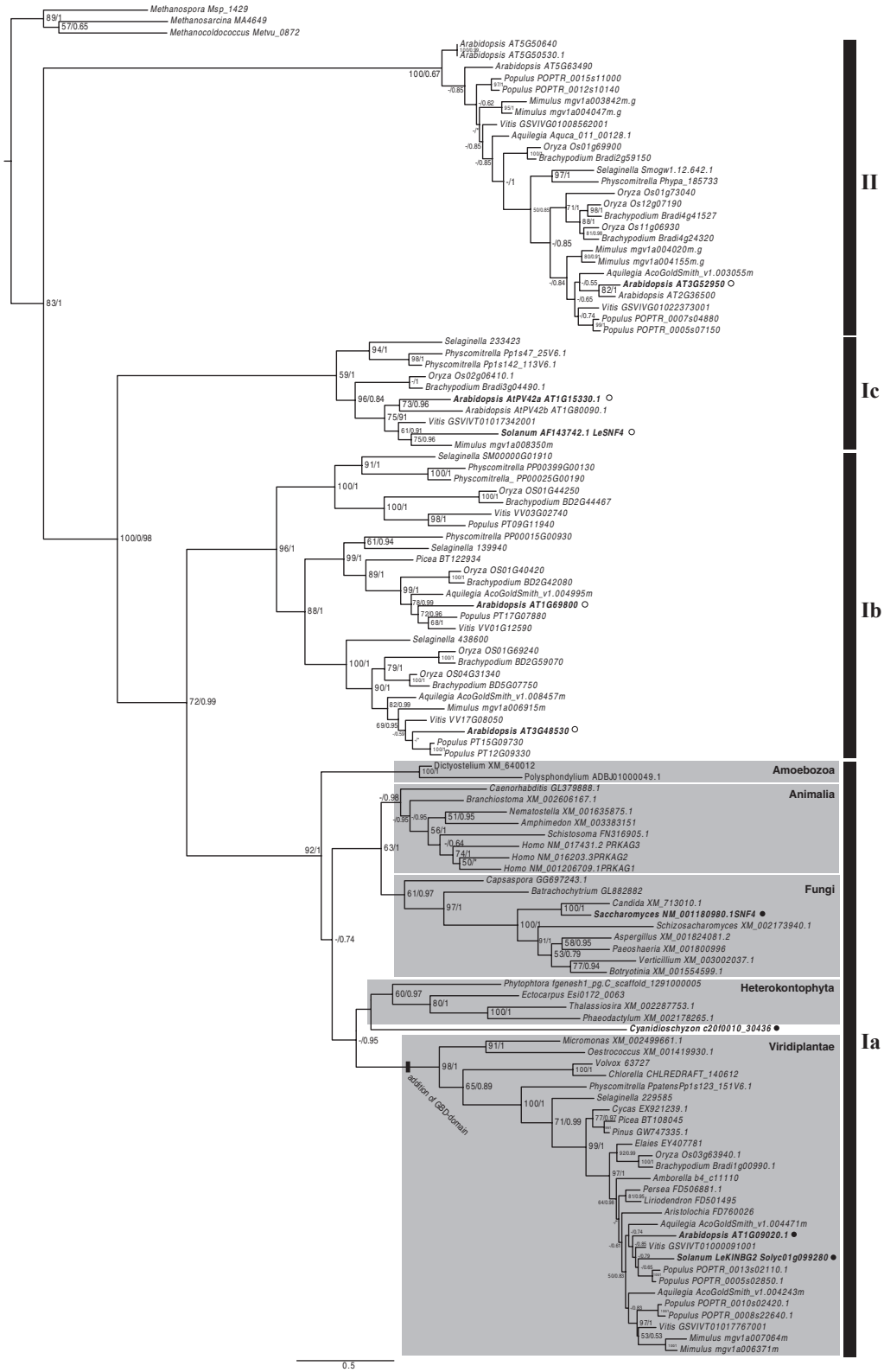

Figure 4. Maximum-likelihood phylogeny of the Four-CBS-domain (FCD) of *SNF4*, *AMPKγ*, *KINβγ*, *KINγ*, *PV42* and *IMDH* genes.
Numbers at the nodes represent maximum-likelihood (ML) bootstrap support values and Bayesian posterior probabilities. Genes in bold correspond to proteins that were assayed 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.

gene from each class was cloned and transformed into the yeast *snf4*Δ 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 *snf4*Δ 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 17 amino acids were retained as conserved in the FCD-Ia proteins and diverged in non-γ FCD proteins (Figures 5, S5 and S6). 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, a finding that suggested that also in tomato the βγ-like FCD-Ia family proteins are the canonical SnRK1 γ subunits (Figure 6a). All proteins were expressed efficiently (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 (Figures 7a and S8). Pull-down experiments in protoplasts showed that KIN10 could still bind efficiently 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. Wild-type (WT) and mutant protoplasts were transfected with *SEN1* promoter-luciferase reporter and KIN10 (SnRK1α) effector constructs. Basal levels and induction of promoter activity were 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 quantitative reverse transcription polymerase chain reaction (qRT-PCR) in a scaled-up experiment (Figure S9). Phenotypic characterization also did not reveal any 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 0.5 × Murashige and Skoog (MS) medium supplemented with 50 mM glucose. After 5 days, the medium was replaced with 0.5 × MS without sugars; samples were taken after 0, 30, 60 or 120 min. 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 h 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 heterotrimeric 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 coimmunoprecipitation (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

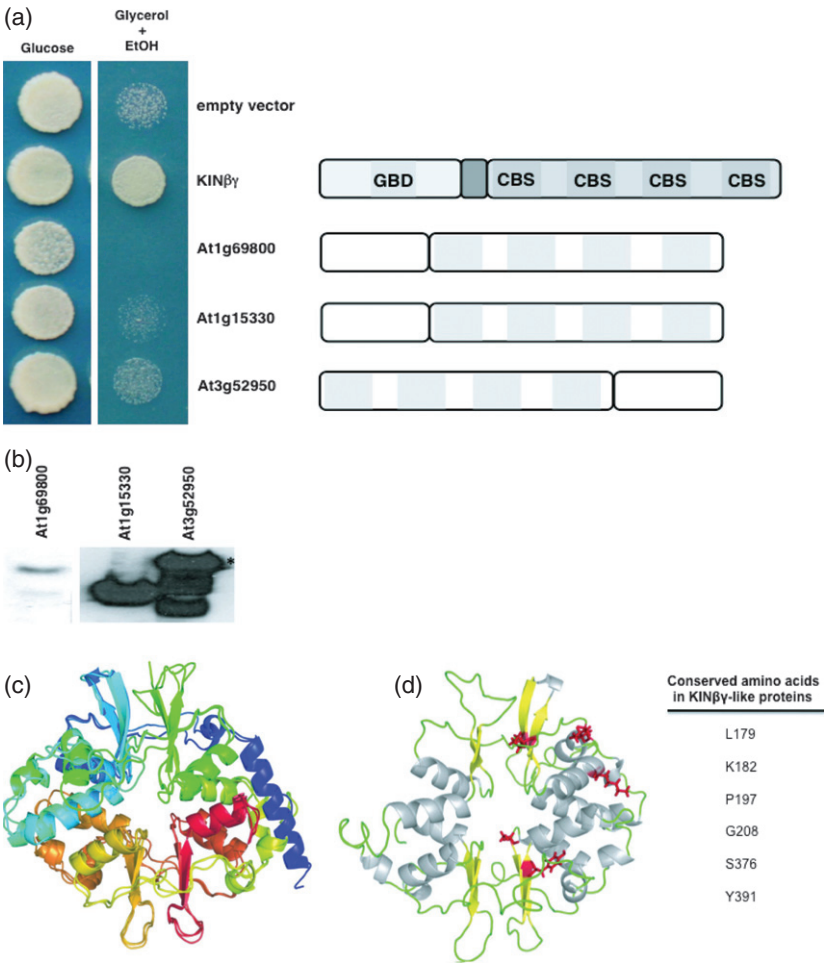

Figure 5. Non-hybrid Arabidopsis Four-CBS-domain (FCD) proteins lack the canonical γ subunit functionality.
(a) Heterologous expression in the yeast *snf4* mutant of Arabidopsis KINβγ (FCD-Ia), At1g69800 (FCD-Ib), At1g15330 (FCD-Ic/AtPV42a) and At3g52950 (FCD-II). Cells growing exponentially in minimal 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. 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 At1 g69800 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 six highly conserved amino acids (highlighted in red) in KINβγ -like hybrid plant proteins and animal and fungal proteins with reported γ subunit functionality.
CBS, cystathionine β-synthase domain; GBD, glycogen-binding domain.

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 (Figures 7 and 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 response (Figure 7(e)). 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 three β subunits have been proposed to exist

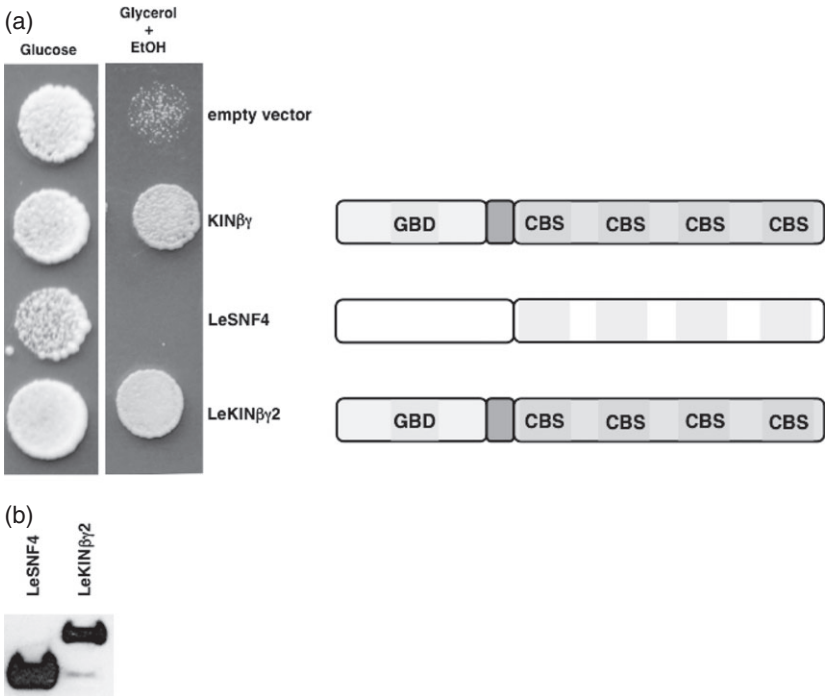

Figure 6. Non-hybrid tomato Four-CBS-domain (FCD) proteins lack the canonical γ subunit functionality.
(a) Heterologous expression in the yeast *snf4* mutant of Arabidopsis KINβγ (FCD-Ia) and tomato LeSNF4 (FCD-Ic) and LeKINβγ 2 (FCD-Ia). Cells growing exponentially in minimal medium (–uracil) with glucose 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. 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.
CBS, cystathionine β-synthase domain; GBD, glycogen-binding domain.

alongside KINγ-containing complexes including KINβ1 or KINβ2, but not KINβ3 (Lumbreras *et al.*, 2001; Gissot *et al.*, 2006). 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 heterotrimeric complexes (Lopez-Paz *et al.*, 2009). Our analyses now indicate that such complexes need to include KINβγ and that KINγ does not assemble in heterotrimeric plant SnRK1 complexes (Figures 1, 2 and 7). Truncations of the KINβ2 subunit, disrupting subunit interaction, further demonstrate that the βγ subunit is absolutely required for heterotrimeric SnRK1 complex formation with α- and β subunits (Figure 1). In addition to a complete ASC truncation (Jiang and 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 (Figures 3c and S7A), but not for γ subunit functionality. Molecular modeling based on the AMPKγ1 subunit confirms conservation of the overall FCD structure in KINγ (Figures 5c and S7A). However, besides this conserved structure, the FCD clearly requires additional features for the canonical γ subunit functionality in yeast (Figure 3a). As 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

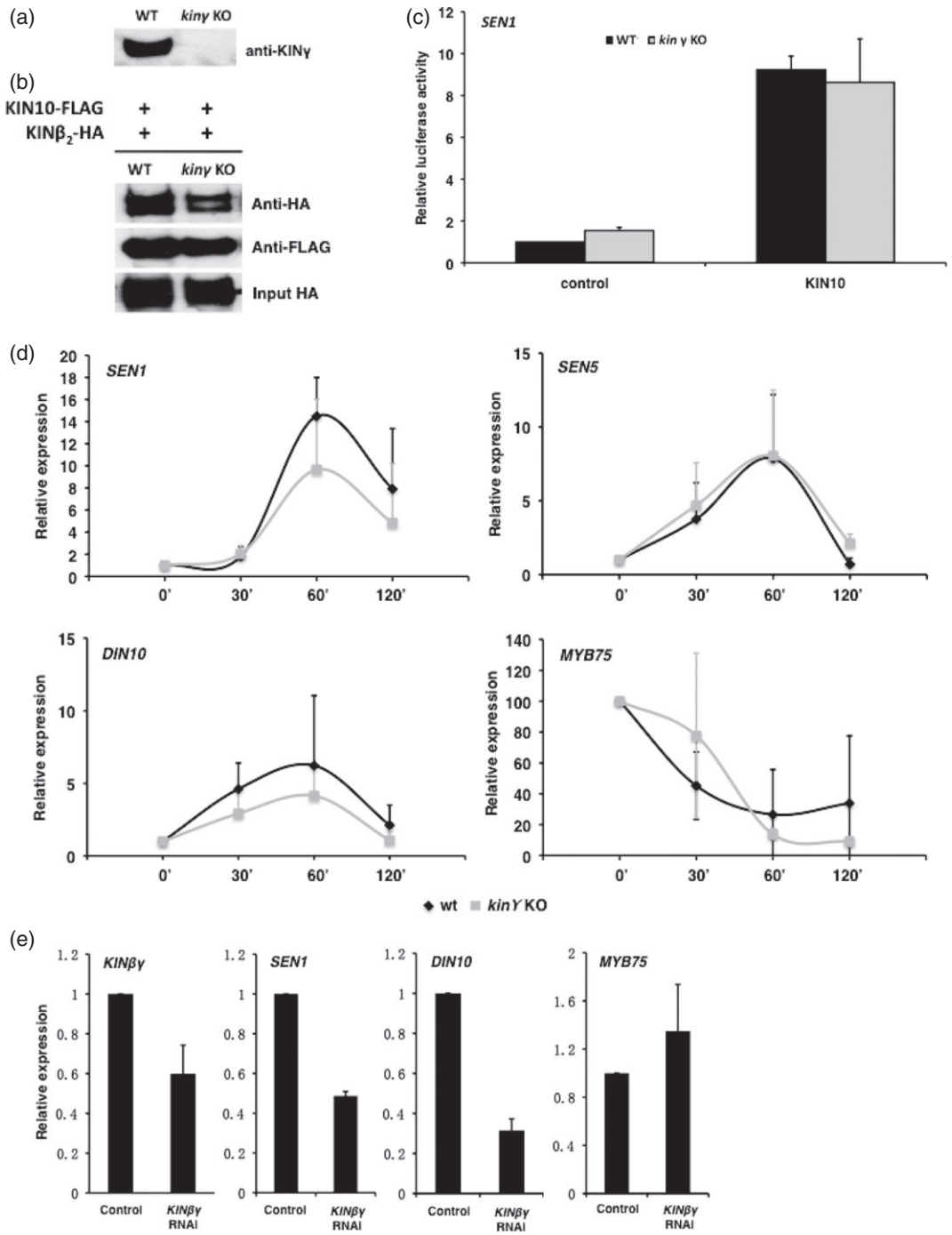

Figure 7. KINγ is not directly involved in SnRK1 signaling.
(a) T-DNA insertion line SALK_074554.52.55 is a complete *kin*γ null mutant as confirmed by western blot analysis on wild type and mutant seedlings.
(b) Efficient co-immunoprecipitation of the hemagglutinin (HA)-tagged β2 subunit with FLAG-tagged KIN10 expressed in Arabidopsis mesophyll protoplasts using FLAG-coupled beads.
(c) Wild-type response of transient KIN10 over-expression in *kin*γ mutant mesophyll protoplasts using a *SEN1/DIN1*-Luciferase reporter construct.
(d) Wild-type SnRK1 target gene responses in the *kin*γ in a seedling sugar starvation assay. Relative *SEN1/DIN1*, *SEN5*, *DIN10* (induced) and *MYB75* (repressed) target gene expression is assayed 0, 30, 60 or 120 min 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.

used docking of AMP in the crystallized structure of AMPKγ1 (17) and in optimized homology models of KINβγ and KINγ (Figures 5c,d and S7A) using Glide in Schrödinger Suite 2011 (Friesner *et al.*, 2004, 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 FCDs from five plant species with AMPKγs and yeast SNF4 revealed 17 conserved amino acids, most of them in the CBS domains; stringent alignment with non-γ FCD proteins retained six (Figures S3, S4, S5 and 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, for which 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 (Sugden *et al.*, 1999; Adams *et al.*, 2004; Momcilovic and Carlson, 2011). 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 (Toroser *et al.*, 2000; Ramon *et al.*, 2008; 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 (Bradford *et al.*, 2003; Rosnoblet *et al.*, 2007; Bolingue *et al.*, 2010; Fang *et al.*, 2011). 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 (Figure 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 ancestrally 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 (Niittylä *et al.*, 2006; Kötting *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 12 h light/12 h dark diurnal cycle with 70 μE light intensity for 4 weeks. Protoplast isolation was performed as described (Yoo *et al.*, 2007; Niu and Sheen, 2012). The *kinγ* SALK_074554.52.55 T-DNA line was obtained from ABRC and homozygous plants were selected on full MS medium with 50 mg ml^{-1} kanamycin. For western blot and PCR confirmation, vapor-sterilized and stratified seedlings were grown in 1 ml 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* knockout seeds were germinated 1 ml half strength MS medium supplemented with 50 mM 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 (*Bam*HI–*Stu*I) in the HBT95 expression vector (Sheen, 1996) in frame with a double hemagglutinin (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: 2.5 μl dNTPs (2.5 mM), 2.5 μl *Pfu* Turbo buffer 10 ×, 25 ng plasmid DNA, 10 ng primer A and B each, and 0.5 μl *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 (30 sec), 55°C (60 sec), 68°C (2 min/kb of plasmid). As a negative control, half of PCR the reaction mix was incubated at room temperature. *Dpn*I was then added to digest the methylated template DNA and 5 μl 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/PRE-CBSβγ.

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 (www.invitrogen.com) according to manufacturer's instructions. 1 μg of total RNA was used for reverse transcription (RT) with the A3500 Reverse Transcription System (www.promega.com). 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_2O, 0.1 μl CXR (5-carboxy-X-rhodamine reference dye) and 2 μl cDNA (5 ng μl^{-1}). The PCR program comprised an initial denaturation for 2 min at 95°C and amplification by 45 cycles of 3 sec at 95°C and 30 sec at 58°C in a StepOnePlus Real Time PCR system (www.appliedbiosystems.com). Expression levels were normalized to *UBIQUITIN10 (UBQ10)*. All qRT-PCR experiments were performed six times and the graph values are means with standard deviation.

Luciferase and β-glucuronidase (GUS) assays

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

Protein expression

For co-immunoprecipitation experiments, around 400 000 leaf mesophyll protoplasts were co-transfected with 40 μg of each (CsCl

gradient purified) construct. After harvesting, cells were lysed with 200 μl IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 1 tablet complete protease inhibitor (Roche 04693159001, www.roche.com) and incubated for 3 h with 30 μl FLAG-conjugated agarose beads (Sigma A2220) (pre-washed five times with IP buffer) at 4°C under gentle rotation. 20 μl lysate was not incubated with agarose beads and used as input control. After incubation, beads were washed five times with IP buffer. 40 μl loading buffer [1 × 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (50 mM MOPS, 50 mM Tris base, 0.1% sodium dodecyl sulphate (SDS), 1 mM EDTA), 16.22 g urea, 11.5 ml glycerol, 9.75 ml 20% SDS] was added to the agarose beads and samples were heated for 5 min at 95°C. 20 μl of bead supernatant and 15 μl of input lysate were loaded on a 10% SDS-PAGE gel and separated in a 1 × MOPS running buffer at 60 V for 15 min and 160 V for 1 h. After running, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon®-P, www.millipore.com) with a semi-dry transfer system (Trans-Blot® SD, www.bio-rad.com) in 1 × MOPS buffer with 10% methanol for 1 h at 12 V. After incubation with 5% skimmed milk, the membrane was incubated with antibody in 1% milk for 2 h (conjugated HA-antibody, conjugated FLAG antibody; Roche). The membrane was washed five times in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20), incubated with Pierce SuperSignal® West Pico chemiluminescent substrate (34078, www.thermoscientific.com) for 1 min and exposed to film for several minutes. To check protein expression in yeast, cells were grown to exponential phase on synthetic defined medium without uracil (SD-ura) and lysed with IP buffer and glass beads three times for 40 sec at 4°C in a FastPrep FP120 Homogenizer (www.thermoscientific.com). Protein concentrations were equalized after Bradford protein concentration measurements and 20 μl was loaded on gel for western blot analysis with conjugated HA-antibody (Roche). For KINγ protein determination in wild type and *kinγ* knockout plants, seedlings were crushed in 200 μl loading buffer and 20 μl 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, homologs 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 *KINβγ*, *KINγ*, *PV42a* and *At3 g52950* 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 and Toh, 2008) and manually refined in MacClade4 taken into consideration their amino acid translation (Maddison and 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 non-parametric bootstrap replicates. Bayesian analysis was carried out using MrBayes 3.2 (Ronquist and Huelsenbeck, 2003). Two independent runs with each four Markov Chain Monte Carlo chains were run for 15 000 000 generations and sampled every 1000 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; Proost *et al.*, 2009; Goodstein *et al.*, 2012). 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 and Huelsenbeck, 2003). Two independent runs for 3 000 000 generations with each four MCMC chains were sampled every 1000 generations. The first 1000 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 and Blundell, 1993). For evaluation of the models the internal discrete optimized protein energy scoring function was used. Figures were made using PyMOL. Optimal structures were imported in MAESTRO 9.2 (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, 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 CLUSTALW (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 (*MATa 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 (*Bam*HI and *Sma*I 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 *Bam*HI and *Sma*I-compatible *Stu*I 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 and 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 3 days at 30°C.

ACKNOWLEDGEMENTS

The authors would like to thank Marian Carlson for yeast strains. Research in the Rolland and Geuten labs is supported by the Fund for Scientific Research – Flanders (FWO). Research in the Sheen laboratory is supported by National Science Foundation (NSF) grant IOS-0843244 and United States National Institutes of Health (NIH) grant R01 GM60493. The authors declare to have no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Kin$\beta\gamma$ displays no β-subunit functionality.

Figure S2. Maximum likelihood phylogeny of *SNF4*/*AMPK*γ and plant *KIN*$\beta\gamma$ FCD-la genes.

Figure S3. Alignment of the FCD of hybrid $\beta\gamma$ proteins from 5 different plant species.

Figure S4. Alignment the FCD of hybrid $\beta\gamma$ proteins (FCD-la) from 5 different plant species together with the human AMPKγ and yeast SNF4 protein sequences, highlighting conservation of the CBS motifs.

Figure S5. Alignment of the non-canonical γ-like FCD proteins with AMPKγ and SNF4 protein sequences.

Figure S6. Alignment of hybrid $\beta\gamma$ proteins (FCD-la) from 5 different plant species with the non-canonical γ-like FCD proteins to identify conserved amino acids in the FCD-la proteins.

Figure S7. *In silico* analysis of nucleotide binding using docking of AMP in the crystallized structure of AMPKγ1 and in optimized homology models of KIN$\beta\gamma$ and KINγ.

Figure S8. The mutant *kin*γ line SALK_074554.

Figure S9. Wild type response of transient KIN10 over-expression in *kin*γ mutant mesophyll protoplasts.

Figure S10. Expression of Arabidopsis canonical and non-canonical γ-subunit FCD genes throughout development.

Figure S11. Bayesian phylogenetic tree of the carbohydrate binding domains of β-subunits, KIN$\beta\gamma$, SEX4 and LSF1 homologs.

Table S1. Oligonucleotides used in this study.

REFERENCES

Adams, J., Chen, Z.P., Van Denderen, B.J., Morton, C.J., Parker, M.W., Witters, L.A., Stapleton, D. and Kemp, B.E. (2004) Intrasteric control of AMPK via the gamma1 subunit AMP allosteric regulatory site. *Protein Sci.*, **13**, 155–165.

Baena-González, E. and Sheen, J. (2008) Convergent energy and stress signaling. *Trends Plant Sci.*, **13**, 474–482.

Baena-González, E., Rolland, F., Thevelein, J.M. and Sheen, J. (2007) A central integrator of transcription networks in plant stress and energy signalling. *Nature*, **448**, 938–942.

Ball, S., Colleoni, C., Cenci, U., Raj, J.N. and Tirtiaux, C. (2011) The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis. *J. Exp. Bot.*, **62**, 1775–1801.

Banks, J.L., Beard, H.S., Cao, Y. *et al.* (2005) Integrated Modeling Program, Applied Chemical Theory (IMPACT). *J. Comput. Chem.*, **26**, 1752–1780.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J. and Wheeler, D.L. (2004) GenBank: update. *Nucleic Acids Res.*, **32** (Database issue), 23–26.

Bitrian, M., Roodbarkelari, F., Horvath, M. and Koncz, C. (2011) BAC-recombineering for studying plant gene regulation: developmental control and cellular localization of SnRK1 kinase subunits. *Plant J.*, **65**, 829–842.

Bolingue, W., Rosnoblet, C., Leprince, O., Vu, B.L., Aubry, C. and Buitink, J. (2010) The MtSNF4b subunit of the sucrose non-fermenting-related kinase complex connects after-ripening and constitutive defense responses in seeds of *Medicago truncatula*. *Plant J.*, **61**, 792–803.

Bouly, J.P., Gissot, L., Lessard, P., Kreis, M. and Thomas, M. (1999) Arabidopsis thaliana proteins related to the yeast SIP and SNF4 interact with AKINalpha1, an SNF1-like protein kinase. *Plant J.*, **18**, 541–550.

Bradford, K.J., Downie, A.B., Gee, O.H., Alvarado, V., Yang, H. and Dahal, P. (2003) Abscisic acid and gibberellin differentially regulate expression of genes of the SNF1-related kinase complex in tomato seeds. *Plant Physiol.*, **132**, 1560–1576.

Buitink, J., Thomas, M., Gissot, L. and Leprince, O. (2003) Starvation, osmotic stress and desiccation tolerance lead to expression of different genes of the regulatory β and γ subunits of the SnRK1 complex in germinating seeds of *Medicago truncatula*. *Plant, Cell Environ.*, **27**, 55–67.

Carling, D., Clarke, P.R., Zammit, V.A. and Hardie, D.G. (1989) Purification and characterization of the AMP-activated protein kinase. Copurification of acetyl-CoA carboxylase kinase and 3-hydroxy-3-methylglutaryl-CoA reductase kinase activities. *Eur. J. Biochem.*, **186**, 129–136.

Comparot-Moss, S., Kötting, O., Stettler, M. *et al.* (2010) A putative phosphatase, LSF1, is required for normal starch turnover in *Arabidopsis* leaves. *Plant Physiol.*, **152**, 685–697.

Fang, L., Hou, X., Lee, L.Y., Liu, L., Yan, X. and Yu, H. (2011) AtPV42a and AtPV42b redundantly regulate reproductive development in *Arabidopsis thaliana*. *PLoS One*, **6**, e19033.

Fordham-Skelton, A.P., Chilley, P., Lumbreras, V., Reignoux, S., Fenton, T.R., Dahm, C.C., Pages, M. and Gatehouse, J.A. (2002) A novel higher plant protein tyrosine phosphatase interacts with SNF1-related protein kinases via a KIS (kinase interaction sequence) domain. *Plant J.*, **29**, 705–715.

Friesner, R.A., Banks, J.L., Murphy, R.B. *et al.* (2004) Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J. Med. Chem.*, **47**, 1739–1749.

Friesner, R.A., Murphy, R.B., Repasky, M.P., Frye, L.L., Greenwood, J.R., Halgren, T.A., Sanschagrin, P.C. and Mainz, D.T. (2006) Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J. Med. Chem.*, **49**, 6177–6196.

Ghillebert, R., Swinnen, E., Wen, J., Vandesteene, L., Ramon, M., Norga, K., Rolland, F. and Winderickx, J. (2011) The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. *FEBS J.*, **278**, 3978–3990.

Gietz, R.D. and Schiestl, R.H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc.*, **2**, 31–34.

Gissot, L., Polge, C., Bouly, J.P., Lemaitre, T., Kreis, M. and Thomas, M. (2004) AKINbeta3, a plant specific SnRK1 protein, is lacking domains present in yeast and mammals non-catalytic beta-subunits. *Plant Mol. Biol.*, **56**, 747–759.

Gissot, L., Polge, C., Jossier, M., Girin, T., Bouly, J.P., Kreis, M. and Thomas, M. (2006) AKINbetagamma contributes to SnRK1 heterotrimeric complexes and interacts with two proteins implicated in plant pathogen resistance through its KIS/GBD sequence. *Plant Physiol.*, **142**, 931–944.

Goodstein, D.M., Shu, S., Howson, R. *et al.* (2012) Phytozome: a comparative platform for green plant genomics. *Nucleic Acids Res.* **40** (Database issue), D1178–D1186.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. and Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.*, **59**, 307–321.

Hardie, D.G., Ross, F.A. and Hawley, S.A. (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat. Rev. Mol. Cell Biol.*, **13**, 251–262.

Hedbacker, K. and Carlson, M. (2008) SNF1/AMPK pathways in yeast. *Front. Biosci.*, **13**, 2408–2420.

Hubbard, E.J., Jiang, R. and Carlson, M. (1994) Dosage-dependent modulation of glucose repression by MSN3 (STD1) in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **14**, 1972–1978.

Hudson, E.R., Pan, D.A., James, J., Lucocq, J.M., Hawley, S.A., Green, K.A., Baba, O., Terashima, T. and Hardie, D.G. (2003) A novel domain in AMP-

activated protein kinase causes glycogen storage bodies similar to those seen in hereditary cardiac arrhythmias. *Curr. Biol.*, **13**, 861–866.

Ignoul, S. and Eggermont, J. (2005) CBS domains: structure, function, and pathology in human proteins. *Am. J. Physiol. Cell Physiol.*, **289**, C1369–C1378.

Janeček, S., Svensson, B. and MacGregor, E.A. (2011) Structural and evolutionary aspects of two families of non-catalytic domains present in starch and glycogen binding proteins from microbes, plants and animals. *Enzyme Microb. Technol.*, **49**, 429–440.

Jiang, R. and Carlson, M. (1997) The Snf1 protein kinase and its activating subunit, Snf4, interact with distinct domains of the Sip1/Sip2/Gal83 component in the kinase complex. *Mol. Cell. Biol.*, **17**, 2099–2106.

Katoh, K. and Toh, H. (2008) Improved accuracy of multiple ncRNA alignment by incorporating structural information into a MAFFT-based framework. *BMC Bioinformatics*, **9**, 212.

Kemp, B.E. (2004) Bateman domains and adenosine derivatives form a binding contract. *J. Clin. Invest.*, **113**, 182–184.

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

Kötting, O., Santelia, D., Edner, C. *et al.* (2009) STARCH-EXCESS4 is a laforin-like phosphoglucan phosphatase required for starch degradation in *Arabidopsis thaliana*. *Plant Cell*, **21**, 334–346.

Lang, T., Yu, L., Tu, Q., Jiang, J., Chen, Z., Xin, Y., Liu, G. and Zhao, S. (2000) Molecular cloning, genomic organization, and mapping of PRKAG2, a heart abundant gamma2 subunit of 5′-AMP-activated protein kinase, to human chromosome 7q36. *Genomics*, **70**, 258–263.

Lopez-Paz, C., Vilela, B., Riera, M., Pages, M. and Lumbreras, V. (2009) Maize AKINbetagamma dimerizes through the KIS/CBM domain and assembles into SnRK1 complexes. *FEBS Lett.*, **583**, 1887–1894.

Lumbreras, V., Alba, M.M., Kleinow, T., Koncz, C. and Pages, M. (2001) Domain fusion between SNF1-related kinase subunits during plant evolution. *EMBO Rep.*, **2**, 55–60.

Maddison, W.P. and Maddison, D.R. (2003) *MacClade 4: Analysis of Phylogeny and Character Evolution 4.06*. Sinauer Associates, Sunderland, MA.

Mangat, S., Chandrashekarappa, D., McCartney, R.R., Elbing, K. and Schmidt, M.C. (2010) Differential roles of the glycogen-binding domains of beta subunits in regulation of the Snf1 kinase complex. *Eukaryot. Cell*, **9**, 173–183.

McBride, A., Ghilagaber, S., Nikolaev, A. and Hardie, D.G. (2009) The glycogen-binding domain on the AMPK beta subunit allows the kinase to act as a glycogen sensor. *Cell Metab.*, **9**, 23–34.

Momcilovic, M. and Carlson, M. (2011) Alterations at dispersed sites cause phosphorylation and activation of SNF1 protein kinase during growth on high glucose. *J. Biol. Chem.*, **286**, 23544–23551.

Neigeborn, L. and Carlson, M. (1984) Genes affecting the regulation of SUC2 gene expression by glucose repression in *Saccharomyces cerevisiae*. *Genetics*, **108**, 845–858.

Niittylä, T., Comparot-Moss, S., Lue, W.L., Messerli, G., Trevisan, M., Seymour, M.D., Gatehouse, J.A., Villadsen, D., Smith, S.M, Chen, J., Zeeman, S.C. and Smith, A.M. (2006) Similar protein phosphatases control starch metabolism in plants and glycogen metabolism in mammals. *J. Biol. Chem.* **281**, 11815–11818.

Niu, Y. and Sheen, J. (2012) Transient expression assays for quantifying signaling output. *Methods Mol. Biol.*, **876**, 195–206.

Oakhill, J.S., Steel, R., Chen, Z.P., Scott, J.W., Ling, N., Tam, S. and Kemp, B.E. (2011) AMPK is a direct adenylate charge-regulated protein kinase. *Science*, **332**, 1433–1435.

Polekhina, G., Gupta, A., Michell, B.J. *et al.* (2003) AMPK beta subunit targets metabolic stress sensing to glycogen. *Curr. Biol.*, **13**, 867–871.

Polge, C. and Thomas, M. (2007) SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? *Trends Plant Sci.*, **12**, 20–28.

Polge, C., Jossier, M., Crozet, P., Gissot, L. and Thomas, M. (2008) Beta-subunits of the SnRK1 complexes share a common ancestral function together with expression and function specificities; physical interaction with nitrate reductase specifically occurs via AKINbeta1-subunit. *Plant Physiol.*, **148**, 1570–1582.

Posada, D. (2008) jModelTest: phylogenetic model averaging. *Mol. Biol. Evol.*, **25**, 1253–1256.

Proost, S., Van Bel, M., Sterck, L., Billiau, K., Van Parys, T., Van de Peer, Y. and Vandepoele, K. (2009) PLAZA: a comparative genomics resource to study gene and genome evolution in plants. *Plant Cell*, **21**, 3718–3731.

Ramon, M., Rolland, F. and Sheen, J. (2008) Sugar sensing and Signaling. *The Arabidopsis Book*. Bio-one: American Society of Plant Biologists, pp. 1–22.

Robaglia, C., Thomas, M. and Meyer, C. (2012) Sensing nutrient and energy status by SnRK1 and TOR kinases. *Curr. Opin. Plant Biol.*, **15**, 301–307.

Ronquist, F. and Huelsenbeck, J.P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rosnoblet, C., Aubry, C., Leprince, O., Vu, B.L., Rogniaux, H. and Buitink, J. (2007) The regulatory gamma subunit SNF4b of the sucrose non-fermenting-related kinase complex is involved in longevity and stachyose accumulation during maturation of *Medicago truncatula* seeds. *Plant J.*, **51**, 47–59.

Rubenstein, E.M., McCartney, R.R., Zhang, C., Shokat, K.M., Shirra, M.K., Arndt, K.M. and Schmidt, M.C. (2008) Access denied: Snf1 activation loop phosphorylation is controlled by availability of the phosphorylated threonine 210 to the PP1 phosphatase. *J. Biol. Chem.*, **283**, 222–230.

Sali, A. and Blundell, T.L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J. Mol. Biol.*, **234**, 779–815.

Sanders, M.J., Grondin, P.O., Hegarty, B.D., Snowden, M.A. and Carling, D. (2007) Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade. *Biochem. J.*, **403**, 139–148.

Santelia, D., Kötting, O., Seung, D. ***et al.*** (2011) The phosphoglucan phosphatase like sex Four2 dephosphorylates starch at the C3-position in *Arabidopsis*. *Plant Cell*, **23**, 4096–4111.

Schultz, J., Copley, R.R., Doerks, T., Ponting, C.P. and Bork, P. (2000) SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.*, **28**, 231–234.

Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G. and Hardie, D.G. (2004) CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. *J. Clin. Invest.*, **113**, 274–284.

Sheen, J. (1996) Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science*, **274**, 1900–1902.

Sugden, C., Crawford, R.M., Halford, N.G. and Hardie, D.G. (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5′-AMP. *Plant J.*, **19**, 433–439.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.*, **22**, 4673–4680.

Toroser, D., Plaut, Z. and Huber, S.C. (2000) Regulation of a plant SNF1-related protein kinase by glucose-6-phosphate. *Plant Physiol.*, **123**, 403–412.

Vander Kooi, C.W., Taylor, A.O., Pace, R.M., Meekins, D.A., Guo, H.F., Kim, Y. and Gentry, M.S. (2010) Structural basis for the glucan phosphatase activity of Starch Excess4. *Proc. Natl Acad. Sci. USA*, **107**, 15379–15384.

Viana, R., Towler, M.C., Pan, D.A., Carling, D., Viollet, B., Hardie, D.G. and Sanz, P. (2007) A conserved sequence immediately N-terminal to the Bateman domains in AMP-activated protein kinase gamma subunits is required for the interaction with the beta subunits. *J. Biol. Chem.*, **282**, 16117–16125.

Wiatrowski, H.A., Van Denderen, B.J., Berkey, C.D., Kemp, B.E., Stapleton, D. and Carlson, M. (2004) Mutations in the gal83 glycogen-binding domain activate the snf1/gal83 kinase pathway by a glycogen-independent mechanism. *Mol. Cell. Biol.*, **24**, 352–361.

Wilson, W.A., Hawley, S.A. and Hardie, D.G. (1996) Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.*, **6**, 1426–1434.

Xiao, B., Heath, R., Saiu, P. *et al.* (2007) Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature*, **449**, 496–500.

Xiao, B., Sanders, M.J., Underwood, E. ***et al.*** (2011) Structure of mammalian AMPK and its regulation by ADP. *Nature*, **472**, 230–233.

Yoo, S.D., Cho, Y.H. and Sheen, J. (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat. Protoc.*, **2**, 1565–1572.

Zhang, Y., Primavesi, L.F., Jhurreea, D., Andralojc, P.J., Mitchell, R.A., Powers, S.J., Schluepmann, H., Delatte, T., Wingler, A. and Paul, M.J. (2009) Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiol.*, **149**, 1860–1871.

