Arabidopsis KIN Gamma subunit 1 (KING1) has potential to regulate activity of SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2s (SnRK2s) in vitro

M. PUNKKINEN[1], K. DENESSIOUK[1, 2], H. FUJII[1*]

Molecular Plant Biology Unit, Department of Biochemistry, University of Turku, Turku, Finland[1]

Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland[2]

Abstract

To respond to environmental changes promptly, plants must regulate their signalling pathways with precision. Sucrose non fermenting1 (SNF1)-related protein kinases (SnRK) 2 are essential kinases in abiotic stress responses, including responses to abscisic acid (ABA). Although homologs of SnRKs in yeast require a γ-subunit for full activation, it has been unclear whether SnRK2s are affected by γ-subunits. In this report, we aimed to show the effect of *Arabidopsis* KIN gamma subunit 1 (KING1), which is a potential γ-subunit, on the activity of SnRK2. Recombinant KING1 bound to SnRK2.6 (a.k.a. OST1) and functionally inhibited its activity *in vitro*. On the other hand, KING1 facilitated the activity of SnRK2.2. Structural models suggest that significant structural changes occur as a result of KING1 binding to the C-terminal tail of SnRK2s. Since KING1 inhibits the kinase activity of a chimeric protein consisting of the N-terminal domain of SnRK2.6 and the C-terminal domain of SnRK2.2, the regulation by KING1 is determined by N-terminal domain of SnRK2s. Together, these results show that KING1 can mediate activity of SnRK2s *in vitro*.

Key words: SnRK2, gamma subunit, ABA, KING1 (AT3G48530), SnRK2.6 (AT4G33950), SnRK2.2 (AT3G50500)

Abbreviations: ABA, abscisic acid; AMPK, AMP-activated protein kinase; AREB, ABA-responsive element binding factor; GST, glutathione S-transferase; KING1, KIN gamma subunit 1; MBP, maltose-binding protein; PP2C, protein phosphatase 2C; SnRK, SNF1-related protein kinase

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* Corresponding author

E-mail: hiroaki.fujii@utu.fi

As sessile organisms, plants have to use their signal transduction pathways to acclimate to harsh environmental conditions, such as reduced availability of nutrients and water. Pathway components, such as kinases, must be regulated precisely to achieve optimal responses.SNF1/AMP-activated protein kinases (AMPKs) are an important kinase family involved in the regulation of energy depletion in yeasts and mammals (Hardie 2011). SNF1 forms a heterotrimeric protein complex with a β-subunit and a γ-subunit (SNF4) for full activation (Celenza and Carlson 1989). In *Arabidopsis thaliana*, the closest homologs of SNF1 are SNF1-related protein kinases (SnRK) 1: SnRK1.1 and 1.2 (a.k.a. KIN10 and 11). As was shown in yeast two-hybrid assays, SnRK1s can bind to three β-subunits (AKINβ 1, 2, and 3) and two γ-subunits: KIN gamma subunit 1 (KING1; Bouly *et al.* 1999) and AtSNF4, the latter of which also contains a β-subunit-like domain (Lumbreras *et al.* 2001, Kleinow *et al.* 2000, Bitrián *et al.* 2011, Gissot *et al.* 2006). Meanwhile, AtSNF4, but not KING1, can make a complex with the α- and β-subunits in co-immunoprecipitation assay (Emanuelle *et al.* 2015). While AtSNF4 has been identified as the functional SnRK1 γ-subunit, KING1 cannot complement the growth defect of a yeast *snf4* mutant on non-glucose plates and might not be involved in SnRK1 signalling (Ramon *et al.* 2013). Therefore, KING1 might be involved in cellular regulation via pathways other than those that involve SnRK1s. In addition to SnRK1s, plants have two other subfamilies of SnRKs: SnRK2 and SnRK3. The involvement of γ-subunits in the activation of either of these subfamilies is unclear. SnRK2s have 10 members in *A. thaliana*, namely, SnRK2.1-SnRK2.10 (Hrabak *et al.* 2003). All SnRK2s in *A. thaliana*, apart from SnRK2.9, are activated by osmotic stress (Boudesocq *et al.* 2004). Three of the *A. thaliana* SnRK2s, SnRK2.2, SnRK2.3 and SnRK2.6, are also strongly activated by phytohormone abscisic acid (ABA; Boudsocq *et al.* 2004), which is an important factor in stress responses and during plant development, such as seed maturation, dormancy, and seedling development (Leung and Giraudat 1998, Sato *et al.* 2009, Umezawa *et al.* 2010, Joshi-Saha *et al.* 2011). SnRK2s are one of the core components of the ABA-signalling pathway with ABA receptor PYRABACTIN RESISTANCE1/PYR1-LIKEs (PYR/PYLs a.k.a. RCARs) and protein phosphatase 2Cs (PP2Cs) (Park *et al.* 2009, Ma *et al.* 2009, Fujii *et al.* 2009). In the absence of ABA, the PP2C molecule physically binds to a SnRK2 and inactivates it by preventing self-phosphorylation of the activation loop (Ng *et al.*, 2011; Yunta *et al.*, 2011, Soon *et al.*, 2012), whereas in the presence of ABA the inhibition of PP2Cs by PYR/PYLs releases SnRK2s

from suppression, allowing them to be activated through phosphorylation (Umezawa *et al.* 2009, Vlad *et al.* 2009, Fujii *et al.* 2009). Thus, modification of SnRK2 activity directly affects ABA responses. Papdi *et al.* (2008) showed in a large-scale overexpression screening that overexpression of KING1 leads to ABA-insensitivity during germination. Involvement of KING1 in the ABA pathway and its homology to the γ-subunit of members of the SNF1 family suggest that KING1 directly regulates the activity of SnRK2s. In this study we analysed the functional interaction between SnRK2s and KING1 in vitro.

First, we analysed whether KING1 physically binds to SnRK2.6. GST-KING1, but not GST, was pulled down with MBP-SnRK2.6, but not with MBP, indicating that KING1 binds directly to SnRK2.6 (Fig. 1A). Next, to determine whether KING1 has any effect on the activity of SnRK2.6, we performed an *in-vitro* kinase assay using recombinant proteins. GST-fused AREB1a was used as a substrate of SnRK2s (Furihata *et al.* 2006). Compared to GST, GST-KING1 prevented both the autophosphorylation and the substrate phosphorylation of SnRK2.6. Intensities of the major AREB1a band and the SnRK2.6 autophosphorylation band decreased in correlation to the amount of KING1 (Fig. 1B), indicating that KING1 inhibits the activity of SnRK2.6 *in vitro*. Besides SnRK2.6, SnRK2.2 and SnRK2.3 are classified as ABA-activated SnRK2s. To determine whether the effects of KING1 on SnRK2.6 activity are consistent among ABA-activated SnRK2s, we performed an *in-vitro* kinase assay using recombinant MBP-fused SnRK2.2. Interestingly, SnRK2.2 incubated with GST-KING1 had higher substrate phosphorylation capacity than SnRK2.2 incubated with GST (Fig. 1C). These results indicate that KING1 facilitates activity of SnRK2.2 *in vitro* and that SnRK2s can be controlled by KING1 in various ways.

Additional plant proteins, such as the β-subunit, were not assumed to exist in the above *in* vitro assays. To speculate on how SnRK2s could interact with KING1, we created a molecular model of the SnRK2.6/KING1 complex, which is based on the crystal structure of the full length human AMPK (PDB ID: 4CFE), a heterotrimeric complex between AMPK subunits α-2 (4CFE:A), β-1, and γ-1 (4CFE:E). The SnRK2.6 sequence was aligned with the AMPK catalytic subunit α-2 with 32% sequence identity (45% sequence similarity, Fig. S1), and KING1 sequence was aligned with AMPK subunit γ-1 with 15% sequence identity (29% sequence similarity, Fig. S2). Two consecutive C-terminal helix/loop

regions of the SnRK2 structure, the SnRK2 box (Gln303-Val317 in SnRK2.6) and the ABA box (Leu333-Met362 in SnRK2.6, Fig. S1), are important for its activation (Yoshida *et al.*, 2006, Ng *et al.*, 2011). α-helix in the ABA box has a well-defined hydrophobic surface Gly331-Leu333-Ile335-Met339-Leu343 pointing inside, towards the core of the structure, and its polar surface Asp334-Asp336-Asp337-Asp338-Glu340-Glu341-Asp342-Glu344-Asp346 pointing outside, which serves to interact with other proteins (Yoshida *et al.*, 2006, Ng *et al.*, 2011). The structure of 4CFE contains two small molecules bound to the N-terminal domain of the catalytic α subunit, an ATP analogue, and a small molecule activator, a benzimidazole derivative (991) (Xiao *et al.*, 2013). Thus, the model contains the SnRK2.6 structure based on the activator-induced active form of the catalytic α subunit (Fig. 2B, shown in grey). Interaction of SnRK2.6 and KING1 occurs between the ABA box in the C-terminal tail of SnRK2.6 and KING1 segments (Gln231-Leu244 and Val95-Ala104) (Figs 2B and S1). This C-terminal tail, including both the SnRK2 box and ABA box, is similar in SnRK2.6 and SnRK2.2 (Fig. S1). Additionally, at least two *A. thaliana* SnRK2.6 structures have been reported in its inactive form. One is the monomeric and ligand-free structure of the *A. thaliana* SnRK2.6 with D59A/E60A mutations which were introduced for the crystallization (PDB ID: 3UC4; Ng *et al.*, 2011). The other is the structure of the SnRK2.6 bound with PP2C (PDB ID: 3UJG, Soon *et al.*, 2012). The two SnRK2.6 structures (3UC4, 3UJG) are conformationally similar. When this inactive form is superposed to the model of SnRK2.6/KING1 complex shown above, the interacting C-terminal tail is turned away from the SnRK2.6/KING1 interface towards N-terminal active site (Fig. 2A, shown in blue). The conformational change of the activation loop (Phe161-Pro180 in SnRK2.6) is significant: it points towards the SnRK2.6/KING1 interface in the active form of SnRK2.6 (Fig. 2B, shown in pink), while it is flipped towards the active site in the inactive form (Fig. 2A, shown in green). Except for the C-terminal tail and the activation loop, there is no difference between the active and inactive models. These data suggest that KING1-binding to the C-terminal region induces conformational changes in the structure of SnRK2, causing SnRK2 to adopt the activator-bound conformation. This could explain how KING1 facilitates the activity of SnRK2.2 (Fig. 1C) in the same manner as γ-subunits of both SNF1 and SnRK1s regulate their kinases positively (Hardie 2011, Ramon *et al.* 2013). On the other hand, KING1 inhibits the activity of SnRK2.6 (Fig. 1B), even though the structural model suggests that the binding sites are located in similar places in the C-terminal

regions of the SnRK2s.

To identify the critical domain of SnRK2s in terms of regulation by KING1, we produced a chimeric protein consisting of the N-terminal (1-312) part of SnRK2.6 and the C-terminal (315-362) part of SnRK2.2 (Fig. S1), and tested the effects of KING1 on its activity in *in vitro* kinase assay. The chimeric SnRK2.6-2.2 protein can be expressed and purified despite there being more degradation bands than in SnRK2.6 or 2.2 (Fig. S3). The chimeric SnRK2.6-2.2 protein reacted similarly to SnRK2.6, with a reduced substrate phosphorylation capacity after treatment with GST-KING1 (Fig. 1D). Although the molecular mechanism is unclear, this result indicates that the effects of KING1 on SnRK2s are not exclusively determined by the C-terminal tail. Multiple pieces of evidence suggest that the N-terminal region is important to this effect: Even though SnRK2.6 and SnRK2.2 have high similarities, several amino acids are different between them (yellow structures in Fig.2 and Fig.S1). As shown by West *et al.* (2013), even the small differences in the N-terminal domains of SnRK2.6 and SnRK2.3 (which has 91% identity to SnRK2.2) result in different stabilities and conformations of the proteins. SnRK2.6 is also a much more stable protein than SnRK2.2 and SnRK2.3 in the thermoshift assay (Ng *et al.*, 2011). We speculate that KING1 binds in a similar manner to SnRK2.2 and SnRK2.6, but cannot induce the same conformational change in SnRK2.6 as the one which activates SnRK2.2. Rather, the binding of KING1 may have an effect on the flexibility of SnRK2.6. This includes the molecular motion of the activation loop, which may happen in SnRK2.6 without KING1 and produce the basal activity of SnRK2.6. There are, however, also other possibilities for the interaction between KING1 and SnRK2s. Because the N-terminal end structure of the AMPK γ-subunit has not been reported, the N-terminal end of KING1 cannot be modelled. The N-terminal end is large enough and close enough to the interphase with SnRK2 to possibly affect the specificity of this protein as both an inhibitor and an activator. The physiological role of KING-mediated inhibition/facilitation of SnRK2s, including the interaction with PP2C in vivo, remains to be clarified in future experiments, while the results in this study show that KING1 has the potential to affect activities of SnRK2s and play a role in regulation of other kinases besides SnRK1s.

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Fig. 1. KING1 binds to SnRK2.6 *in vitro* (A) and affects autophosphorylation and substrate phosphorylation of SnRK2.6 (B), SnRK2.2 (C), and chimeric SnRK2.2-2.6 (D) in different ways *in vitro*. Binding and kinase assays were performed with recombinant proteins produced in *E.coli*. For binding assay (A), amylose beads with MBP or MBP-fused SnRK2.6 were incubated with GST or GST-fused KING1. Proteins in the bound fraction (left) and in the input fraction (middle, 10%) were detected using anti-GST-antibody. Coomassie staining was used to show the amounts of proteins on the beads (right). Expected sizes of GST-KING1 and GST are indicated by a black and a white arrowhead, respectively. Some GST-KING1s were degraded (curly bracket). For kinase assays (B-D), MBP-fused SnRK2s were incubated with either GST (white arrow) or GST-fused KING1 (black arrow) in the presence of [γ-^{32}P] ATP. GST-fused AREB1a was used as a phosphorylation substrate for SnRK2s.

Autophosphorylation (black arrowhead) and substrate phosphorylation (white arrowhead) were detected by autoradiography (left). Coomassie staining was used to show the amounts of protein (right). GST ran out from the bottom of gel in the SnRK2.2 experiment.

Fig 2. Structural model of the "Activated" SnRK2.6/KING1 complex, in comparison with the "Inactive" SnRK2.6.

(A) A model of KING1 (gold , based on the γ-1 subunit of AMPK, PDB ID: 4CFE:E) in complex with the "Inactive form" of SnRK2.6 (blue, PDB ID: 3UC4). (B) A model of KING1 (gold, same as in A) in complex with the "Activated form" of SnRK2.6 (grey, based on the AMPK structure PDB ID: 4CFE:A). Amino acids F161 and P180 show ends of the SnRK 2.6 activation loop (green in A, pink in B). In A, position of the SnRK2 box and the estimated position of the ABA box (not present in 3UC4) are shown. In B, estimated position of the SnRK2 box (not present in 4CFE:A) and the position of the ABA box are also indicated. The N-terminal 86 amino acids of KING1 could not be modelled. The putative PP2C binding site is positioned on the "top" of the inactive form of SnRK2.6 structure across the activation loop. Yellow structures indicate different amino acid residues between SnRK2.2 and SnRK2.6.

Supporting information

S1 Fig. Hybrid sequence/structural alignment of SnRK2.6.

S2 Fig. Hybrid sequence/structural alignment of KING1

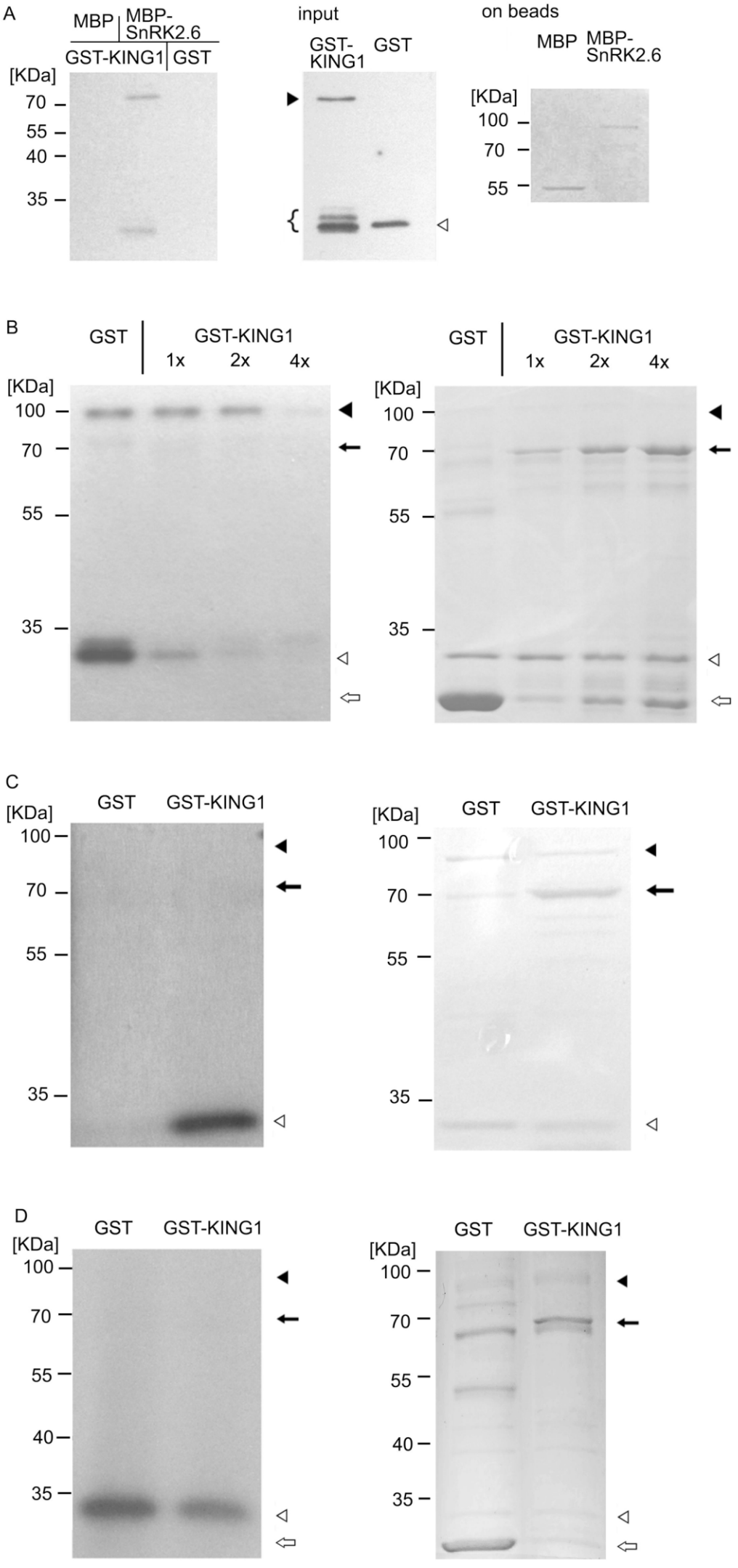
S3. Purified proteins used for *in vitro* kinase assays. Proteins were run in SDS-PAGE gels and stained with Coomassie stain. The predicted size are indicated by black arrowheads.

S4 Fig. Ramachandran plots demonstrate results of the structural validation of models with only three amino acids with bad geometries in each structure.

S5. Additional Materials and Methods

255 **S6 Table. PCR-primers.**

Fig.1


A
MBP
MBP-SnRK2.6
GST-KING1
GST
[KDa]
70
55
40
35
input
GST-KING1
GST
on beads
MBP
MBP-SnRK2.6
[KDa]
100
70
55
B
GST
GST-KING1
1x
2x
4x
[KDa]
100
70
55
35
C
GST
GST-KING1
[KDa]
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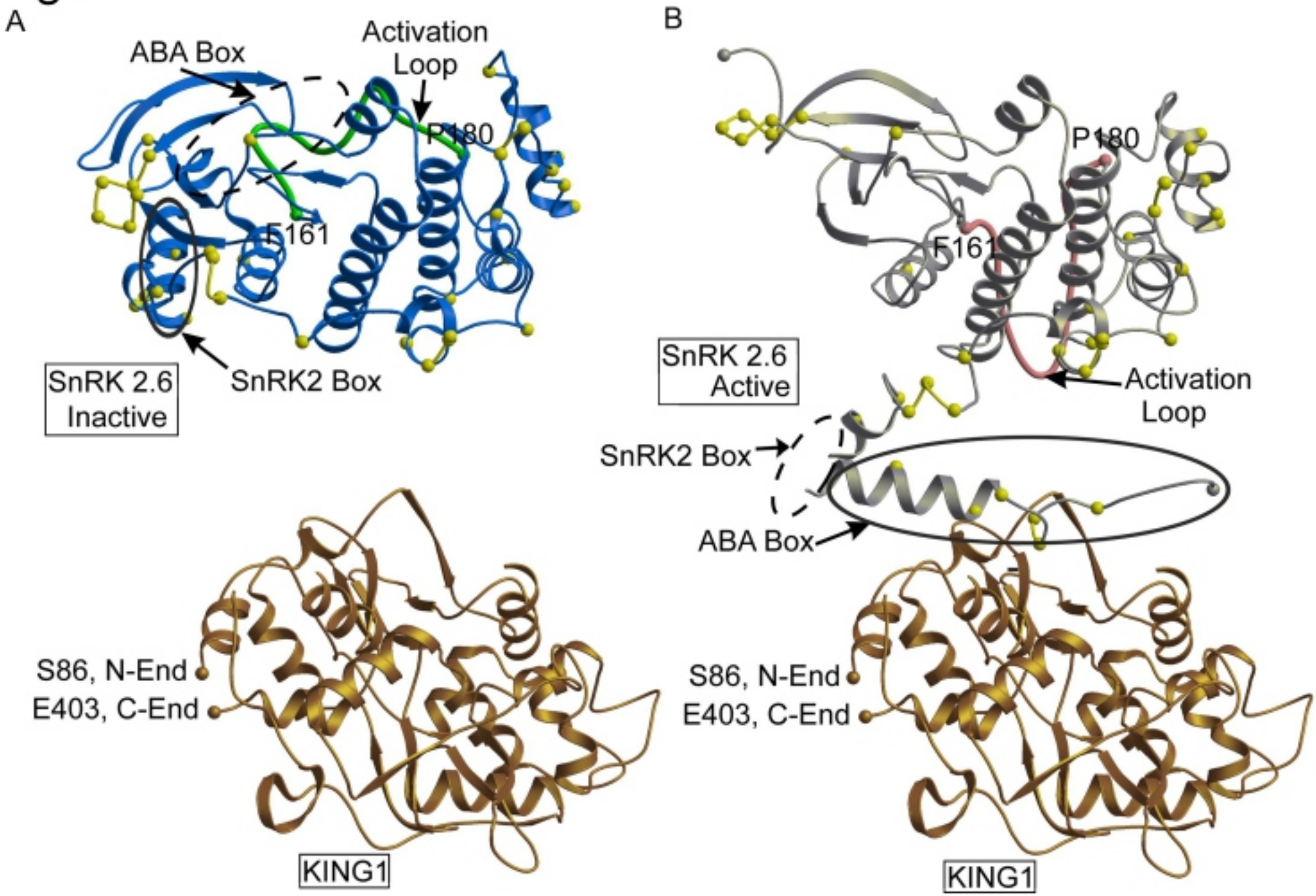
70
55
35
D
GST
GST-KING1
[KDa]
100
70
55
40
35

Fig.2


A
ABA Box
Activation Loop
P180
F161
SnRK2 Box
SnRK 2.6 Inactive
B
P180
F161
SnRK 2.6 Active
Activation Loop
SnRK2 Box
ABA Box
S86, N-End
E403, C-End
KING1
S86, N-End
E403, C-End
KING1

Supplementary Figure S1. Hybrid sequence/structural alignment of *A. thaliana* SnRK2.6 (UniProtKB - Q940H6), *A. thaliana* SnRK2.2 (UniProtKB - Q39192), and *H. sapiens* AMPK Catalytic Subunit α-2 (PDB ID: 4CFE, chain A). Numbering of 4CFE:A sequence is according to the Protein Data Bank file. Sequence similarity is highlighted.

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                                          Activator binding site in 4CFE:A
                                              ----------------------
SnRK2.6  1   --------MDRPAVSG--PMDLPIMHDS----DRYELVKDIGSGNFGVARLMRDKQSNELVAVKYIERGE----KIDENVKREIINHRSLRHPNIVRFKE 82
SNRK2.2  1   --------MDPATNSPIMPIDLPIMHDS----DRYDFVKDIGSGNFGVARLMTDRVTKELVAVKYIERGE----KIDENVQREIINHRSLRHPNIVRFKE 84
4CFE:A   8   -------------------------DGRVKIGHYVLGDTLGVGTFGKVKIGEHQLTGHKVAVKILNRQKIRSLDVVGKIKREIQNLKLFRHPHIIKLYQ 81

SnRK2.6  83  VILTPTHLAIVMEYASGGELFERICNAGRFSEDEARFFFQQLISGVSYCHAMQVCHRDLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGTPAY 182
SNRK2.2  85  VILTPSHLAIVMEYAAGGELYERICNAGRFSEDEARFFFQQLISGVSYCHAMQICHRDLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGTPAY 184
4CFE:A   82  VISTPTDFFMVMEYVSGGELFDYICKHGRVEEMEARRLFQQILSAVDYCHRHMVVHRDLKPENVLLDAHM--NAKIADFGLSNMMSDGEFLRTSCGSPNY 179

SnRK2.6  183 IAPEVLLKKEYDGKVADVWSCGVTLYVMLVGAYPFEDPEEPKNFRKTIHRILNVQYAIPDYVHISPECRHLISRIFVADPAKRISIPEIRNHEWFLKNLP 282
SNRK2.2  185 IAPEILLRQEYDGKLADVWSCGVTLYVMLVGAYPFEDPQEPRDYRKTIQRILSVTYSIPEDLHLSPECRHLISRIFVADPATRITIPEITSDKWFLKNLP 284
4CFE:A   180 AAPEVISGRLYAGPEVDIWSCGVILYALLCGTLPFDDEHVPTLFKK----IRGGVFYIPEYLNRSVAT--LLMHMLQVDPLKRATIKDIREHEWFKQDLP 273

                        Deletion in 4CFE:A (partially reconstructed)
                        ------------------------
                                      SnRK2 box                                   ABA box
SnRK2.6  283 ADLMNDNTMTTQFDESDQPGQSIEEIMQIIAEATVPPAGTQNLNHYLTGS--LDIDDDMEEDLESDLDDLDIDSSGEIVYAM----------------- 362
SNRK2.2  285 GDLMDENRMGSQFQEPEQPMQSLDTIMQIISEATIPTVRNRCLDDFMADN--LDLDDDMD-DFDSE-SEIDVDSSGEIVYAL----------------- 362
4CFE:A   267 SYLFPEDPSYDANVIDDEAVKEVCEKFECTESEVMNSLYSGDPQDQLAVAYHLIIDNRRIMNQASEFYLASSPPSGSFMDDSAMHIPPGLKPHPERMPPL 373
                                     swapping point in the chimera

SnRK2.6      ------------------------------------------------------------------------------------------------------
SNRK2.2      ------------------------------------------------------------------------------------------------------
4CFE:A   374 IADSPKARCPLDALNTTKPKSLAVKKAKWHLGIRSQSKPYDIMAEVYRAMKQLDFEWKVVNAYHLRVRRKNPVTGNYVKMSLQLYLVDNRSYLLDFKSID 473

SnRK2.6      ------------------------------------------------------------------------
SNRK2.2      ------------------------------------------------------------------------
4CFE:A   474 DEVVEQRSGSSTPQRSCSAAGLHRPRSSFDSTTAESHSLSGSLTGSLTGSTLSSVSPRLGSHTMDFFEMCASLITTLA 551
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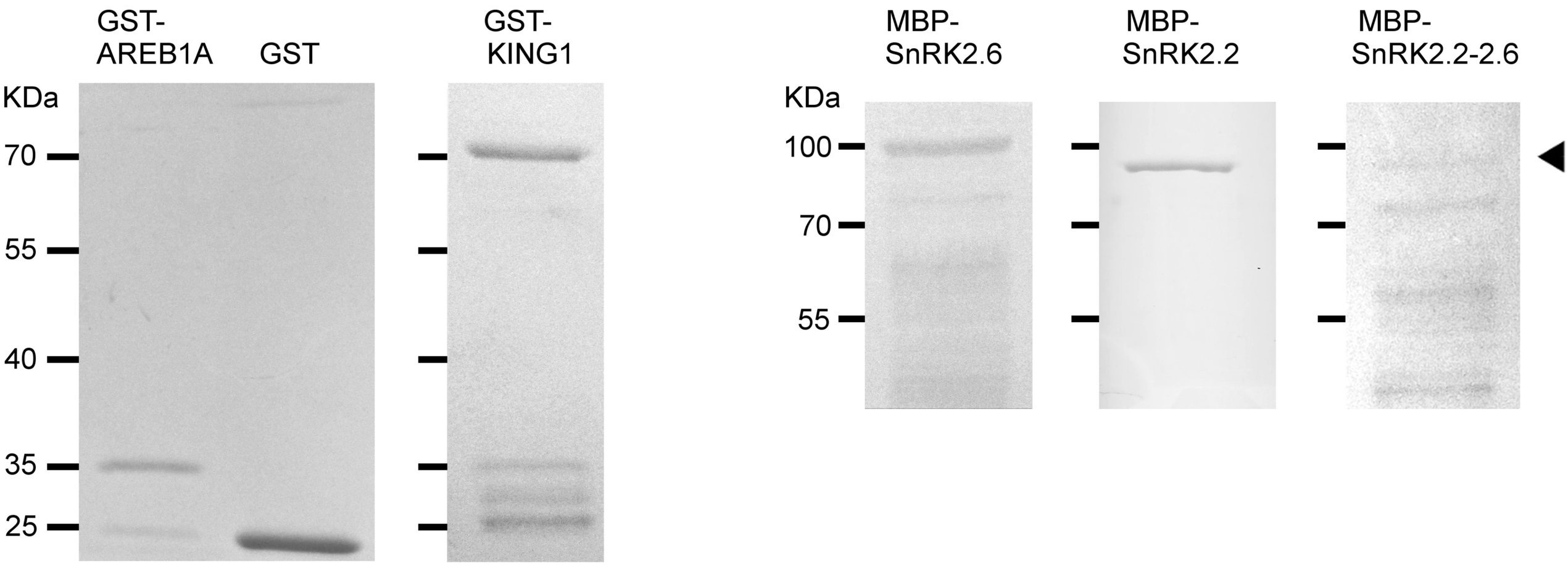
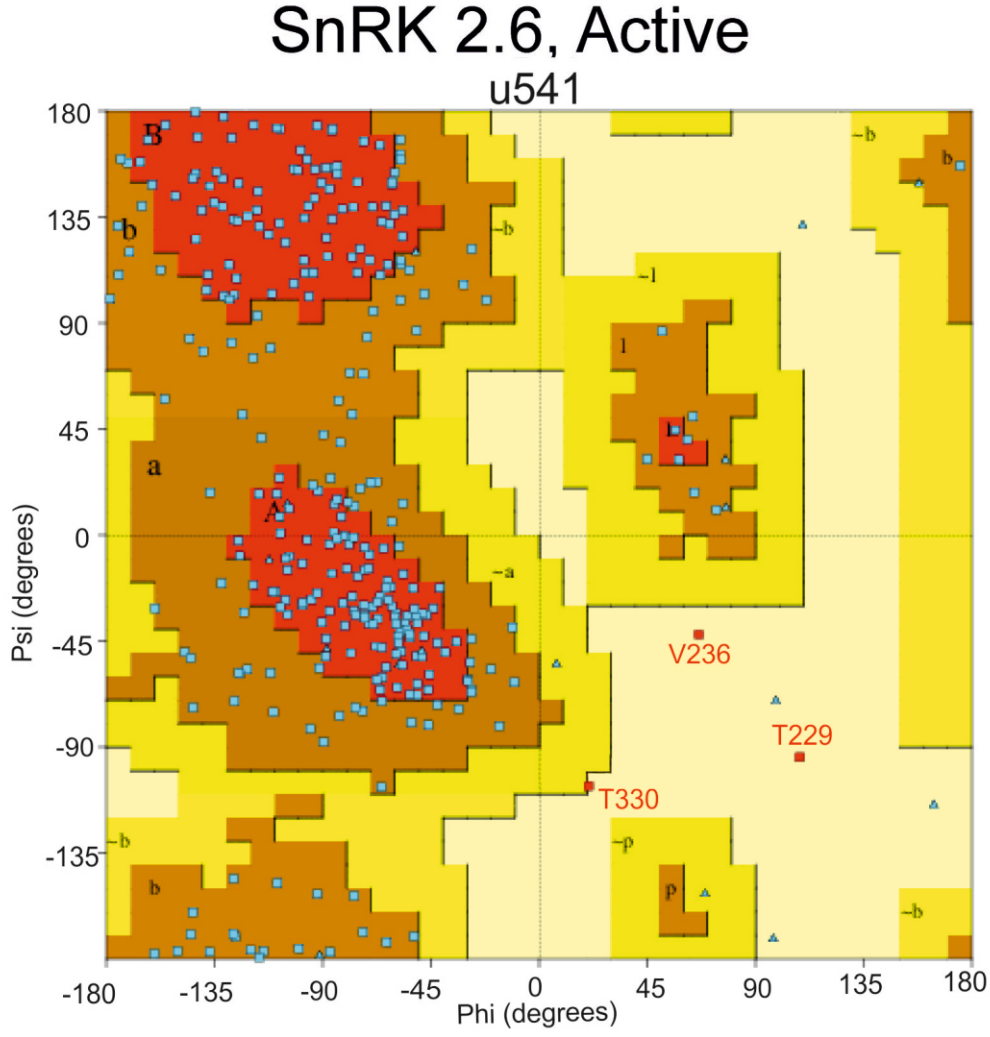
Supplementary Figure S2. Hybrid sequence/structural alignment of *A. thaliana* KING1 (UniProtKB - Q8LBB2), and *H. sapiens* AMPK Subunit γ-1 (PDB ID: 4CFE, chain E). Numbering of 4CFE:E sequence is according to the Protein Data Bank file. Sequence similarity is highlighted.

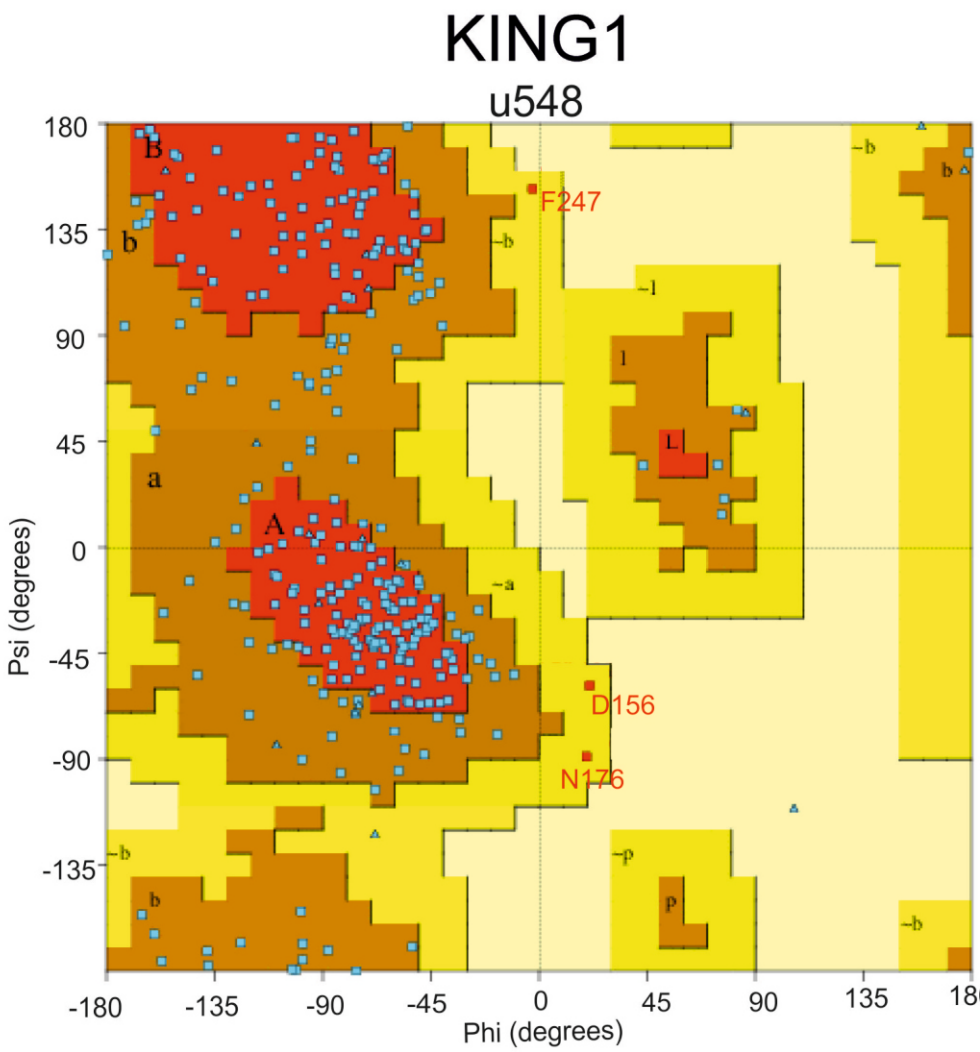
```
KING1     1 MATVPEIKIMRSESLGHRSDVSSPEAKLGMRVEDLWDEQKPQLSPNEKLNACFESIPVSAFPLSSDSQDIEIRSDTSLAEAVQTLSKFKVLSAPVVDVDA 100
4CFE:E   27 ----------------------------------------------------------------------------SVYTSFMKSHRCYDL 41

KING1   101 PEDASWIDRYIGIVEFPGIVVWLLHQLEPPSPRSPAVAASNGFSHDFTTDVLDNGDSAVTSGNFFEVLTSSELYKNTKVRDISGTFRWAPFLALQKENSF 200
4CFE:E   42 IPTSSKLVVFDTSLQVKKAFFALVTNGVRAAPLWDSKKQS--FVGMLTITDFINILHRYYKSALVQIYELEE-HKIETWREVYLQDSFKPLVCISPNASL 138

KING1   201 LTMLLLLSKYKMKSIPVVDLGVAKIENIITQSGVIHMLAECAGLLWFEDWGIKTLSEVGLPIMSKDHIIKIYEDEPVLQAFKLMRRKRIGGIPVIERNSE 300
4CFE:E  139 FDAVSSLIRNKIHRLPVIDPESGNTLYILTHKRILKFLKLFITEFPKPEFMSKSLEE--LQIGTYANIAMVRTTTPVYVALGIFVQHRVSALPVVDEKGR 236

KING1   301 KPVGNISLRDVQFLLTAPEIYHDYRSITTKNFLVSVREHLEKCGDTSAPIMSGVIACTKNHTLKELILMLDAEKIHRIYVVDDFGNLEGLITLRDIIARL 400
4CFE:E  237 --VVDIYSKFDVINLAAEKTYN--------NLDVSVTKALQH----RSHYFEGVLKCYLHETLETIINRLVEAEVHRLVVVDENDVVKGIVSLSDILQAL 322

KING1   401 VHEPSGYFGDFFDGVMPLPENYRV 424
4CFE:E  323 VLT--------------------- 325
```



S3. Purified proteins used for *in vitro* kinase assays.

Proteins were run in SDS-PAGE gels and stained with Coomassie stain.
The predicted size are indicated by black arrowheads.





S4 Fig. Ramachandran plots demonstrate results of the structural validation of models with only three amino acids with bad geometries in each structure.

Materials and methods

Plasmid constructs

KING1 cDNA was cloned by PCR using a cDNA library (Fujii *et al.* 2007) as template and the primers listed in Table S5 in Supporting Information. The PCR fragment was cloned into the BamHI and EcoRI sites of pGEX4T1 (Amersham, Buckinghamshire, UK). Maltose-binding protein (MBP)-fused SnRK2.6 is described in Fujii et al (2009). Primer pairs in Supplemental Table S5 were used for the first PCR in production of the chimeric protein. Second amplification was performed using the PCR products as templates. The final product was cloned into pMALc2x (*BamHI-SalI* sites).

Purification of recombinant proteins from *E. coli* and *in vitro* binding assays

Recombinant proteins were purified as described previously (Fujii and Zhu 2009). Glutathione S-transferase (GST)-fused proteins were eluted from beads with 40 mM reduced glutathione (pH 8.0). The eluted proteins were dialysed in STE solution (150 mM NaCl, 10mM Tris-HCl, pH8.0, 1 mM EDTA) at 4 °C overnight. MBP-fused proteins were incubated with the eluted proteins in 100 µl of the STE solution at 23 °C for 1 h with slow rocking. After the beads were washed six times with 1 ml of the STE solution, Laemmli sample buffer was added on the beads. Proteins were separated on an SDS-PAGE gel and subjected to western blot analysis with anti-GST antibody (Upstate/EMD Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Purified proteins are shown in Fig. S3.

In vitro kinase assay

Recombinant proteins were produced in *E. coli* as described previously (Fujii and Zhu 2009). MBP-fused SnRK2s were incubated with GST-fused AREB1a and either GST-fused KING1 or GST in 30 µl of reaction mixture (25 mM Tris, pH 7.4, 12 mM $MgCl_2$, 1 µM cold ATP, 5 µCi [γ-^{32}P] ATP, 1 mM DTT, 1 mM Na_3VO_4, 5 mM NaF, and 0.03 µg/µl BSA). Reaction to SnRK2.6 was tested with different amounts of GST-KING1. After incubation (45 min, 30 °C), reactions were stopped by adding Laemmli sample buffer. After separation of the proteins by SDS-PAGE, radioactive bands

were detected by autoradiography on the dried gel. The experiments were replicated three times. GIMP (https://www.gimp.org/) was used for generating the figures for the *in vitro* assays.

Modeling of the SnRK2.6/KING1 complex

The heterotrimeric AMPK complex (PDB ID: 4CFE) was used as hetero oligomeric template. Because of the moderate-to-low level of sequence similarity, we used a combined sequence/structural approach to ensure that all the "key" domain residues would be aligned at the correct domain positions. This approach involved a combination of multiple sequence alignments made with ClustalW (Larkin et al., 2007) and Multalin (Corpet, 1988), combined with the data from the Conserved Domains Database (CDD; Marchler-Bauer et al., 2017), and manual adjustment. Modeller (Sali and Blundell, 1993) and Homodge (in Bodil; Lehtonen et al., 2004) were used for homology modeling of domains based on known related structures. BIOVIA Discovery Studio (http://accelrys.com) and the Schrödinger Suite for molecular modeling (http://www.schrodinger.com/) were used for structure preparation and adjustments, additional structure modeling, superposition, viewing and analysis. Models of KING1 and SnRK2.6 in its "activated" form were minimized and evaluated by PROCHECK (Laskowski et al., 1993) through the PDBsum database (de Beer et al., 2014). Analysis of the resulting Phi/Psi Ramachandran Plots indicated that only three amino acids in each protein, Asp156, Asn176 and Phe247 in KING1, and Thr229, Val236 and Thr330 in SnRK2.6, had a disallowed geometry (see Ramachandran plots in Fig. S4). Fig.S4 was manually modified with CorelDRAWX7 to change fonts. Influence of these small number of amino acids on the conclusions derived in this manuscript can be considered as negligible.

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Supplemental table S6. PCR-primers

KING1 forward	AAGGATCCATGGCGACTGTTCCGGAG
KING1 reverse	AGAATTCAGACTCGGTAGTTTTCGG
2622CtermForward	AGAAGAAATTATGCAGATAATATCGGAGGCTAC
pMAL Reverse	GCGATTAAGTTGGGTAACGCC
2622NtermReverse	CCGATATTATCTGCATAATTTCTTCTATGCTTTGG
pMAL Forward	CGCAGACTAATTCGAGCTCGAAC