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# Domain fusion between SNF1-related kinase subunits during plant evolution

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**Members of the conserved SNF1/AMP-activated protein kinase (AMPK) family regulate cellular responses to environmental and nutritional stress in eukaryotes. Yeast SNF1 and animal AMPKs form a complex with regulatory SNF4/AMPKγ and SIP1/SIP2/GAL83/AMPKβ subunits. The β-subunits function as target selective adaptors that anchor the catalytic kinase and regulator SNF4/γ-subunits to their kinase association (KIS) and association with the SNF1 complex (ASC) domains. Here we demonstrate that plant SNF1-related protein kinases (SnRKs) interact with an adaptor-regulator protein, AKINβγ, in which an N-terminal KIS domain characteristic of β-subunits is fused with a C-terminal region related to the SNF4/AMPKγ proteins. AKINβγ is constitutively expressed in plants, suppresses the yeast *Δsnf4* mutation, and shows glucose-regulated interaction with the *Arabidopsis* SnRK, AKIN11. Our results suggest that evolution of AKINβγ reflects a unique function of SNF1-related protein kinases in plant glucose and stress signalling.**

## INTRODUCTION

Many biological processes depend on interactions between proteins acting in metabolic and signalling pathways or in the assembly of cellular structures. Despite this universal role of protein–protein interactions, our knowledge of how pairs, or even networks of proteins develop physical associations during evolution is rather limited. One possibility is that such associations arise spontaneously between two non-interacting proteins through accumulation of mutations that generate binding sites. Another hypothesis (known as the Rosetta Stone model) argues that a protein interaction develops preferentially between two domains of a single polypeptide, and that subsequent splitting of this protein generates a pair of binding factors (Enright *et al.*, 1999; Marcotte *et al.*, 1999). This model predicts the existence of pairs of interacting proteins that are fused within a single polypeptide in another organism. Here, we report the identification of such a mosaic protein during evolution of the SNF1/AMP-activated protein kinase (AMPK) family.

The yeast SNF1 kinase is a prototype of AMPKs that regulate cellular responses to a variety of nutritional and environmental stresses (Hardie *et al.*, 1998). In *Saccharomyces cerevisiae*, SNF1 is essential for adaptation to glucose starvation and the utilization of alternative carbon sources (Schuller and Entian, 1987; Carlson, 1999). SNF1 controls other basic processes, such as sporulation, glycogen storage, thermotolerance and peroxisome biogenesis (Carlson, 1998). In mammals, AMPKs mediate cellular responses to different types of stress (e.g. nutrient starvation, heat shock and hypoxia) by inhibiting key enzymes of ATP-consuming biosynthetic pathways (Hardie *et al.*, 1998). SNF1 and AMPK probably exert their related functions in different organisms by phosphorylating a conserved set of target proteins (Woods *et al.*, 1994).

SNF1/AMPK kinases function in a complex with other factors, including a regulatory subunit (SNF4 in yeast and AMPKγ in mammals) that binds to and activates the kinase catalytic subunit under stress conditions (Celenza and Carlson, 1989; Woods *et al.*, 1994). Another known component of SNF1/AMPK complexes is a family of interchangeable subunits including yeast SIP1, SIP2 and GAL83, and mammalian AMPKβ proteins. In yeast, SIP1/SIP2/GAL83 bind to SNF1 and SNF4 via two independent domains, the KIS and ASC domains, respectively (Jiang and Carlson, 1997). Thus, the SIP1/SIP2/GAL83/AMPKβ proteins function as adaptors that bring SNF1 and SNF4 into proximity and also contribute to substrate specificity of the kinase complex (Jiang and Carlson , 1996, 1997).

Here, we identify a novel class of plant SNF1-related subunits that show a unique structural organization of protein domains. These factors, designated as AKINβγ, are similar to the SNF4/AMPKγ

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proteins, but contain an extended N-terminal region related to the KIS domain of SIP1/SIP2/GAL83/AMPKβ. Functional analyses indicate that AKINβγ proteins retain a number of conserved activities, including the ability to suppress the yeast Δ*snf4* mutation and physically associate with plant SNF1 orthologs. These results provide an intriguing example of the evolution of interacting regulatory proteins within eukaryotes.

## RESULTS

### Identification of *AKINβγ* genes in *Zea mays* and *Arabidopsis thaliana*

We have used the yeast two-hybrid screen for maize factors that interact with Rab28, a late embryogenesis abundant protein that accumulates in response to osmotic stress in vegetative tissues (Niogret *et al.*, 1996). These experiments identified a novel protein similar to yeast and animal SNF4/AMPKγ factors that we have named AKINβγ-1. Although a functional interaction of AKINβγ-1 with Rab28 has not been confirmed yet, full-length cDNA clones were characterized leading to the identification of two related genes, *ZmAKINβγ-1* and *ZmAKINβγ-2*. These genes encode predicted proteins of 54.9 and 54.8 kDa, respectively, which are 95% identical and are homologous to SNF4/AMPKγ proteins (e.g. 30% identity with human AMPKγ, Figure 1). Surprisingly, ZmAKINβγ-1 and -2 are also similar in their N-terminal domains to the conserved KIS domain of SIP1/SIP2/GAL83/AMPKβ proteins (~42% identity with the KIS domain of human AMPKβ; Figure 1). This organization of KIS and SNF4/γ-like domains within the same polypeptide has not been previously found in components of SNF1/AMPK complexes, suggesting that ZmAKINβγ proteins could participate in the assembly of plant SNF1 complexes with a novel composition.

To determine whether the unexpected structure of ZmAKINβγ proteins was unique to maize or more widespread in plants, we examined the available sequences of SNF4 orthologs in *Arabidopsis*. One such ortholog, AKINγ (Bouly *et al.*, 1999), showed ~20% identity to yeast SNF4 and mammalian AMPKγ proteins, but no similarity to KIS-like sequences. A second SNF4 ortholog encoded by the *AtSNF4* gene (Kleinow *et al.*, 2000), also lacked a KIS motif. However, re-examination of the *AtSNF4* genomic sequence (DDBJ/EMBL/GenBank accession No. AC000106) revealed two putative exons encoding a KIS-related motif ~540–780 bp upstream of the predicted ATG initiation codon. We confirmed that such exons are indeed part of an AKINβγ transcript by PCR amplification of the relevant cDNA sequences (not shown). Thus, *AtSNF4* encodes a longer protein of 487 amino acids that is closely related to ZmAKINβγ-1 and ZmAKINβγ-2 (Figure 1), implying that AKINβγ factors are evolutionarily conserved in plants.

### Evolutionary relationships of SNF4-like and KIS domains

The structure of AKINβγ proteins suggests a remarkable reorganization of functional domains during evolution of SNF1/AMPK in different phyla. Phylogenetic analysis of KIS-domain sequences of AKINβγ factors and AMPKβ-like proteins from different species indicated that the KIS domains of AKINβγ and plant AMPKβ-like factors (Bouly *et al.*, 1999; Lakatos *et al.*, 1999) belong to distant

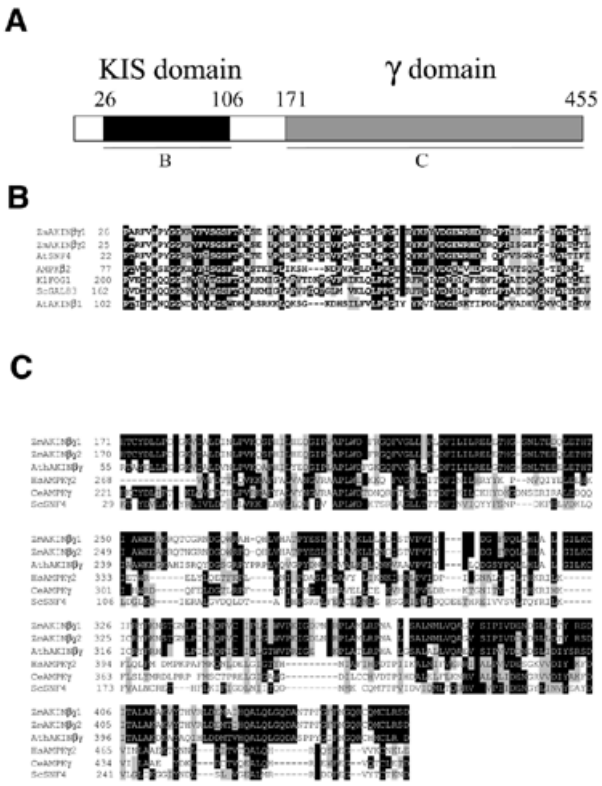


**Fig. 1.** AKINβγ factors are a novel family of plant proteins. (**A**) Diagram of the general structure of AKINβγ factors, which include conserved KIS and SNF4/AMPKγ domains. Numbers indicate amino acid positions for ZmAKINβγ-1. DDBJ/EMBL/GenBank sequence accession Nos for *AKINβγ* genes are as follows: AF276085 (*ZmAKINβγ-1*), AF276086 (*ZmAKINβγ-2*) and AF250335 (*AtSNF4*). (**B**) Alignment of the KIS regions from three AKINβγ proteins (ZmAKINβγ-1, -2 and AtSNF4) and other SNF1/AMPK β-subunits including human AMPKβ2, *K. lactis* FOG1, *S. cerevisiae* GAL83 and *A. thaliana* AKINβ1. (**C**) Alignment of the SNF4 regions from the three AKINβγ proteins and other SNF4/AMPKγ-subunits including human AMPKγ2, *C. elegans* AMPKγ and *S. cerevisiae* SNF4. Numbers indicate amino acid positions.

sequence classes (Figure 2A). The KIS domains of AKINβγ proteins share 40–45% identity with the KIS domains of mammalian AMPKβ proteins, but show only 29–32% identity with the equivalent region of *Arabidopsis* AKINβ2. Thus, plants contain two divergent classes of KIS domains, one in fusion with SNF4-like sequences in the AKINβγ proteins, and another in combination with the ASC domain of conserved AMPKβ subunits.

SNF4/AMPKγ-type factors other than the AKINβγ proteins may exist in plants. In *Arabidopsis*, a SNF4/AMPKγ-related protein, AKINγ, which does not possess a KIS motif has been identified (Bouly *et al.*, 1999). However, AKINγ shows only marginal sequence identity with animal AMPKγ and yeast SNF4 proteins (~17 and 20%, respectively) and is unable to suppress the yeast Δ*snf4* mutation (Bouly *et al.*, 1999), suggesting that AKINγ is a distant SNF4/AMPKγ ortholog. In contrast, AKINβγ proteins show a sequence identity of ~30% with mammalian AMPKγ proteins and of 26% with yeast SNF4 along the entire region compared, indicating that AKINβγ proteins may represent closer relatives of SNF4/AMPKγ than the AKINγ factors. The phylogenetic tree shows separate clusters for animal, yeast and plant proteins

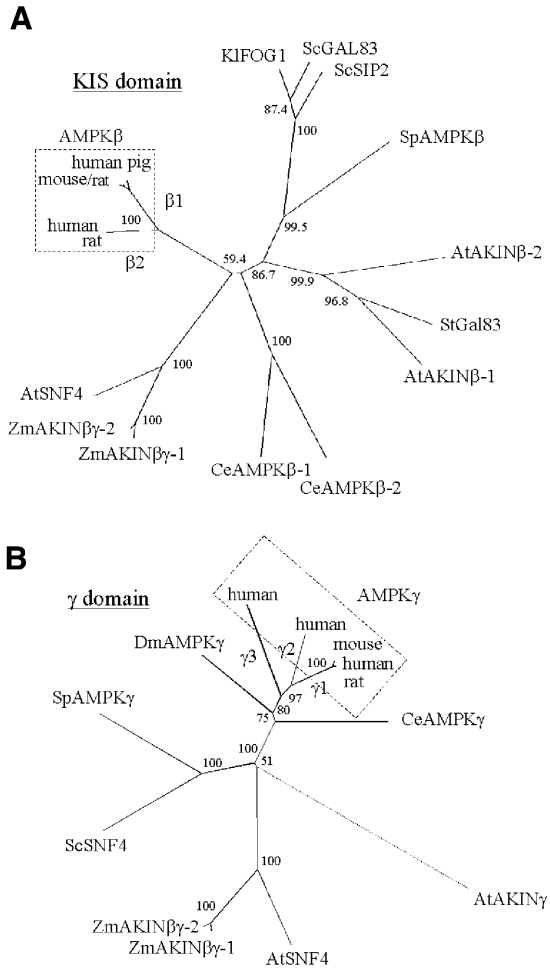


**Fig. 2.** Neighbour-joining tree showing the relationships between KIS (**A**) and SNF4/AMPKγ (**B**) sequences from different species. Numbers at the nodes represent bootstrap values. The results indicate the existence of two classes of KIS domains in plants, one present in AMPKβ-like proteins such as AtAKINβ-1 and -2, and the other in AKINβγ proteins (right side and bottom of tree in A, respectively). Similarly, plants appear to have two types of SNF4/AMPKγ sequences, one found in isolation as in AtAKINγ, and the other in combination with a KIS domain in AKINβγ factors.

(Figure 2B). The long branch for *Arabidopsis* AKINγ indicates its high sequence divergence from the rest of the proteins.

## Expression of *ZmAKINβγ* genes in maize

Northern blot analyses were performed to examine the expression of *ZmAKINβγ-1* and *-2* genes. The *ZmAKINβγ-1* cDNA probe detected a single band of 2.2 kb of similar intensity in root, stem and leaf (Figure 3A). This reflects the combined expression of *ZmAKINβγ-1* and *-2* since RT–PCR experiments indicate that both genes are expressed at similar levels (data not shown). We find that *ZmAKINβγ* expression is regulated during embryogenesis, being high in young embryos but barely detected at late stages of maturation (Figure 3A). We also tested the effects of different stress stimuli on *ZmAKINβγ* expression, but none of the experimental conditions analyzed (including heat, cold, water and salt stress) resulted in a significant induction of *ZmAKINβγ* transcription (Figure 3B). Hence, if *ZmAK-INβγ-1* and *-2* function in the control of stress responses, this is likely to be related to the activity of their encoded proteins as seen in yeast and mammals, where modulation of stress responses by the SNF1/AMPK complexes results from a regulated interaction of subunits, rather than from transcriptional regulation of the corresponding genes.

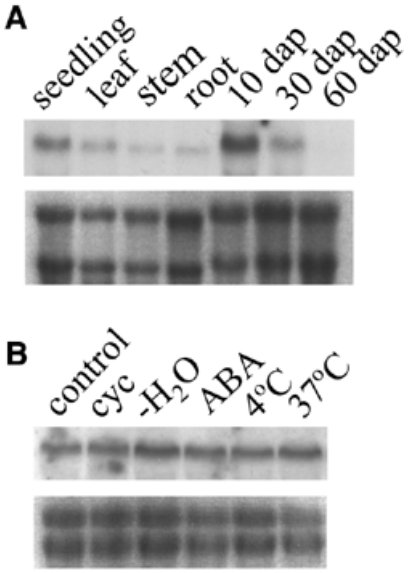


**Fig. 3.** Expression analysis of *ZmAKINβγ* genes. (**A**) Combined *ZmAKINβγ-1* and *-2* mRNA levels in different tissues (leaf, stem and root) and during embryogenesis (dap: days after pollination). (**B**) *ZmAKIN*βγ*-1* and *-2* expression in seedlings under different stress conditions: cycloheximide (cyc), desiccation ($-H_2O$), abscisic acid (ABA), cold (4°C) and heat (37°C). A control of RNA loading is shown in both panels.

## Complementation of the Δ*snf4-2* yeast mutant by ZmAKINβγ-1 and ZmAKINβγ-2

To examine the functional conservation of ZmAKINβγ-1 and -2 proteins, a yeast suppressor assay was performed by testing the ability of ZmAKINβγ-1 and -2 to rescue the glucose dependence of Δ*snf4-2* mutant cells. Δ*snf4-2* cells expressing either ZmAK-INβγ-1, -2 or the full-length AtSNF4, grew equally well on media containing glucose or glycerol at 30°C, whereas control mutant cells only grew in the presence of glucose (Figure 4, and T. Kleinow and C. Koncz, unpublished observations). Thus, AKINβγ proteins are sufficiently similar to SNF4 to substitute for this factor during glucose starvation of yeast cells. As expected, this function requires only the SNF4-like sequences present in ZmAKINβγ-1 and -2, because deletion derivatives of ZmAKINβγ-1 and -2 lacking the KIS domain also rescued the Δ*snf4-2* mutation (Figure 4).

## Glucose regulation of ZmAKINβγ interaction with a plant SNF1-related kinase

The yeast SNF4 subunit binds to and activates SNF1 during glucose starvation, whereas both the SNF4 and SNF1 subunits interact constitutively with SIP1/SIP2/GAL83 through the ASC

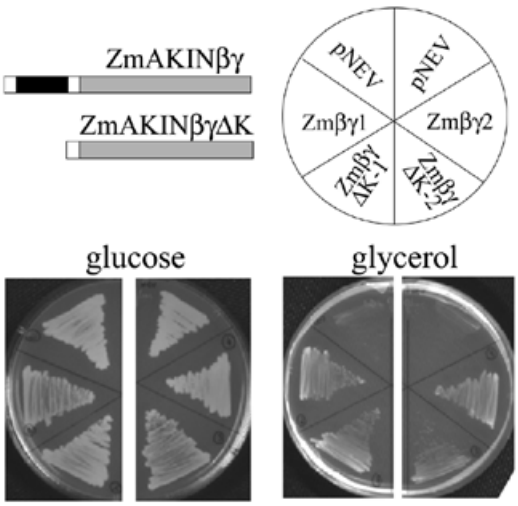


**Fig. 4.** Complementation of yeast Δ*snf4-2* mutant by ZmAKINβγ proteins. ZmAKINβγ-1 and -2 proteins and two derivatives lacking the KIS domain were expressed in yeast using the pNEV vector and tested for their ability to rescue the growth of Δ*snf4-2* cells in glucose-depleted medium containing 2% glycerol. Rescue is observed for all derivatives.

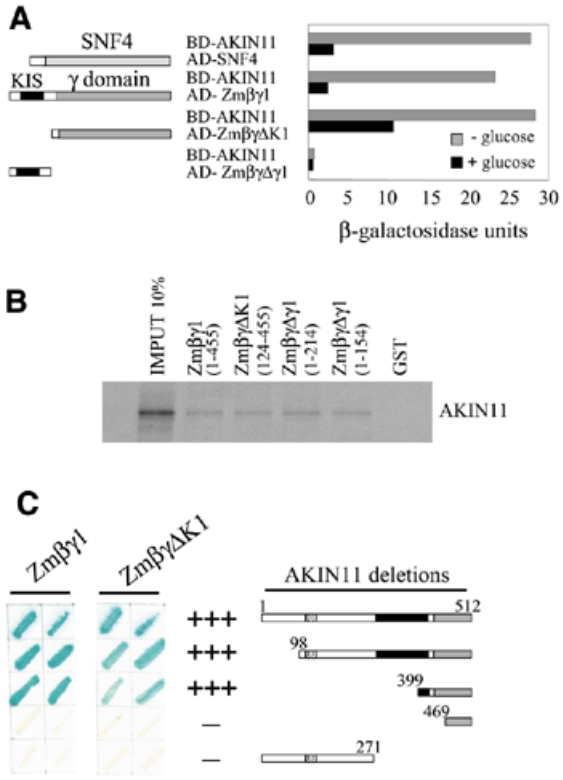


**Fig. 5.** ZmAKINβγ-1 interacts with SNF1-related protein AKIN11 from *Arabidopsis*. (**A**) Yeast two-hybrid assays for binding of *Arabidopsis* AKIN11 to control yeast SNF4 and three ZmAKINβγ-1 derivatives (full-length, and KIS and γ-like truncations). Binding to a control KIS domain from AtAKINβ2 is also shown. Binding assays were performed on low glucose (2% glycerol–galactose–ethanol plus 0.05% glucose) and high glucose (2%) media. AD- and BD- denote fusions with GAL4 activation and DNA-binding domains, repectively. (**B**) Equal amounts of [$^{35}$S]methionine-labelled AKIN11 incubated with GST, GST–ZmAKINβγ-1, GST–ZmAKINβγΔK-1, GST–ZmAKINβγΔγ-1 (1–214) and GST–ZmAKINβγΔγ-1 (1–154) proteins coupled to gluthathione-Sepharose show specific retention on ZmAKINβγ-1 and derivatives matrices *in vitro*. (**C**) Mapping of AKIN11 domains that bind to ZmAKINβγ-1. Cells were grown on low glucose medium. Black and grey domains of AKIN11 denote regions with similarity to yeast SNF1 domains that interact with SNF4p and SIP2p, respectively; the catalytic-centre is indicated by a dotted box. Numbers indicate amino acid positions.

and KIS domains, respectively (Jiang and Carlson, 1997). To test whether AKINβγ proteins establish similar associations with plant kinases, we assayed for interaction of ZmAKINβγ-1 with *Arabidopsis* SNF1-related kinase AKIN11 (Bhalerao *et al.*, 1999). Full-length ZmAKINβγ-1 and two derivatives lacking the SNF4-like domain (ZmAKINβγΔγ-1) or the KIS motif (ZmAKINβγΔK-1) were expressed in yeast in fusion with the GAL4-activation domain (AD) and tested for their ability to interact with full-length AKIN11 fused to the GAL4 DNA-binding domain (BD). ZmAKINβγ-1 and ZmAKINβγΔK-1 interacted with AKIN11, whereas ZmAKINβγΔγ-1 did not recognize the AKIN11 bait (Figure 5A). It should be noted that the minimal region of Gal83 shown to interact with SNF1 comprises residues 198–350 (Jiang and Carlson, 1997), whereas the conserved KIS domain of AKINβγ proteins is shorter and corresponds to residues 152–243 of Gal83.

However, this conserved AKINβγ KIS domain is sufficient for *in vitro* binding experiments using ZmAKINβγ-1 derivatives fused to glutathione *S*-transferase (GST) and radiolabelled AKIN11 protein. As shown in Figure 5B, all ZmAKINβγ-1 derivatives, including two fragments comprising the KIS region, bound AKIN11 with similar efficiency. This binding appears specific because the same KIS fragments do not bind another kinase, CK2α, in the same assay (data not shown). Together, these results suggest that both the SNF4-like and KIS domains of ZmAKINβγ-1 mediate interactions with plant SNF1-related kinases that parallel those described for SNF1-complex subunits in yeast.

We also tested the effect of glucose availability on the binding of AKINβγ-1 to AKIN11 using the two-hybrid assay. These proteins bound to each other more strongly in the absence than in the presence of glucose. Similar results have been obtained for yeast SNF4 and SNF1 proteins (Jiang and Carlson, 1996), suggesting that interactions between plant SNF1-subunits are sensitive to glucose-dependent signals in yeast. Interestingly, a deletion of the KIS domain from ZmAKINβγ-1 resulted in stronger binding to AKIN11 in the presence of glucose, one possibility is that this domain may affect the regulation of subunit interactions by glucose. The ZmAKINβγ-binding site in AKIN11 was mapped by assaying the interaction of ZmAKINβγ-1 with a series of BD-baits carrying N- and C-terminal deletions of AKIN 11. As reported for *Arabidopsis* (Kleinow *et al.*, 2000), these experiments identified a region in AKIN11 (amino acids 399–469) that is involved in binding to ZmAKINβγ-1, irrespective of the presence of the KIS domain of ZmAKINβγ-1 and the level of glucose in the medium (Figure 5C and data not shown). This critical region of AKIN11 overlaps the conserved domains of SNF1 proteins responsible for binding to SNF4 and GAL83/SIP1/SIP2 in yeast (Jiang and Carlson, 1997).

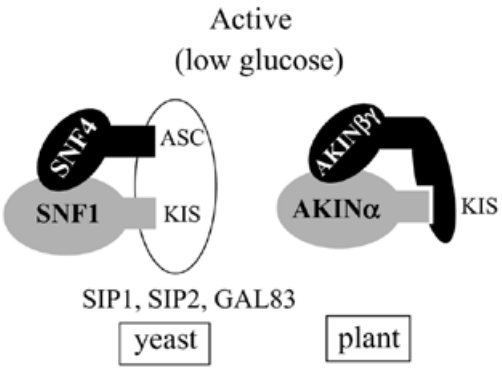


**Fig. 6.** Comparative models for protein interactions in the SNF1 complex in yeast and plants. The interactions shown are thought to occur in activated complexes. In yeast, the complex contains three subunits, SNF1 (α), SNF4 (γ) and SIP1/SIP2/GAL83 (β). Plants are likely to contain SNF1 complexes with a similar composition (see main text). In addition, plants contain AKINβγ factors that appear to be a fusion of γ- and β-like subunits, suggesting the existence of SNF1 complexes made of two subunits only.

## DISCUSSION

It has been proposed that protein–protein associations can evolve in the context of a single polypeptide that subsequently splits to generate a pair of interacting proteins. This model is supported by examples of partner proteins that are fused in another organism, usually involving sequences of prokaryotic origin (Marcotte *et al.*, 1999). Here, we have reported an example of such a mosaic protein between components of the SNF1 complex in plants.

The AKINβγ proteins could be the product of a fusion event of the KIS domain of SIP1/SIP2/GAL83/AMPKβ kinase subunits and the SNF4/AMPKγ subunits in the plant lineage. Alternatively, they could represent a primordial protein that subsequently split into two separate proteins during the evolution of other major eukaryotic phyla, according to the Rosetta Stone model. It should be noted that this model predicts the interacting ASC region to be present in the AKINβγ proteins. Perhaps, the ASC sequences have been lost during evolution of plant AKINβγ factors. Also, the presence in plants of other proteins related to SIP1/SIP2/GAL83 and to SNF4 could indicate an early duplication of these subunits prior to the fusion of domains or, alternatively, a duplication of the mosaic protein prior to the splitting event. Although it is not possible at present to fully delineate the evolutionary events leading to the diversity of SNF1 subunits in plants and other eukaryotes, the existence of AKINβγ proteins indicate that domain rearrangements have been important in the evolution of this family of proteins.

One important implication of our results is the apparent existence of SNF1 complexes in plants that may only contain two components: an AKINβγ and an SNF1-related kinase subunit (Figure 6). Previous studies suggested that plants may also have SNF1-like complexes with the general three-subunit composition (Bouly *et al.*, 1999; Lakatos *et al.*, 1999). However, the *Arabidopsis* SNF4-like protein AKINγ shares weak sequence similarity with yeast SNF4 and animal AMPKγ, and does not rescue the yeast *snf4* mutant (Bouly *et al.*, 1999), indicating a possible functional divergence of yeast and plant SNF1 complexes. Although we do not know yet the relative importance and specificity of different types of SNF1 complexes in plants, it is possible that such a diversity allows for an increased flexibility and regulation of SNF1-dependent responses in plants. Indeed, plant SNF1 kinases have been recently observed to associate with a regulatory WD-protein, PRL1, which is not present in budding yeast (Bhalerao *et al.*, 1999).

What could be the mechanism of action of plant SNF1-like complexes made of AKINβγ and SNF1-like kinase subunits? In yeast, glucose-regulated binding of SNF4 subunit to SNF1 is a key step in the regulation of the kinase activity, whereas the SIP1/SIP2/GAL83 subunits act as docking factors to increase the proximity of SNF4 and SNF1 (Figure 6). The interaction of the KIS region with SNF1-related kinases could be interpreted as a constitutive association in the complex (Jiang and Carlson, 1997).

We have shown that each of the the conserved KIS and SNF4-like domains of AKINβγ proteins bind to the *Arabidopsis* SNF1-related kinase AKIN11. Consistent with the yeast model, we find that binding of AKINβγ-1 to AKIN11 is dependent on glucose availability in yeast (Figure 5, Jiang and Carlson, 1996). However, our results suggest that the KIS domain may influences the regulation of interactions by glucose, suggesting that there are some differences between the function of yeast and plant SNF1 complexes. Further studies of the AKINβγ subunits and their interacting partners should help to uncover important specific aspects of SNF1-mediated glucose signalling during plant stress responses and development.

## METHODS

**Sequence and expression analyses of *ZmAKINβγ* genes.** A partial *ZmAKINβγ-1* cDNA clone was used to isolate full-length clones of *ZmAKINβγ-1* and *-2* (AF276085; AF276086) from a maize water stressed leaf cDNA library (Clontech). Similarity searches were performed using psi-BLAST and alignments were made using CLUSTAL-W. Distance matrices were derived from alignments using the neighbour-joining method of CLUSTAL-W, invoking the BLOSUM series and run with 100 bootstraps. Trees were viewed with DRAWTREE from the PHYLIP package (Felsenstein, 1995). A complete list of database sequences used in these comparisons are available on request. Northern analyses were performed using total maize RNA isolated from 5-day-old seedlings, leaves, stems and roots, and embryos of 10, 30 and 60 (dry embryo) days post-pollination. For stress experiments, RNA was isolated from seedlings exposed to heat, cold, desiccation, salt, abscisic acid (ABA, 100 μM) and cycloheximide (50 μM).

**Yeast experiments.** Complementation of the yeast *snf4* mutation with wild type and truncated ZmAKINβγ derivatives was carried out as described (Bhalerao *et al.*, 1999). To assay for protein–protein interactions, relevant coding sequences were cloned in-frame with GAL4 DNA binding and activation sequences in the pAS2 and pGAD424 vectors (Clontech), repectively. Yeast strain Y187 was transformed with various combinations of these plasmids and tested for the ability to grow on trp⁻leu⁻his⁻ plates. Interactions were also analysed by growing cells on solid medium containing either 2% glucose or 2% glycerol–galactose–

ethanol plus 0.05% glucose and assaying for *lacZ* reporter expression (Jiang and Carlson, 1996, 1997). β-galactosidase liquid assays were performed as described (Bhalerao *et al.*, 1999). Details on the construction of the plasmids used in these experiments are available on request.

***In vitro* binding experiments.** AKIN11 and different AKINβγ-1 cDNA fragments were cloned into pET28a (Promega) and pZex (Jiménez *et al.*, 1997) expression vectors, respectively. Expression of GST fusion proteins and binding assays were performed as described (Jiménez *et al.*, 1997).

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