AKIN$\beta\gamma$ Contributes to SnRK1 Heterotrimeric Complexes and Interacts with Two Proteins Implicated in Plant Pathogen Resistance through Its KIS/GBD Sequence[1]

Lionel Gissot, Cécile Polge[2], Mathieu Jossier, Thomas Girin[3], Jean-Pierre Bouly[4], Martin Kreis, and Martine Thomas*

Institut de Biotechnologie des Plantes, Unité Mixte de Recherche Centre National de la Recherche Scientifique 8618, Université Paris-Sud, F–91405 Orsay cedex, France (L.G., C.P., M.J., T.G., J.-P.B., M.K., M.T.); and Laboratoire de Biologie Cellulaire, Laboratoire Commun de Cytologie, Institut National de la Recherche Agronomique Versailles, RD10, F–78026 Versailles cedex, France (L.G.)

The sucrose nonfermenting-1 protein kinase (SNF1)/AMP-activated protein kinase subfamily plays a central role in metabolic responses to nutritional and environmental stresses. In yeast (*Saccharomyces cerevisiae*) and mammals, the β- and γ-noncatalytic subunits are implicated in substrate specificity and subcellular localization, respectively, and regulation of the kinase activity. The atypical $\beta\gamma$-subunit has been previously described in maize (*Zea mays*), presenting at its N-terminal end a sequence related to the KIS (kinase interacting sequence) domain specific to the β-subunits (Lumbreras et al., 2001). The existence of two components, SNF1-related protein kinase (SnRK1) complexes containing the $\beta\gamma$-subunit and one SnRK1 kinase, had been proposed. In this work, we show that, despite its unusual features, the Arabidopsis (*Arabidopsis thaliana*) homolog AKIN$\beta\gamma$ clearly interacts with AKINβ-subunits in vitro and in vivo, suggesting its involvement in heterotrimeric complexes located in both cytoplasm and nucleus. Unexpectedly, a transcriptional analysis of *AKIN$\beta\gamma$* gene expression highlighted the implication of alternative splicing mechanisms in the regulation of *AKIN$\beta\gamma$* expression. A two-hybrid screen performed with AKIN$\beta\gamma$ as bait, together with in planta bimolecular fluorescence complementation experiments, suggests the existence of interactions in the cytosol between AKIN$\beta\gamma$ and two leucine-rich repeats related to pathogen resistance proteins. Interestingly, this interaction occurs through the truncated KIS domain that corresponds exactly to a GBD (glycogen-binding domain) recently described in mammals and yeast. A phylogenetic study suggests that AKIN$\beta\gamma$-related proteins are restricted to the plant kingdom. Altogether, these data suggest the existence of plant-specific SnRK1 trimeric complexes putatively involved in a plant-specific function such as plant-pathogen interactions.

The Suc nonfermenting-1 protein kinase (SNF1) from yeast (*Saccharomyces cerevisiae*) and its mammalian homolog, the AMP-activated protein kinase (AMPK), are Ser/Thr kinases implicated in global metabolic regulation in response to cellular and environmental stresses (Hardie, 2004). Yeast SNF1 is implicated in cell adaptation to Glc deprivation (Carlson, 1999) by derepression of Glc-repressed genes in the absence of Glc. It is also involved in developmental processes (Honigberg and Lee, 1998) or cellular functions (Carlson, 1999). In mammals, AMPK is activated by an increase in the cellular AMP to ATP ratio. In response to nutrient starvation, exercise, or hypoxia, AMPK negatively regulates ATP-consuming pathways, including fatty acid and cholesterol synthesis, by phosphorylating metabolic key enzymes and promotes fatty acid β-oxidation to produce ATP (Hardie, 2004). Furthermore, AMPK is implicated in the modulation of Glc-regulated gene expression (Leclerc et al., 1998; Woods et al., 2000).

Some convergent results suggest the implication of SNF1-related protein kinase (SnRK1) in sugar signaling in plants (Bhalerao et al., 1999; Bradford et al., 2003; Halford et al., 2003; Lovas et al., 2003) and in the control of carbohydrate and starch metabolism (Purcell et al., 1998; Geigenberger, 2003; Laurie et al., 2003; Thelander et al., 2004). In vitro kinase assays suggest that SnRK1 might play a role in controlling metabolic pathways by phosphorylating key enzymes such as

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[2] Present address: Laboratoire de Physiologie Cellulaire Végétale, UMR5168, CEA-CNRS-INRA, Univ. Joseph Fourier, F–38054 Grenoble, France.

[3] Present address: Laboratoire Intégration des fonctions nutritives, UMR Biochimie et Physiologie Moléculaire des Plantes, ENSA-M/INRA/CNRS/UM2, place Viala, F–34060 INRA Montpellier cedex 1, France.

[4] Present address: Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes, Université Paris 6, bâtiment Le Raphaël, 3 rue Galilée, F–94200 Ivry-sur-Seine, France.

* Corresponding author; e-mail martine.thomas@u-psud.fr; fax 33 01–69–15–34–24.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Martine Thomas (martine.thomas@u-psud.fr).

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HMGCoA reductase, Suc phosphate synthase, and nitrate reductase (Sugden et al., 1999). More recently, the SNF1 kinase was also shown to participate in innate antiviral defenses (Hao et al., 2003) and in resource reallocation so that plants better tolerate herbivory (Schwachtje et al., 2006).

It is now well established that these kinases are associated with two types of noncatalytic proteins belonging to heterotrimeric kinase complexes composed of one catalytic subunit, one β-type subunit (SIP1/SIP2/GAL83, AMPKβ, and SnRKβ), and one γ-type protein (SNF4, AMPKγ, and SnRKγ; Halford et al., 2000).

The members of the SIP1/SIP2/GAL83 family might play an essential role in the specificity of recognition between the kinase complex and its targets (Vincent and Carlson, 1999) and also in the subcellular localization of the complex in yeast (Vincent et al., 2001; Warden et al., 2001; Hedbacker et al., 2004). The noncatalytic β-subunits from yeast (Celenza et al., 1989), mammals (Stapleton et al., 1994; Gao et al., 1996), and plants (Bouly et al., 1999; Lakatos et al., 1999; Bradford et al., 2003; Buitink et al., 2004) share a common structure composed of a variable N-terminal domain associated with the two highly conserved KIS (kinase interacting sequence) and ASC (association with SNF1 complex) domains, which mediate the interaction with α- and γ-subunits, respectively (Yang et al., 1992, 1994). Recently, a new domain implicated in glycogen binding and overlapping part of the KIS domain was characterized in AMPKβ1 (Hudson et al., 2003; Polekhina et al., 2003) and GAL83 (Wiatrowski et al., 2004), and thus named GBD (glycogen-binding domain). Interestingly, such a domain has also been identified in the protein phosphatase PTP-KIS (Fordham-Skelton et al., 2002). In Arabidopsis, AKINβ1 and β2 share the classical N-terminal/KIS/ASC structure (Bouly et al., 1999), while AKINβ3 presents only a truncated KIS domain, lacking the region related to the GBD, fused to the ASC domain (Gissot et al., 2004).

In yeast, SNF4 plays an essential role in the Glc regulation of SNF1 activity (Jiang and Carlson, 1996). Results obtained from yeast, mammals, *Drosophila melanogaster*, and plants (Gao et al., 1996; Stapleton et al., 1996; Bouly et al., 1999; Yoshida et al., 1999; Cheung et al., 2000) have shown that the SNF4/AMPKγ/SnRKγ family of proteins contains four in-tandem CBS (cystathionine β-synthase) motifs (Bateman, 1997). Bateman domains formed by tandems of CBS represent the AMP- and ATP-binding sites of the AMPK complex (Kemp, 2004; Scott et al., 2004). Interestingly, in mammals, several mutations localized within the CBS domains of the three AMPKγ-subunits result in the modification of the kinase activity and of its regulation by AMP (Cheung et al., 2000; Milan et al., 2000; Hamilton et al., 2001; Daniel and Carling, 2002; Scott et al., 2004; Burwinkel et al., 2005). Two unusual maize (*Zea mays*) SnRK1 γ-related proteins (*Zm*AKIN$\beta\gamma$-1 and -2) have been characterized by Lumbreras et al. (2001). These proteins present in their N-terminal end a sequence related to the KIS domain of the β-subunits (Lumbreras et al., 2001). *Zm*AKIN$\beta\gamma$-1 and -2 have been shown to complement the yeast *snf4* mutant and to interact with Arabidopsis AKIN11 (AKINα2) kinase, but at present nothing is known about their partners or their function (Kleinow et al., 2000; Lumbreras et al., 2001). Due to the presence of a KIS domain in AKIN$\beta\gamma$, the existence of two SnRK1 complexes containing only the AKIN$\beta\gamma$-type subunit and one SnRK1 kinase has been proposed by Lumbreras et al. (2001). In Arabidopsis, AKIN$\beta\gamma$ is the ortholog of *Zm*AKIN$\beta\gamma$-1 and -2 and corresponds to the initial annotation *At*SNF4 (Kleinow et al., 2000).

In this article, we show that AKIN$\beta\gamma$ interacts with other members of the AKIN complex in the two-hybrid system, suggesting their involvement in heterotrimeric alternative complexes. Bimolecular fluorescence complementation (BiFC) experiments, used to confirm these interactions in planta, have also allowed us to get the first data, to our knowledge, concerning the subcellular localization of plant SnRK1 proteins. Unexpectedly, a transcriptional study of *AKIN$\beta\gamma$* highlights the implication of alternative splicing mechanisms in the regulation of *AKIN$\beta\gamma$* expression. Finally, a two-hybrid screen performed with AKIN$\beta\gamma$ as bait suggests the interaction of AKIN$\beta\gamma$ with Leu-rich repeat (LRR)-rich proteins related to pathogen resistance proteins through their truncated KIS domain. Altogether, these data suggest the existence of plant-specific SnRK1 trimeric complexes putatively involved in plant-specific functions such as plant-pathogen interactions.

RESULTS

AKIN$\beta\gamma$ Gene Is Differentially Spliced

Mammalian AMPKγ1, γ2, γ3, and yeast SNF4 were used as probes to screen the National Center for Biotechnology Information (NCBI) databases (nr and dbest restricted to Arabidopsis). Some of the retrieved sequences correspond partially to the *At*SNF4 cDNA initially published by Kleinow et al. (2000), but their 5′ end differs from the annotations of the corresponding bacterial artificial chromosome (BAC) and of *At*SNF4 cDNA. Actually, 5′ RACE experiments revealed the existence of two other exons and of a long 5′ untranslated region (UTR) located upstream of the first predicted ATG of *At*SNF4 related to a KIS domain. Such a structure has been previously described by Lumbreras et al. (2001) in maize (*ZmAKIN$\beta\gamma$-1* and *ZmAKIN$\beta\gamma$-2*) and the Arabidopsis ortholog was named *AKIN$\beta\gamma$*. Interestingly, the KIS domain of these subunits is truncated compared to the KIS domains of the β-subunits and corresponds exactly to the GBD domain recently described in AMPKβ1 (Hudson et al., 2003; Polekhina et al., 2003) and GAL83 (Wiatrowski et al., 2004). For this reason, we propose to name this domain KIS/GBD. The *AKIN$\beta\gamma$* Arabidopsis gene contains 13 exons and 12 introns encoding a 2.3-kb long cDNA corresponding to a protein of 487 amino acids (53 kD; Fig. 1A). The length of the *AKIN$\beta\gamma$* cDNA is in accordance with

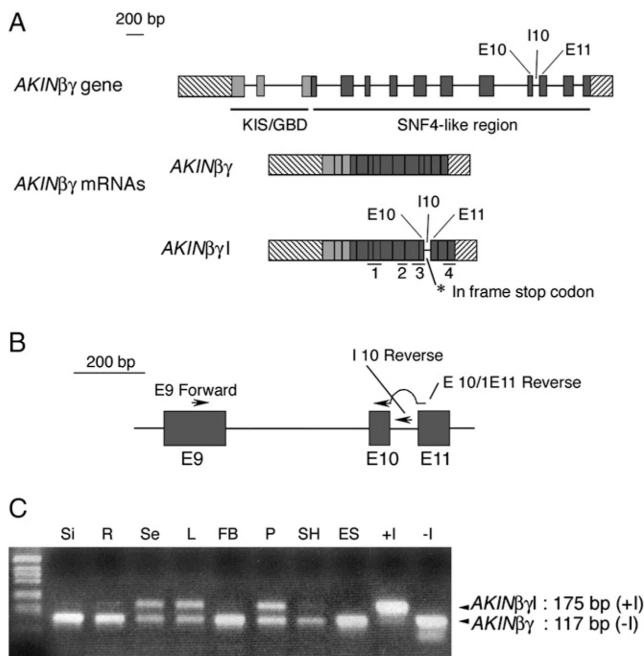

Figure 1. Structure of the *AKINβγ* gene and analysis of its expression. A, Intron/exon structure of the *AKINβγ* gene and the corresponding mRNAs. The 5′ and 3′ UTRs are represented by hatched boxes, exons (E) by colored boxes, and introns (I) by horizontal bars. Exons corresponding to the KIS/GBD domain (light gray boxes) and SNF4 domain (dark gray boxes) are represented by different gray levels. The four CBS domains are positioned on *AKINβγ* and *AKINβγI*, the two mRNAs derived from alternate splicing. B, Position of the oligonucleotides on exons 9, 10, and 11 and intron 10. The oligonucleotide E9Forward, positioned on exon 9, is used either with the oligonucleotide I10Reverse on intron 10 to amplify 175 bp of the *AKINβγI* cDNA or with the oligonucleotide E10/E11Reverse on the exon 10/intron 10 junction to amplify 117 bp of the *AKINβγ* cDNA. C, Analysis of the presence of *AKINβγ* and *AKINβγI* mRNAs. PCR experiments were performed using DNA of several cDNA libraries as template. Si, Siliques; R, roots; Se, seeds; L, leaves; FB, floral buds; P, pollen; SH, hypocotyls; ES, etiolated seedlings; +I, pGEMT vector (Promega) containing the *AKINβγI* cDNA; −I, pGEMT vector (Promega) containing the *AKINβγ* cDNA.

the 2.2-kb length found for *ZmAKINβγ-1* and *βγ-2* in maize (Lumbreras et al., 2001). Two kinds of PCR products were obtained and cloned after a 3′ RACE PCR, the largest including the unspliced intron 10. The two full-length cDNAs were named *AKINβγ* and *AKINβγI* (with and without intron 10, respectively). The protein sequence deduced from the *AKINβγI* cDNA is reduced to 394 amino acids (43.3 kD) due to the presence of a stop codon located 6 bp downstream of the beginning of intron 10. Therefore, one of the most conserved regions of the protein, mainly corresponding to the fourth CBS domain, is deleted in AKINβγI.

AKINβγI and *AKINβγ* Are Expressed in the Same Organs

To determine if the presence of the two cDNAs corresponds to an artifact product obtained during the building of the shoot library or to an alternative splicing, an analysis of the expression of these two cDNAs has been performed. The small difference in size observed between the two cDNAs (100 bp) did not allow us to separate *AKINβγ* and *AKINβγI* transcripts by northern-blot experiments (data not shown). Therefore, PCR experiments were performed using two pairs of oligonucleotides designed to selectively amplify each of the two putative cDNAs. One oligonucleotide (E9Forward), common to both cDNAs, is located on the exon 9, while a second is located either on the exon10/intron10 junction (E10/E11Reverse) to amplify a fragment of 117 bp corresponding to *AKINβγ* cDNA (without intron 10), or in intron 10 (I10Reverse) to amplify a 175-bp fragment corresponding to *AKINβγI* cDNA (including intron 10; Fig. 1B). DNA from eight cDNA libraries was used as templates. The sizes of the two PCR products of 117 and 175 bp correspond to the fragments amplified using the two control cDNAs [pGEMT vectors containing either *AKINβγ* (−I) or *AKINβγI* (+I) cDNA; Fig. 1C]. *AKINβγ* mRNA is ubiquitously expressed. On the other hand, a high level of *AKINβγI* mRNA is observed when using the libraries prepared from seeds, leaves, and pollen, while it is almost undetectable in siliques, roots, hypocotyls, and etiolated seedlings. Nevertheless, whatever the organs or conditions tested, *AKINβγI* mRNA is always present. These data, also obtained by reverse transcription-PCR experiments (data not shown), confirm the existence of an alternative splicing event.

CBS4 Deletion in AKINβγI Prevents the Interaction with the Kinases and the Complementation of the Yeast *snf4* Mutant

Two-hybrid experiments and in vitro coimmunoprecipitation assays were performed to study protein-protein interactions between AKINβγ/βγI and the AKINα1/α2 kinases (Fig. 2). The whole coding sequences of AKINβγ or AKINβγI were fused in-frame with the HA epitope and the GAL4 activation domain (AD) by cloning into the pGADT7 vector. The AKINα1 and α2 kinases were fused in-frame with the GAL4-binding domain (BD) and c-Myc epitope by cloning into the pGBKT7 vector. The two-hybrid experiments and in vitro binding assays show as expected that AKINβγ interacts with both kinases AKINα1 and α2, while no interaction was detected between AKINβγI and the kinases (Fig. 2). Therefore, the interaction between AKINβγ and the kinases appears to be lost when a region containing the fourth CBS domain (CBS4) is absent. Nevertheless, no interaction with the kinases could be detected using the CBS4 domain alone either in two-hybrid experiments or in in vitro binding assays. Thus, the domain absent in AKINβγI appears necessary but not sufficient for the interaction with the kinases.

To test whether, despite the absence of interaction with the AKINα kinases, AKINβγI protein was still able to act as a γ-subunit, we performed yeast *snf4*

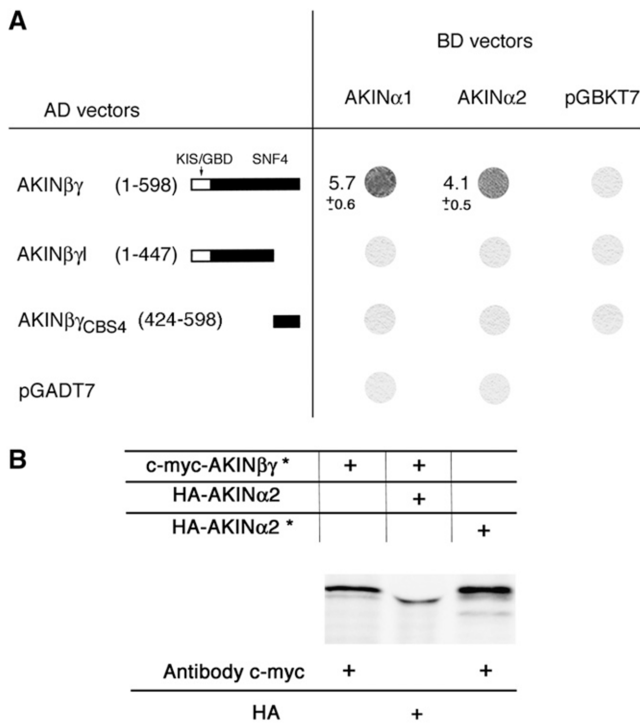

Figure 2. Interactions between AKIN$\beta\gamma$/$\beta\gamma$I and the AKINα1 and α2 kinases by two-hybrid experiments and in vitro binding assays. A, Qualitative β-galactosidase enzyme assays were performed between full-length AKIN$\beta\gamma$/$\beta\gamma$I or AKIN$\beta\gamma$ CBS4 domain and AKINα1 and α2 kinases. B, In vitro binding assays. Due to their similar molecular masses, AKIN$\beta\gamma$ and AKINα2 proteins were synthesized by in vitro transcription/translation system (Promega) without or with (*) ^{35}S-Met labeling and incubated alone or by pairs, respectively. After coimmunoprecipitation with HA or c-Myc antibodies, proteins were separated by 10% SDS-PAGE and exposed to an x-ray film.

complementation experiments. While the full-length AKIN$\beta\gamma$ protein complements the yeast *snf4* mutant on medium lacking Glc as carbon source, AKIN$\beta\gamma$I protein does not (data not shown).

The SNF4 Domain of AKIN$\beta\gamma$ Interacts with the AKINβ-Subunits to Form SnRK1 Heterotrimeric Complexes Located Both in the Cytoplasm and in the Nucleus

The KIS/GBD of AKIN$\beta\gamma$ has been previously shown to interact with the kinase without the requirement of a β-subunit, suggesting the existence of dimeric complexes composed by AKIN$\beta\gamma$ and one of the kinases in which this KIS/GBD domain could play the role of a β-subunit (Lumbreras et al., 2001). To test if the existence of the KIS/GBD in a γ-type subunit prevents the formation of trimeric complexes, two-hybrid, in vitro coimmunoprecipitation and in planta BiFC experiments were performed using AKIN$\beta\gamma$, AKIN$\beta\gamma$I, and each domain of AKINβ-subunits (Fig. 3). Interactions have been detected between AKIN$\beta\gamma$ and each of the three β-subunits (AKINβ1, β2, and β3; Fig. 3B, lanes FL) in vitro. The β-galactosidase activity measured for the interaction involving AKIN$\beta\gamma$ was 2.3- and 11.2-fold higher with AKINβ2 than with AKINβ3 and β1, respectively. However, no interaction was detected with these proteins when using AKIN$\beta\gamma$I in two-hybrid experiments (data not shown). These results were confirmed by BiFC experiments performed between AKIN$\beta\gamma$ fused to the N-terminal domain of the yellow fluorescent protein (YFP; YN-AKIN$\beta\gamma$) and the AKINβ-subunits fused with the C-terminal domain of the YFP (YC-AKINβ; Fig. 3A). All combinations led to a YFP signal showing a clear in planta interaction between YN-AKIN$\beta\gamma$- and YC-AKINβ-subunits. Interestingly, the YFP signal is located both in the cytoplasm and the nucleus, as presented in Figure 3A, for a classical β-subunit (YN-AKIN$\beta\gamma$/YC-AKINβ2 interaction) and for the plant-specific one (YN-AKIN$\beta\gamma$/YC-AKINβ3 interaction). No YFP signal was detected when YN-AKIN$\beta\gamma$ and YC-AKINβ1/2/3 were independently infiltrated (data not shown). Moreover, as a negative control, the PAS1 protein (Faure et al., 1998) that does not interact with SnRK1 subunits was fused to YC and YN and cotransformed in *Nicotiana benthamiana* leaves with YN-AKIN$\beta\gamma$ and YC-AKINβ1/2/3, respectively. No YFP fluorescence was detected in any case (data not shown), confirming the relevance of the previously shown interactions.

To precisely determine the domains involved in these interactions, the sequences encoding SNF4, KIS/GBD, and CBS4 domains of AKIN$\beta\gamma$ and the N-terminal, KIS, and ASC domains of AKINβ1/2/3 were subcloned into pGADT7 and pGBKT7 vectors and used in similar experiments (Fig. 3B). The ASC domains of all AKINβ-subunits were sufficient to direct the interaction with the SNF4 domain of AKIN$\beta\gamma$, while the KIS/GBD of AKIN$\beta\gamma$ does not interact with any AKINβ-subunits. No interaction was detected using other domains of AKIN$\beta\gamma$ or AKINβ1/2/3 (data not shown).

AKIN$\beta\gamma$-Subunit Is Restricted to the Plant Kingdom

At present, the AKIN$\beta\gamma$-type subunits have been described only in maize, Arabidopsis, and *Medicago truncatula* (Lumbreras et al., 2001; Buitink et al., 2004). To look for AKIN$\beta\gamma$-related sequences in other organisms, the NCBI databases were screened using the AKIN$\beta\gamma$ sequence as a probe. The retrieved sequences from plants, mammals, yeast, *D. melanogaster*, and *Caenorhabditis elegans* were aligned using ClustalX (Thompson et al., 1997). The corresponding γ-related sequences were restricted to the SNF4 region and used to draw a phylogenetic tree (Fig. 4). The plant γ-subunits appear separated from other organisms and distributed into three subgroups corresponding to three γ-subunits previously published: PV42p from *Phaseolus vulgaris* (Abe et al., 1995) and AKINγ (Bouly et al., 1999) and AKIN$\beta\gamma$ from Arabidopsis. The group including

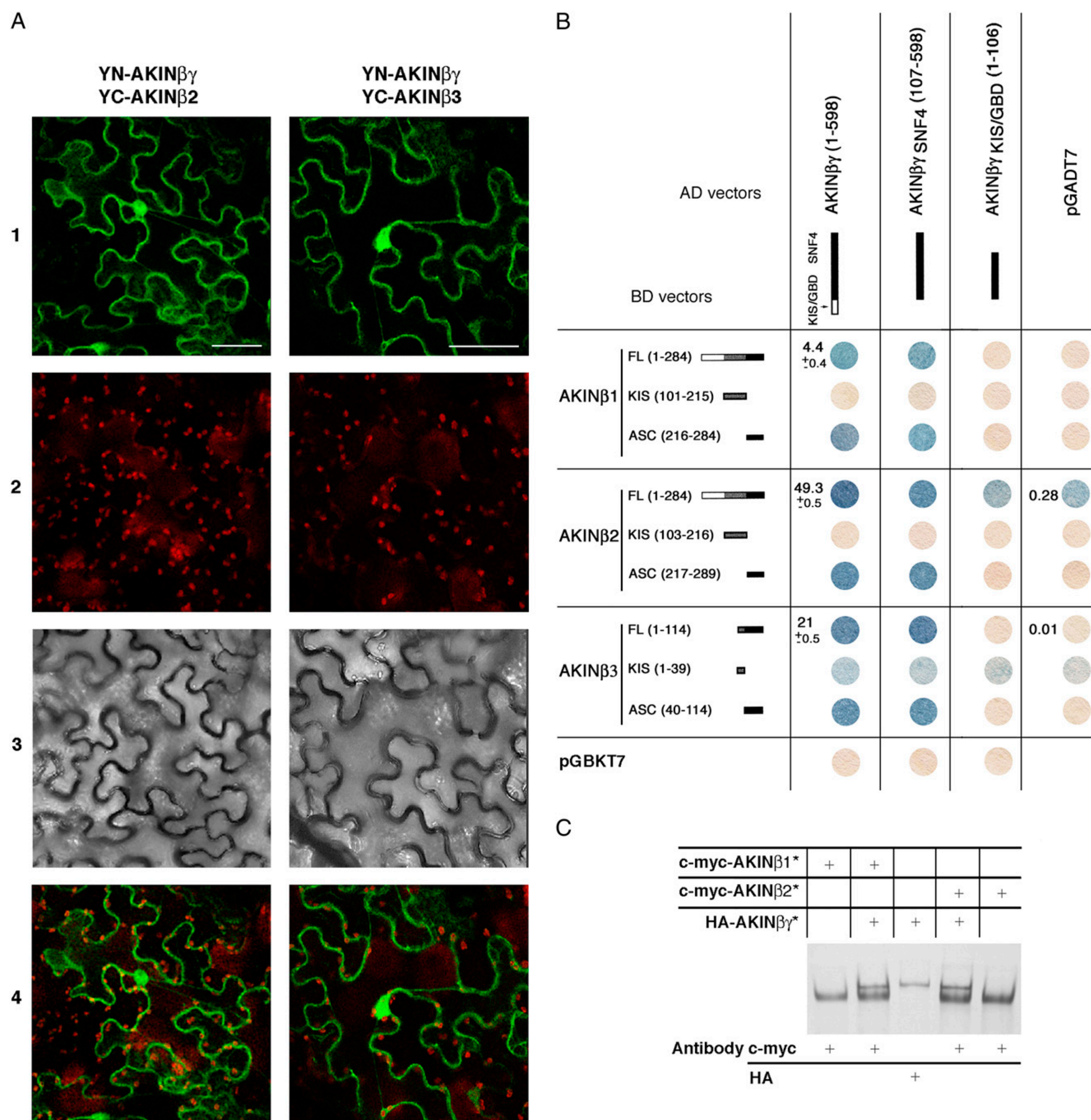

Figure 3. Protein-protein interactions between AKIN$\beta\gamma$ and the AKINβ-subunits. A, Subcellular localization of reconstructed YFP complexes determined in leaf epidermis of *N. benthamiana*. Left, YN-AKIN$\beta\gamma$/YC-AKINβ2 interaction; and right, YN-AKIN$\beta\gamma$/ YC-AKINβ3 interaction. Section 1, YFP fluorescence (green); section 2, chlorophyll autofluorescence (red); section 3, bright field; section 4, YFP/chlorophyll autofluorescence overlay. Scale bars correspond to 50 μm. B, Two-hybrid experiments between AKIN$\beta\gamma$ and AKINβ subdomains. Qualitative and quantitative β-galactosidase enzyme assays have been represented for the full-length proteins (FL). Activities were measured at least twice from six independent colonies grown with 2% Glc. C, AKIN$\beta\gamma$ in vitro protein binding assays. ^{35}S-Met labeled proteins (*) were synthesized by in vitro transcription/translation system (Promega) and incubated alone or by pairs. After coimmunoprecipitation with c-Myc or HA antibodies, proteins were separated by 10% SDS-PAGE and exposed to an x-ray film.

AKIN$\beta\gamma$ appears closer to the nonplant group containing the mammalian, yeast, *D. melanogaster*, and *C. elegans* sequences than to the other plant γ-proteins. Interestingly, the proteins corresponding to the nine sequences grouped with AKIN$\beta\gamma$ present the same AKIN$\beta\gamma$ atypical structure with a KIS/GBD fused to an SNF4 domain. Moreover, proteins presenting similar structures have never been found in other groups, neither in the plant PV42p and AKINγ subgroups nor in nonplant organisms.

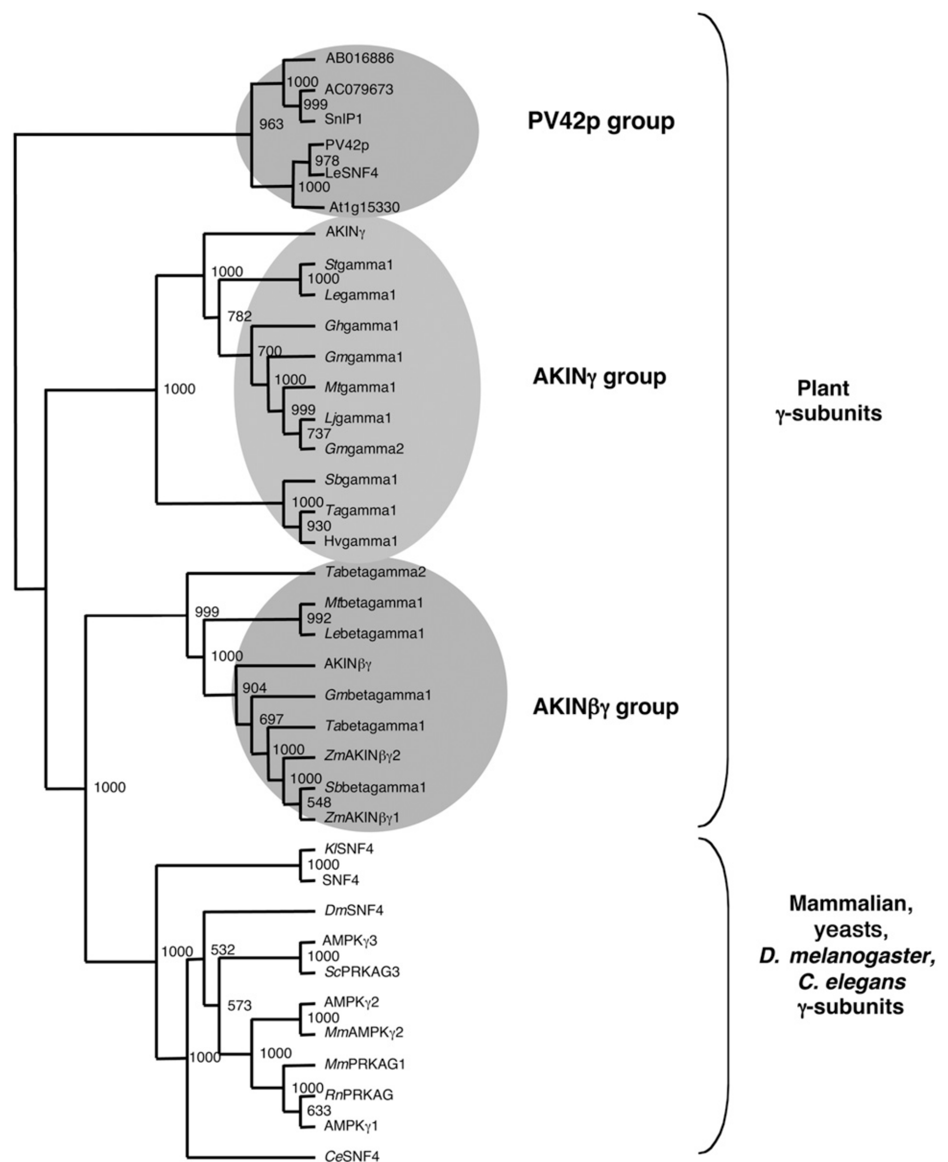

Figure 4. Phylogenetic analysis of the γ-subunits from various organisms. Alignment of the SNF4 domain of γ-related proteins was created by ClustalX (Thompson et al., 1997) and bootstrapped 1,000 times. The tree was visualized by Treeview (Page, 1996). Bootstrap values >500 are shown to the right of each branch point. Numbers appearing in the plant PV42p group correspond to the accession numbers of the Arabidopsis proteins (AB016886, AC079673) or the AGI number (At1g15330). Sequences shown are human AMPKγ1, γ2, and γ3 (accession nos. U42412, AJ249976, and NM_017431), *S. scrofa* (*Sc*PRKAG3, AF214520), *R. norvegicus* (*Rn*AMPKγ, U42413), *M. musculus* (*Mm*AMPKγ1, NM_016781; and *Mm*AMPKγ2, BC015283), yeasts *S. cerevisiae* (SNF4, M30470) and *K. lactis* (*Kl*SNF4, AJ277480), maize (*Zm*AKIN$\beta\gamma$1, AF276085; and *Zm*AKIN$\beta\gamma$2, AF276086), and *Lycopersicon esculentum* (*Le*SNF4, AF143742).

The large number of sequences analyzed in this work show that this type of protein is present in most plant organisms and emphasizes the hypothesis proposed by Lumbreras et al. (2001) that AKIN$\beta\gamma$-type subunits could be specific to the plant kingdom.

AKIN$\beta\gamma$ Interacts with Two Hs1$^{pro\text{-}1}$ Orthologs Implicated in Pathogen Resistance

To get information about the function of the AKIN$\beta\gamma$-type subunits in plants, we decided to search for partners of this protein by performing a two-hybrid screen of a cDNA library from Arabidopsis 3-week-old rosettes using the full-length *AKIN$\beta\gamma$* cDNA as bait. Thirty-two clones presenting a β-galactosidase activity higher than three units corresponding to the basal β-galactosidase activity level of BD-AKIN$\beta\gamma$ were further analyzed and sequenced. In this article, we present the characterization of 22 of these clones.

NCBI databases (nr and dbest) were screened with BLASTN and TBLASTX algorithms (Altschul et al., 1990) to find sequence similarities with the preys. Nine clones corresponded to two already-known members of the AKIN complex: AKINβ1 (one clone) and AKINβ2 (eight clones; Bouly et al., 1999). The clone corresponding to AKINβ1 is truncated to one-half of the ASC domain previously described by Bouly et al. (1999; position +236 after the first Met), thus lacking the Nterm, KIS domain, and part of the ASC domain.

The 13 other clones encode two Arabidopsis unknown proteins that present strong similarities with the Hs1$^{pro\text{-}1}$ protein encoded by a nematode resistance gene isolated in sugar beet (*Beta vulgaris*; Cai et al., 1997). One clone, named *At*HSPRO1, is assigned to

BAC AL163832 on chromosome III (At3g55840). Analysis of the corresponding genomic sequence and of 13 overlapping expressed sequence tags (ESTs) found in the NCBI database suggests that this clone is full length. The 12 other clones found during the two-hybrid screen are assigned to BAC AF002019 on chromosome II (At2g40000). This cDNA, named *At*HSPRO2, appears full length according to the analysis of the BAC and of the 93 available ESTs. *AtHSPRO1* and *AtHSPRO2* are the two Arabidopsis orthologs of the sugar beet *Hs1^{pro-1}* gene and no other related sequences have been characterized in the Arabidopsis genome. The location of these two genes in duplicated regions of chromosomes II and III could explain their high percentage of similarity (http://www.tigr.org/tdb/e2k1/ath1/Arabidopsis_genome_duplication.shtml). The two Arabidopsis predicted proteins *At*HSPRO1 and 2 are 75% identical and 86% similar. Both of them share 59% identity and 78% similarity with the sugar beet Hs1^{pro-1} protein (Fig. 5). Moreover, three sequences from *Glycine max*, *Pisum sativum*, and *Hordeum vulgare* were available in the database. The alignment of the Hs1^{pro-1}-related proteins found in Arabidopsis, *G. max* (AAG44839), and *P. sativum* (AAF67003) highlights an N-terminal extension compared to the Hs1^{pro-1} protein from sugar beet (Fig. 5). The two Arabidopsis proteins present imperfect LRR but no nucleotide-binding site (NBS) or kinase domain previously described for Hs1^{pro-1} (Cai et al., 1997; Fig. 5). The existence of a putative transmembrane domain in Hs1^{pro-1} has also been reported by Cai et al. (1997). Nevertheless, the corresponding regions in the two Arabidopsis proteins present several differences, and the Munich Information Center for Protein Sequences (MIPS) Arabidopsis database does not predict the presence of any transmembrane domain in these proteins.

Northern-blot experiments were performed to analyze the expression patterns of *AKIN$\beta\gamma$* and of the two isolated preys and to determine if they were expressed in the same organs or conditions (Fig. 6). *AKIN$\beta\gamma$* gene is expressed constitutively in all the organs tested,

Figure 5. Alignment of the sugar beet Hs1^{pro-1} protein and the plant Hs1^{pro-1}-related proteins available in the database. Alignments were realized using the ClustalX program (Altschul et al., 1990) and the Hs1^{pro-1} protein from sugar beet (U79733), the Hs1^{pro-1}-like proteins from *Glycine max* (AAG44839) and *Pisum sativum* (AAF67003), and from the two Arabidopsis AtHSPRO1 and AtHSPRO2 proteins. Identical amino acids are visualized with an asterisk (*) and similar ones with a colon (:). The imperfect LRR repeats (red amino acids and regions delimited by red lines) and the transmembrane domain (blue) defined in the Hs1^{pro-1} protein are positioned.

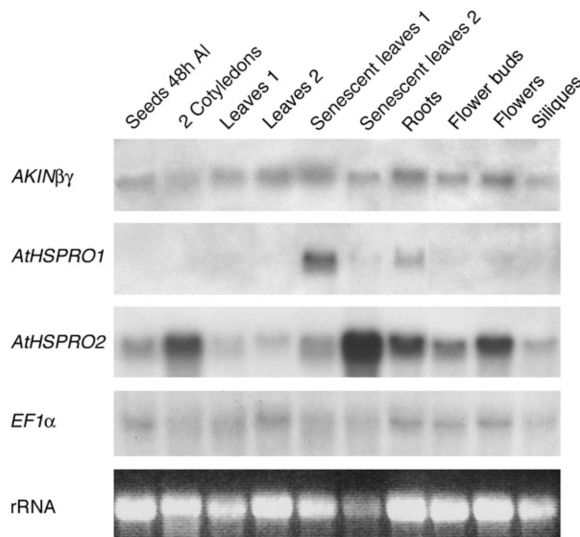

Figure 6. Expression patterns of *AKIN*$\beta\gamma$ and *AtHSPRO1/2* by northern-blot experiments. Seeds 48 h AI, Germinated seedlings 48 h after imbibition. Leaves 1 and 2, Leaves from 3- and 5-week-old rosettes. Senescent leaves 1, Leaves from mature rosettes before flowering. Senescent leaves 2, Yellow leaves from mature rosettes. Roots, Four-week-old roots grown under aeroponic conditions. Flowers, Open flowers. Siliques, One-centimeter green siliques. rRNA, EtBr visualization of ribosomic 28S. EF1α, Elongation Factor 1α. Hybridizations were performed at least twice on two independent membranes using the full-length cDNAs of *AKIN*$\beta\gamma$ and *AtHSPRO1/2* as probes.

while *AtHSPRO1* and *AtHSPRO2* appear to be differentially expressed. *AtHSPRO2* mRNA is ubiquitous, high in cotyledons, roots, and flower organs, and increases dramatically during senescence (senescent leaves 1 and 2). At the contrary, the *AtHSPRO1* messenger remains undetectable or at a very low level except in roots and in senescent leaves. These data are in accordance with the number of ESTs reported for these two proteins in the MIPS Arabidopsis database.

AKIN$\beta\gamma$/*At*HSPRO Interaction Is Located in the Cytoplasm and Occurs via the KIS/GBD

To confirm the putative interactions between AtHSPRO1/2 and AKIN complexes and to precisely determine the domains of interaction with AKIN$\beta\gamma$, two-hybrid experiments and in vitro binding assays were performed using the full-length sequences of the preys and the AKIN$\beta\gamma$ subdomains described above cloned in pGADT7 and pGBKT7 vectors (Fig. 7). No interaction could be detected between these two proteins and AKINα, AKINβ, or AKIN$\beta\gamma$I either in two-hybrid experiments or in in vitro binding assays (data not shown). Concerning their interaction with AKIN$\beta\gamma$, two-hybrid (Fig. 7B) and coimmunoprecipitation experiments (Fig. 7C) were confirmed by BiFC experiments performed between YN-AKIN$\beta\gamma$ and YC-HSPRO1 or YC-HSPRO2. Indeed, YFP signals were detected in the cytoplasm for both YN-AKIN$\beta\gamma$/YC-HSPRO1 and YN-AKIN$\beta\gamma$/YC-HSPRO2, confirming the in planta interaction between AKIN$\beta\gamma$ and HSPRO1/2 (Fig. 7A). Moreover, two-hybrid and in vitro binding assays show that the AtHSPRO1 and AtHSPRO2 proteins interact preferentially with the KIS/GBD domain of AKIN$\beta\gamma$, while only a weak interaction remains with the SNF4 domain alone (Fig. 7, B and C).

DISCUSSION

The isolation and characterization of *Zm*AKIN$\beta\gamma$-1 and -2, two maize proteins interacting with the Arabidopsis AKINα2-subunit, corresponding to a γ-subunit fused in the N terminal to a truncated KIS domain, have been previously published by Lumbreras et al. (2001). Our work presents several breakthroughs concerning its Arabidopsis ortholog AKIN$\beta\gamma$ at the level of gene regulation, structure of the complex containing this unusual protein, function, and subcellular localization.

We have shown that the atypical KIS extension of AKIN$\beta\gamma$ is related to and corresponds exactly to a domain named GBD characterized in the mammalian AMPKβ1 (Hudson et al., 2003; Polekhina et al., 2003, 2005) and in the yeast GAL83 (Wiatrowski et al., 2004). To easily distinguish it from the classical KIS domain present in β-subunits, this domain has been named KIS/GBD in this article, even if it is unlikely to bind glycogen in plants. The interaction of this fragment with the AKINα2 kinase had previously led to the hypothesis of the existence of dimeric complexes (Lumbreras et al., 2001). Interestingly, our data show that AKIN$\beta\gamma$ interacts strongly also with the three Arabidopsis β-subunits (AKINβ1, β2, and the plant-specific AKINβ3 missing the KIS/GBD). This interaction occurs between the AKIN$\beta\gamma$ SNF4 region and the ASC domains of the AKINβ-subunits, while no interaction could be detected either with the KIS/GBD or the N-terminal domains. These data are in accordance with the binding of SNF4 to SIP1, SIP2, and GAL83 previously described in yeast (Jiang and Carlson, 1997) and of Arabidopsis AKINγ with AKINβ1/β2 (Bouly et al., 1999). Furthermore, the existence of trimeric complexes including AKIN$\beta\gamma$ is strengthened by the isolation of several AKINβ1/2 clones during the course of the two-hybrid screen using AKIN$\beta\gamma$ as bait.

BiFC experiments, an imaging technique recently adapted to the plant field (Walter et al., 2004; Bracha-Drori et al., 2004), allowed us to get the in planta confirmation of the interactions and also gave us the first data concerning the subcellular localization of SnRK1 subunits in plants. A clear localization of YN-AKIN$\beta\gamma$/YC-AKINβ interactions was shown both in the cytoplasm and the nucleus. The nuclear localization of SnRK1 complex is particularly interesting because it is in accordance with the implication of the yeast SNF1

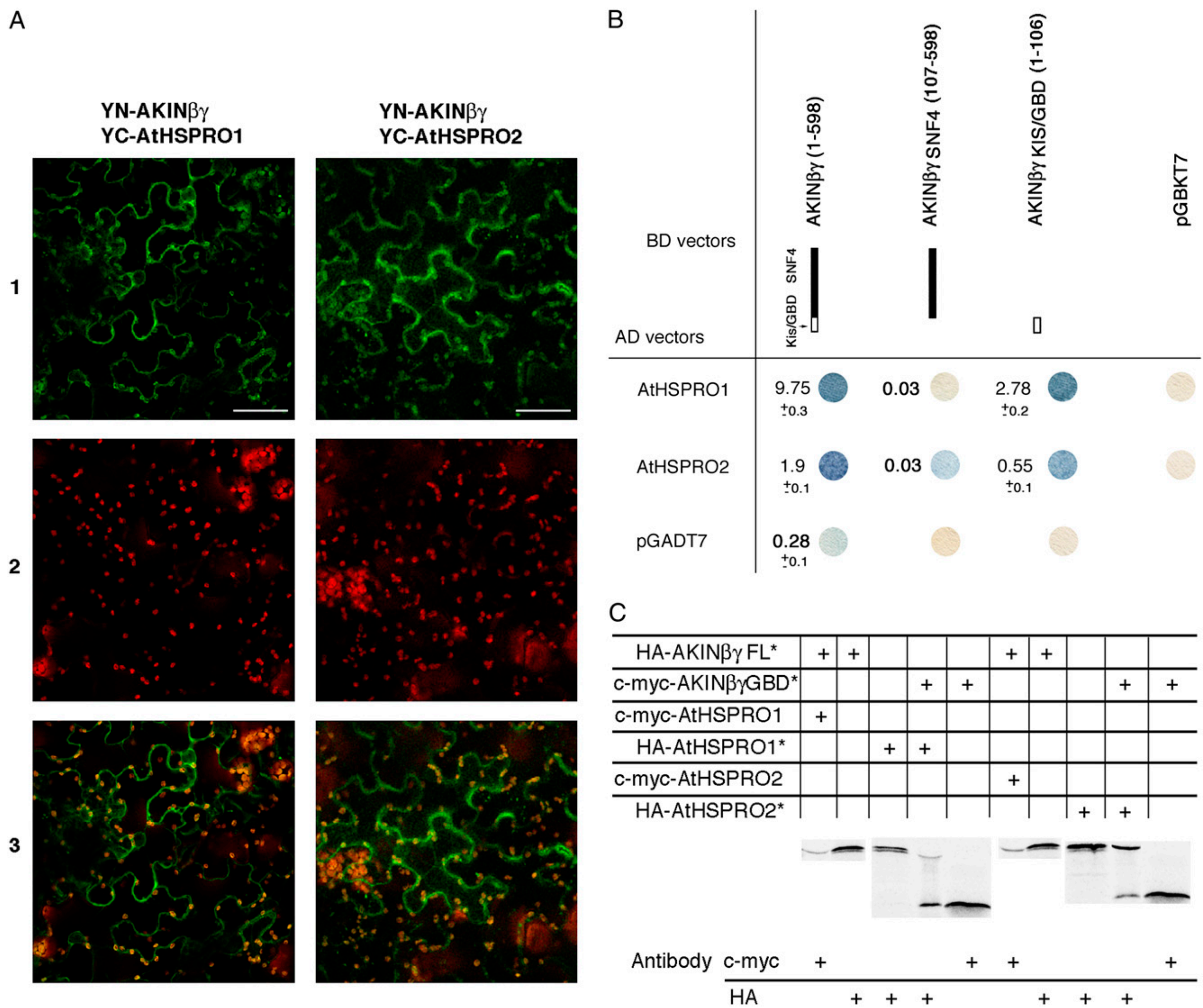

Figure 7. Protein-protein interaction between AtHSPRO1/2 and AKINβγ/βγKIS. A, Subcellular localization of reconstructed YFP complexes determined in leaf epidermis of *N. benthamiana*. Left, YN-AKINβγ/YC-*At*HSPRO1 interaction; and right, YN-AKINβγ/YC-*At*HSPRO2 interaction. Section 1, YFP fluorescence (green); section 2, chlorophyll autofluorescence (red); section 3, YFP/chlorophyll autofluorescence overlay. Scale bars correspond to 50 μm. B, Two-hybrid experiments between AKINβγ subdomains and *At*HSPRO1/*At*HSPRO2. Qualitative and quantitative β-galactosidase enzyme assays have been represented. Activities were measured at least twice from six independent colonies grown with 2% Glc. C, In vitro protein binding assays between AtHS1PRO1/AtHS1PRO2 and AKINβγ/βγKIS. AKINβγ proteins were synthesized by in vitro transcription/translation system (Promega) with ^{35}S-Met labeled (*) and incubated alone or by pairs. AtHSPRO1 and AtHSPRO2 were synthesized without ^{35}S-Met when used with the full-length AKINβγ due to their similar molecular masses. After coimmunoprecipitation with c-Myc or HA antibodies, proteins were separated by 10% SDS-PAGE and exposed to an x-ray film.

kinase at different levels of gene regulation as demonstrated in yeast (Hardie et al., 1998; Lo et al., 2001; Schuller, 2003).

Taken together, these data do not exclude the existence of dimeric complexes composed by AKINβγ and one of the two kinases proposed by Lumbreras et al. (2001), but strongly suggest that unusual trimeric complexes never described in the literature can also be formed by the association of one AKINα-, one AKINβ-, and one AKINβγ-subunit and located both in the cytoplasm and the nucleus.

The PCR-based cDNA isolation of two *AKINβγ* cDNAs, differing by the presence of the unspliced intron 10 in *AKINβγI*, highlighted the existence of alternative splicing events. Very few examples of alternative splicing have been reported yet in plants (Kazan, 2003), and in these cases the splicing is often regulated in a tissue-, environmental-, or organelle-specific manner (Lazar and Goodman, 2000; Kobayashi et al., 2001; Xu and Johnson, 2001; Shi et al., 2002). In our case, both mRNA seem to coexist in all the conditions tested but vary by their related levels. The

existence of intron 10 alternative splicing produces a C-terminal end truncated putative protein of 394 amino acids that lacks mainly the fourth CBS domain (CBS4). CBS4 is the most conserved CBS domain found between plants, mammals, and yeast γ-subunits (Bradford et al., 2003). Our data have shown that this domain seems necessary for the interaction with α- and β-subunits. Yeast complementation, two-hybrid, and CoIP experiments indicate that AKIN$\beta\gamma$I would not be implicated in the formation of an active SnRK1 kinase complex. Further experimental supports will be necessary to test the simultaneous presence of both proteins in the plant and analyze the function of such a truncated $\beta\gamma$-protein. Alternative splicing could be one way of regulating these kinase complexes because splice variants have also been reported in mammals (Hardie, 2004).

To search for orthologs of AKIN$\beta\gamma$ in other organisms, a phylogenetic analysis of the sequences encoding all types of γ-subunits was performed. Plant γ-type subunits appear strictly separated from the mammal, yeast, and *C. elegans* γ-subunits and can be divided into three groups containing PV42P, AKINγ, or AKIN$\beta\gamma$, respectively. The AKIN$\beta\gamma$ group is exclusively composed by plant proteins presenting the AKIN$\beta\gamma$ characteristics (SNF4 region fused in N terminus with a KIS/GBD), while such atypical subunits do not exist in yeasts, mammals, *C. elegans*, or *D. melanogaster*. Despite its unusual features, this group is the closest to the mammals and yeast. This observation is supported by the functional ability of three members of this group (*Zm*AKIN$\beta\gamma$-1 and -2, AKIN$\beta\gamma$) to complement the yeast *snf4* mutant. *Le*SNF4 is the only protein belonging to one of the two other groups reported to complement the *snf4* mutant (Bradford et al., 2003).

What could be the function of this plant-specific subunit with its unusual extension? Interestingly, when searching for *AKIN$\beta\gamma$* orthologs in other organisms, more than 20 ESTs similar to *AKIN$\beta\gamma$* were found in a sorghum (*Sorghum bicolor*) cDNA library prepared from mRNA extracted from a plant attacked by pathogens, suggesting that AKIN$\beta\gamma$ expression is regulated by plant-pathogen interaction.

In this work, a two-hybrid screen performed using AKIN$\beta\gamma$ as a bait led to the isolation of two types of cDNAs, *AtHSPRO1* and *AtHSPRO2*, that encode proteins similar to the sugar beet Hs1^{pro-1} (Cai et al., 1997). The Hs1^{pro-1} protein has previously been shown to confer the resistance to the nematode *Heterodora schachtii* Schmidt on the basis of gene-for-gene relationship in sugar beet (Cai et al., 1997). Moreover, its mRNA expression is induced during nematode infection in sugar beet and Arabidopsis (Thurau et al., 2003). The absence of any NBS suggests that this protein does not belong to the NBS-LRR class of plant resistance genes but could be a member of another class of R genes (Jung et al., 1998). At present, nothing to our knowledge has been reported on the two Arabidopsis orthologs. An alignment between the sugar beet Hs1^{pro-1} and the predicted orthologs from several plants shows that the sugar beet Hs1^{pro-1} is smaller than its orthologs with fewer putative LRR repeats (Fig. 5). This difference in size can be explained either by the result of a divergence of these proteins during the evolution or by a wrong annotation of the sugar beet cDNA. Actually, the latter is more likely because northern-blot experiments performed by Cai et al. (1997) revealed a 1.6-kb mRNA that is close to the size of our two Arabidopsis full-length clones.

The expression patterns of the two *AtHSPRO* genes are very different and only slightly overlapping. *AtHSPRO2* appears more expressed than *AtHSPRO1* in most stages throughout Arabidopsis development. *AtHSPRO1* and *2* mRNAs accumulate in the roots as previously shown for Hs1^{pro-1} in sugar beet (Cai et al., 1997). Interestingly, the mRNA level is dramatically increased during the two stages of senescence tested, with a higher level of *AtHSPRO1* in the early senescent stage (leaf 3) and of *AtHSPRO2* in the late senescent stage (senescent leaves) in the absence of pathogens. A similar observation had been reported previously for *NIT2* and *AtOSM34*, two defense-related genes induced during leaf senescence in pathogen-free plants (Quirino et al., 1999). The induction of the two *AtHSPRO* genes putatively involved in plant defense response could be a component of the leaf senescence program.

Furthermore, an analysis of the microarray experiments available on the The Arabidopsis Information Resource site (www.arabidopsis.org) shows that the *AtHSPRO2* mRNA is up-regulated by a factor comprised between 6 and 10 after inoculation by the bacteria *Xanthomonas campestris* pv campestris. Altogether, these data suggest that these two Arabidopsis Hs1^{pro-1} orthologs could be implicated in a more general resistance process and not only in response to a nematode attack. The interaction between the AKIN$\beta\gamma$-subunit and these two Hs1^{pro-1}-related proteins suggests that the SnRK1 complexes are implicated in plant-pathogen interactions. These results are in accordance with the data published by Hao et al. (2003). The authors have shown that the enhanced susceptibility of the transgenic plants expressing the geminivirus AL2 and L2 proteins is due to the interaction between these proteins and AKINα2, their interaction resulting in the inactivation of this kinase (Hao et al., 2003).

We have shown that the AtHSPRO1 and AtHSPRO2 proteins interact mainly with the AKIN$\beta\gamma$ KIS/GBD but not with the KIS subdomains of AKINβ1, β2, and β3 (data not shown), highlighting a specificity of interaction between these proteins. Recent data present evidence that the carbohydrate BD present in the dual-specificity protein phosphatase PTP-KIS (At3g52180) can bind starch (Kerk et al., 2006). This phosphatase, closely related to laforin, a mammalian glucan-binding protein phosphatase required for the metabolism of glycogen, binds to starch granules during the day and dissociates at night (Sokolov et al.,

2006). Mutants of this dual-specificity protein phosphatase present a dramatic increase in their level of starch (Niittylä et al., 2006; Sokolov et al., 2006). Interestingly, the identified key conserved residues for this binding (W19, K39) are conserved in AKIN$\beta\gamma$ KIS/GBD. Because we have demonstrated here that this domain can mediate protein-protein interactions, more work will be necessary to understand if such a domain can mediate both protein-protein and carbohydrate-protein interactions.

Taken together, in vitro and in vivo results led us to propose that at least three different types of SnRK1 complexes could exist in plants: (1) dimeric complexes formed by AKIN$\beta\gamma$ and one of the two kinases as proposed by Lumbreras et al., (2001); (2) trimeric complexes composed by one AKINα-subunit, AKINβ1/β2-subunit, and AKINγ; and (3) other trimeric complexes composed by one AKINα, one AKINβ, and AKIN$\beta\gamma$. The isolation of AtHSPRO1/2 proteins reinforces the latter hypothesis. In this case, the SNF4 domain of AKIN$\beta\gamma$ would interact with the AKINβ-subunits and with the kinase, leaving its KIS/GBD free for the interaction with AtHSPRO1/2. BiFC experiments show that this interaction is located exclusively in the cytoplasm, suggesting that the interaction with AtHSPRO proteins could retain the targeted complex in this compartment.

Altogether, the data presented in this article suggest that the SnRK1 heterotrimeric complex containing AKIN$\beta\gamma$ could be implicated in plant pathogen resistance through the interaction in the cytoplasm of the KIS/GBD domain of AKIN$\beta\gamma$ with AtHSPRO1/2 proteins.

MATERIALS AND METHODS

Plant Material

Wild-type Arabidopsis (*Arabidopsis thaliana*), ecotype Columbia, were grown in a culture room and were maintained in soil with a 15-h-light and 9-h-dark regime (long day). Relative humidity was 65% and temperature was 20°C/17°C during the light/dark cycle. Germinated seedlings (used for RNA extraction) were grown in the dark on a Whatman Number 1 paper imbibed by water, relative humidity was 65%, and temperature was 20°C.

Genomic Analysis of AKIN$\beta\gamma$

The yeast (*Saccharomyces cerevisiae*) SNF4 (accession no. M30470) and mammalian AMPKγ1, γ2, and γ3 (U42412, AJ249976, and NM_017431) were used to screen the NCBI database (nr and dbest restricted to Arabidopsis) with the TBLASTN algorithm (Altschul et al., 1990). Three ESTs (AI999237, AV540740, and AV552460) were selected. The corresponding gene was assigned to the BAC clone AC000106 of chromosome I. NetPlantGene (Hebsgaard et al., 1996) and SIM4 (Florea et al., 1998) programs were used, respectively, to determine the intron/exon structure of the gene and align the different ESTs with the genomic sequence. Amplification of the 5′ and 3′ UTRs was realized by PCR experiments performed on an Arabidopsis shoot cDNA library as previously described by Bouly et al. (1999).

PCR Experiments

To determine the expression of the two *AKIN$\beta\gamma$* mRNA containing (or not) the 10th intron, PCR experiments were performed on DNA of several cDNA libraries using three specific oligonucleotides. The downstream oligonucleotide (E9Forward) 5′-ACGCCTCTTTGGGTTCTGC-3′, localized in exon 9, was used with 5′-TATCATCAGAACACAAGGACG-3′ (I10Reverse) localized into the 10th intron for the amplification of a 175-bp fragment of the cDNA containing the 10th intron or with the oligonucleotide (E10/E11Reverse) 5′-GAGCAGTTCTATCACTTCGAGA-3′ localized on the exon10/intron10 junction in order to amplify the corresponding region of the AKIN$\beta\gamma$ cDNA spliced. cDNA libraries were previously described by Aubourg et al. (1999).

Northern-Blot Hybridization

Total Arabidopsis RNA were isolated in the middle of the light treatment from 48-h-old germinated seedlings (seeds 48 h after imbibition) and from green siliques according to Kay et al. (1987). Total RNA from 7-d-old germinated seedlings with two cotyledons (2 Cotyledons), leaves from 3- and 5-week-old rosettes (Leaves 1 and 2), leaves from mature rosettes before flowering (Senescent Leaves 1), yellowish mature leaves during flowering (Senescent Leaves 2), stems, floral buds, flowers, and 4-week-old roots grown under aeroponic conditions (Roots) were isolated according to Lessard et al. (1997). Samples have been taken in the middle of the light regime. RNAs (20 μg) were electrophoresed, blotted, and hybridized as previously described by Bouly et al. (1999). The probes have been obtained by PCR amplification using specific primers and correspond to the full-length *AKIN$\beta\gamma$*, *AtHSPRO1*, *AtHSPRO2*, and *EF1α* cDNAs.

Protein-Protein Interaction Analysis by the Yeast Two-Hybrid System and in Vitro Binding Assays

AKIN$\beta\gamma$ full-length cDNA was cloned downstream of the Gal4 AD or BD in pGBT9 and pGAD424 vectors (CLONTECH) with specific oligonucleotides modified by 5′*Eco*RI and 3′*Bam*HI restriction sites (5′ATGTTTGGTTCTAC$_{3′}$ and 5′TCAAAGACCGAGCAG$_{3′}$) to produce BD-AKIN$\beta\gamma$ and AD-AKIN$\beta\gamma$. Specific oligonucleotides (5′-ATGGTTCCTGCTGGT-3′ and 5′-TCAAAGA-CCGAGCAG-3′, 5′-ATGTTTGGTTCTAC-3′ and 5′-TATTGTATTCACTAC-3′, 5′-ATGTTTGGTTCTAC-3′ and 5′-TACCTTCGAGAGTATATGTC-3′, and 5′-AGTGATATAACTGCTCTGGC-3′ and 5′-TCAAAGACCGAGCAGGAA-TTG-3′) modified with 5′*Eco*RI and 3′*Bam*HI restriction sites were used to amplify AKIN$\beta\gamma$ SNF4$_{107\text{-}598}$ domain, AKIN$\beta\gamma$ KIS/GBD$_{1\text{-}106}$ domain, AKIN$\beta\gamma_{1\text{-}403}$ (truncated in the 10th intron), and AKIN$\beta\gamma_{424\text{-}598}$ (after intron 10 including the last CBS domain), respectively, and cloned into pGBKT7 and pGADT7 vectors (CLONTECH) to produce BD-$\beta\gamma_{107\text{-}598}$/AD-$\beta\gamma_{107\text{-}598}$, BD-$\beta\gamma_{1\text{-}106}$/AD-$\beta\gamma_{1\text{-}106}$, BD-$\beta\gamma_{1\text{-}403}$/AD-$\beta\gamma_{1\text{-}403}$, and BD-$\beta\gamma_{424\text{-}498}$/AD-$\beta\gamma_{424\text{-}498}$, respectively. All these AKIN$\beta\gamma$ constructs were tested against each α- and β-member of the AKIN complex as described by Bouly et al. (1999). The BD and AD constructs were used to transform the yeast strain Y190, and protein interactions were assayed using LacZ-filter lift and *o*-nitrophenyl-β-D-galactopyranoside assays and by monitoring growth on SD medium without Leu, Trp, and His (SD-LTH) containing 25 mM 3-aminotriazole (3-AT).

The vectors described above were used to produce ^{35}S-Met-labeled proteins fused with the c-Myc or the HA epitope. These fusion proteins were prepared by in vitro transcription/translation using a TNTT7 Quick Transcription/Translation Lysate system (Promega). A total of 5 μL of each marked protein (or peptide) was incubated together into 400 μL of immunoprecipitation buffer (25 mM MOPS, 50 mM NaCl, 10% glycerol, 6 mg/mL bovine serum albumin, 1 mM EDTA, 0.5% Tween 20, 0.02% NaN_3) at 4°C for 1 h. After 2 h of incubation at 4°C with 1 μL of c-Myc monoclonal antibody or 10 μL of HA-Tag polyclonal antibody (CLONTECH), the mixture was incubated with 6 mg of protein A-Sepharose (Sigma P3391) at 4°C for 1 h. The Sepharose was then washed eight times with 1 mL of the immunoprecipitation buffer. Proteins were then detached from the protein A-Sepharose in 20 μL of Laemmli buffer at 85°C for 5 min and separated in SDS-PAGE (15% acrylamide). The gel was dried and exposed to an x-ray film overnight at room temperature.

Two-Hybrid Screen Using AKIN$\beta\gamma$ as Bait

The yeast strain HF7C (Feilotter et al., 1994) carrying BD-AKIN$\beta\gamma$ bait was transformed with 500 μg of DNA from a pGAD424 cDNA library (AD-prey) from Arabidopsis 3-week-old rosettes (CLONTECH). The cells were then grown on SD-LTH (Durfee et al., 1993). A total of 6,000 transformants were streaked on SD-LTH plates in the presence of 25 mM 3-AT. A total of 286

transformants were grown on nitrocellulose filters (Whatman No. 5) and placed on SD-LTH plates containing 10, 25, or 50 mM 3-AT to check their $LacZ^+$ phenotype by β-galactosidase assays. Yeast DNA was extracted according to Kaiser and Auer (1993) and used to transform bacterial strain MC1066. Bacteria were then streaked on minimum medium without Leu to select only the library AD plasmid. Each AD-prey isolated was used to cotransform the yeast strain Y190 (Harper et al., 1993) with BD-AKIN$\beta\gamma$. β-Galactosidase measurement (as described previously by Bouly et al. [1999]) and a monitoring of growth on SD-LTH containing 25 or 50 mM 3-AT allowed a quantification of protein interaction and the selection of clones on the basis of high LacZ+ phenotypes and their capacity to grow on SD-LTH with a high concentration of 3-AT. After selection, the selected clones were sequenced and database screening was used to characterize the putative partners.

Finally, each AD-prey was cloned in the opposite vector pGBKT7 (CLONTECH) to produce BD-prey. After transformation of the yeast strain Y190, the β-galactosidase activity was measured.

BiFC and Confocal Microscopy

AKINβ2, AKINβ3, AKIN$\beta\gamma$, AtHSPRO1, and AtHSPRO2 full-length cDNAs were cloned in the GATEWAY-compatible vector pDONR207 (Invitrogen) prior to being transferred in the BiFC GATEWAY-modified vector developed by F. Parcy (CEA-Grenoble, France). AKIN$\beta\gamma$ was fused to the N-terminal part of the YFP (YN) to produce 35S::YN-AKIN$\beta\gamma$. AKINβ2, AKINβ3, AtHSPRO1, and AtHSPRO2 were fused to the C-terminal part of the YFP (YC) to produce 35S::YC-AKINβ2, 35S::YC-AKINβ3, 35S::YC-AtHSPRO1, and 35S::YC-AtHSPRO2. *Nicotiana benthamiana* plants were grown in the greenhouse under 13 h light, 25°C day temperature, and 17°C night temperature for all the agroinfiltration experiments. Leaves were infiltrated (Rathjen et al., 1999) with an exponential phase culture *Agrobacterium tumefaciens* strain C58C1 resuspended to an OD_{600} of 0.5 with the infiltration medium (10 mM MES, pH 5.6, 10 mM $MgCl_2$, 200 μM acetosyringone). For the coinfiltration, equal volumes of both cultures were mixed before agroinfiltration. Observations were performed 48 h after infiltration. Confocal microscopy was performed on an inverted Leica TCS-SP2-AOBS spectral confocal laser scanning microscope. Samples were excited with a 514-nm argon laser (50%) with an emission band of 510 to 550 nm for YFP detection and 640 to 700 nm for chlorophyll autofluorescence. Measurements of fluorescence emission spectra were carried out with an excitation at 488 nm (25%), and emission was detected between 500 and 600 nm at 5-nm intervals.

Eukaryotic SNF4/AMPKγ/SnRKγ-Subunit Analysis

The AKINγ sequence from Arabidopsis (CAB64720), *Phaseolus vulgaris* PV42p (AAA91175), yeast SNF4 (accession no. M30470), human AMPKγ1, γ2, and γ3 (U42412, AJ249976, and NM_017431), and *Drosophila melanogaster* (*Dm*SNF4:AF094764) were used to search for overlapping ESTs and genomic sequences from different plant or nonplant organisms in the NCBI database (dbest and nr) using the TBLASTN algorithm (Altschul et al., 1990).

Sequences related to the γ-subunits from *Caenorhabditis elegans* (*Ce*SNF4: CAC35836) were retrieved from the genomic sequence analysis using HMM (Krogh, 1997) and annotations of the BAC. Published mammals AMPKγ (*Sus scrofa*, *Sc*PRKAG3: AF214520; *Rattus norvegicus*, *Rn*AMPKγ: U42413; *Mus musculus*, *Mm*AMPKγ1; NM_016781; and *Mm*AMPKγ2; BC015283) and yeast *Kluyveromyces lactis* *Kl*SNF4 (AJ277480) sequences were used. For each organism, overlapping sequences were used to create contigs and then translated. Full-length sequences were aligned using ClustalX (Thompson et al., 1997) and phylogenetic trees were generated, bootstrapped 1,000 times, and visualized by Treeview (Page, 1996). ''Exclusions for positions with gaps'' and ''corrections for multiple sequences'' were both set to ''off.'' The tree was outgrouped with PV42p and SnIP1 groups. Sequences related to SnIP1 correspond to a new group of SNF4-like protein isolated by Crawford et al. (2001). Names of the protein sequences deduced from EST contigs were given arbitrarily in bold, starting with the initials of the organism, ''gamma'' or ''betagamma'' for γ- or $\beta\gamma$-subunits, and one number when several sequences were found in the same organism. ESTs accession numbers used for each organism are listed in alphabetical order: *Gh*gamma1: AI730544, BF274631, AW187178; *Gm*gamma1: AW507983, AW348746, AI965571, AW350665, BG508480, BG041147, BI315858, BE474422, AI460404, AI495211, BF425409, BE661899, BE802388, AW100253, BE611594, AW755822; *Gm*gamma2: AW234285, BG726542, BF594973, BG790832, AW832024; *Gm*betagamma1: BE329598, AW761384, BG045823, BI471330, AI938551, BF067362; *Hv*gamma1: BE413386, AL503884, BF620756; *Le*gamma1: AW031797, AW930464, AW030495, AW931734, AW930111, AI896552, AW030567, AW221409, AW934655, BG132919, AW221379, AW616814, BE432801, AW034443, AW033795, AW033948, AW931052, AW931884, AI778239, BE435549, AI896954, BF052148, AW224492, BE441128, AW221367, AW221368, AW221366, BF051899, AI486513, AI485033, BE432466, AW222755, AW930016, BI209432, BI209432, BI207710, BG631518, BE433740; *Le*betagamma1: AI896937, BE434764, AW933231, BE434544, AW033267, BG12450; *Lj*gamma1: AV410347, AV410863, AV412990, AV423982, AV420137, AV426298, AV428789; *Mt*gamma1: AA660841, AL284884, AL375470, AL375471, AL384885, AL388323, AW560279, AW573932, AW684384, AW685042, AW685140, AW686841, AW689677, AW773651, BE319326, BE942814, BF003163, BF003164, BF646632, BF649373, BG447936, BG589026, BG647811, BI266830; *Mt*betagamma1: BG452241, AW774494; *St*gamma1: BG590178, BE342824, BE341103, BG600892, BE342642, BG098543, BF890379; *Sb*gamma1: AW28645, BE363968, BE356406, AW564195, BE356340, BF421349, BE595667, BE563959, BF421265, BG465396, BG463490, BG464559, BG464134, BE594283, BG463409, BE363898, BE5945416; *Sb*betagamma1: BG560098, BG241716, BF585763, AW744961, BI140637, BG412843, BG050314, BF481119, AW285543, AW285557, AW565509, AW565523, AW676933, AW745014, BE353014, BE598031, BE598304, BE598571, BE598914, BF421885, BF585679, BG050385, BG560098; *Ta*gamma1: BE403487, BE426463, BG314395; *Ta*betagamma1: BG907542, BE489373, BI479562, BE415403, BE498573, BG605114, BE492292, BE445860, BG907543, BF293342; and *Ta*betagamma2: BE416528, BE417856, BE416027, BE417134, BE416979, BE416980.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers DQ132632 (AKIN$\beta\gamma$), DQ132633 (AKIN$\beta\gamma$I), DQ132634 (*At*HSPRO1), and DQ132634 (*At*HSPRO2).

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