

Materials and methods

Plasmid constructs: A KING1 cDNA was cloned by PCR using a cDNA library (Fujii *et al.* 2007) as a template and primers are listed in Table 1 Suppl. The PCR fragment was cloned into the BamHI and EcoRI sites of pGEX4T1 (Amersham, Buckinghamshire, UK). The maltose-binding protein (MBP)-fused *Sucrose non fermenting1* (SNF1)-related protein kinase (SnRK)2.6 was described in Fujii *et al.* (2009). Primer pairs (Table 1 Suppl.) were used for the first PCR in production of the chimeric protein. The second amplification was performed using the PCR products as templates. The final product was cloned into pMALc2x (BamHI-Sall sites).

Purification of recombinant proteins from *Escherichia 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 dialyzed in an STE (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA_{Na2}) solution at 4 °C overnight. The MBP-fused proteins were incubated with the eluted proteins in 0.1 cm³ of the STE solution at 23 °C for 1 h with slow rocking. After the beads were washed six times with 1 cm³ of the STE solution, a Laemmli sample buffer was added on the beads. Proteins were separated on an SDS-PAGE gel and subjected to Western blot analysis with an anti-GST antibody (Upstate/EMD, Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Purified proteins are shown in Fig. 3 Suppl.

***In vitro* kinase assay:** Recombinant proteins were produced in *E. coli* as described previously (Fujii and Zhu 2009). The MBP-fused SnRK2s were incubated with GST-fused ABA-responsive element binding factor (AREB)1a and either GST-fused KIN γ subunit (KING)1 or GST in 0.03 cm³ of a reaction mixture (25 mM Tris, pH 7.4, 12 mM MgCl₂, 1 μ M cold ATP, 0.185MBq [γ -³²P] ATP, 1 mM dithiothreitol, 1 mM Na₃VO₄, 5 mM NaF, and 0.03 mg cm⁻³ bovine serum albumin). Reaction to SnRK2.6 was

tested with different amounts of GST-KING1. After incubation (30 °C for 45 min), reaction was stopped by adding a 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. The *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 AMP-activated protein kinase (AMPK) complex (PDB ID: 4CFE) was used as a hetero-oligomeric template. Because of the moderate-to-low 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* (Lehtonen *et al.* 2004) were used for homology modeling of domains based on known related structures. The *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 indicates 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. 4 Suppl.). Fig. 4 Suppl. was manually modified with *CorelDRAWX7* to change fonts. The influence of this small number of amino acids on the conclusions derived in this manuscript can be considered as negligible.

References

- Corpet, F.: Multiple sequence alignment with hierarchical clustering. - Nucl. Acids Res. **16**: 10881-10890, 1988.
- De Beer, T.A.P., Berka, K., Thornton, J.M., Laskowski, R.A.: PDBsum additions. - Nucl. Acids Res. **42**: D292-D296, 2014.
- Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.-Y.: *In vitro* reconstitution of an ABA signaling pathway. - Nature **462**: 660-664, 2009.
- Fujii, H., Verslues, P.-E., Zhu, J.-K.: Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. - Plant Cell **19**: 485-494, 2007.
- Fujii, H., Zhu, J.-K.: An autophosphorylation site of the protein kinase SOS2 is important for salt tolerance in *Arabidopsis*. - Mol. Plant. **2**: 183-190, 2009.
- Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G.: Clustal W and Clustal X version 2.0. - Bioinformatics **23**: 2947-2948, 2007.
- Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M.: PROCHECK - a program to check the stereochemical quality of protein structures. - J. appl. Crystal. **26**: 283-291, 1993.
- Lehtonen, J.V., Still, D.J., Rantanen, V.V., Ekholm, J., Bjorklund, D., Iftikhar, Z., Huhtala, M., Repo, S., Jussila, A., Jaakkola, J., Pentikäinen, O., Nyrönen, T., Salminen, T., Gyllenberg, M., Johnson, M.S.: BODIL: a molecular modeling environment for structure-function analysis and drug design. - J. Comput. Aided Mol. Des. **18**: 401-419, 2004.
- Marchler-Bauer, A., Bo, Y., Han, L., He, J., Lanczycki, C.J., Lu, S., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., Gwadz, M., Hurwitz, D.I., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Geer, L.Y., Bryant, S.H.: CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. - Nucl. Acids Res. **45**: D200-D203, 2017.
- Sali, A., Blundell, T.L.: Comparative protein modelling by satisfaction of spatial restraints. - J. mol. Biol. **234**: 779-815, 1993.

Table 1 Suppl. PCR-primer sequences.

Primers	Forward	Reverse
KING1	AAGGATCCATGGCGACTGTTCCGGAG	AGAATTCAGACTCGGTAGTTTTCGG
pMAL	CGCAGACTAATTCGAGCTCGAAC	GCGATTAAGTTGGGTAACGCC
2622Nterm	AGAAGAAATTATGCAGATAATATCGGAGGCTAC	CCGATATTATCTGCATAATTTCTTCTATGCTTTGG

		Activator binding site in 4CFE:A	
SnRK2.6	1	-----MDRPAVSG--PMDLPIMHDS-----DRYELVKDIGSGNFGVARLMRDQSNELVAVKYIERGE-----KIDENVKREIINHRSLRHPNIVRFKE	82
SNRK2.2	1	-----MDPATNSPIMPIDLPIHDS-----DRYDFVKDIGSGNFGVARLMTDRVTKELVAVKYIERGE-----KIDENVQREIINHRSLRHPNIVRFKE	84
4CFE:A	8	-----DGRVKIGHYVLGDTLGVGTGFKVKIGEHQLTGHKVAVKILNRQKIRSLDVVGKIKREIQNLKLFHHPHIKLYQ	81
SnRK2.6	83	VILTPTHLAIVMEYASGGELFERICNAGRFSEDEARFFFQQLISGVSYCHAMQVCHRDLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGTTPAY	182
SNRK2.2	85	VILTPSHLAIVMEYAAGGELYERICNAGRFSEDEARFFFQQLISGVSYCHAMQICHRLKLENTLLDGSPAPRLKICDFGYSKSSVLHSQPKSTVGTTPAY	184
4CFE:A	82	VISTPTDFFMMEYVSGGELFDYICKHGRVEEMEARRLFQQILSAVDYCHRMVVRDLKPENVLDAHM--NAKIADFGLSNMMSDGEFLRTSCGSPNY	179
SnRK2.6	183	IAPEVLLKKEYDGKVDVWVSCGVTLYVMLVGAYPFEDPEEPKNFRKTIHRLNVQYAIPTYVHISPECRHLISRI FVADPAKRISIPERINHEWFLKNLP	282
SNRK2.2	185	IAPEILLRQEYDGKLADVWVSCGVTLYVMLVGAYPFEDPQEPDRYRKTIIQRILSVTYSIPEDLHLSPECRHLISRI FVADPATRITIPETSDKWFKNLP	284
4CFE:A	180	AAPEVISGRLYAGPEVDIWSGVIYALLCGTLPFDDHVPITLFFK----IRGGVFYIPEYLNRSVAT--LLMHMLQVDPLKRATIKDIREHEWFKQDLP	273
		Deletion in 4CFE:A (partially reconstructed)	

SnRK2.6	283	ADLMNDNTMTTQFDESDDQOSTEEIMQITAEATPPAGTONLNHYLTGS--LDIDDDMEEDLESDDLDDIDSSGEIVYAM-----	362
SNRK2.2	285	GDLMNDNRMGSOQFQEPQMSLDTIMOTISEATIPTVRNRCLDDFMADN--LDLDDMD-DFDSE-SEIDVDSSGEIVYAL-----	362
4CFE:A	267	SYLFPEDPSYDANVIDDEAVKEVCEKFECTESEVMNSLYSGDQDQLAVAYHLTIIDNRRIMNQASEFYLAASSPPSGSFMDDSAMHIPPGLKPHPERMPPL	373
		swapping point in the chimera	
SnRK2.6		-----	
SNRK2.2		-----	
4CFE:A	374	IADSPKARCPLDALNTTKPKSLAVKKAKWHLGIRSQSKPYDIMA EVYRAMKQLDFEWKVVNAYHLRVRKKNPVTGNYVKMSLQLYLVDNRSYLLDFKSID	473
SnRK2.6		-----	
SNRK2.2		-----	
4CFE:A	474	DEVVEORSGSSTPQRSCSAAGLHRPRSSFDSTTAESHLSGSLTGSTLSSVSPRLGSHTMDFEMCASLITTLA	551

Fig 1 Suppl. The hybrid sequence and structural alignment of SnRK2.6.

KING1	1	MATVPEIKIMRSESLGHRSDVSSPEAKLGMRVEDLWDEQKPLSPNEKLNACFESIPVSAPPLSSDSQDIEIRSDTSLAEAVQTLISKFKVLSAPVVDVDA	100
4CFE:E	27	-----SVYTSFMKSHRCYDL	41
KING1	101	PEDASWIDRYIGIVEFPGIVVWLLHQLEPPSPRPAPAAASNGFSHDFITDVLNDNGDSAVTSGNFFEVLTSSELYKNTKVRDISGTFRWAPFLALQKENSF	200
4CFE:E	42	IPTSSKL VVFDTS LQVKKAFFALVTNGVRAAPLWDSKKQS--FVGMLTITDFINILHRYYSALVQTYELEEHKTIETWREVLQDSFKPLVCISPNASL	138
KING1	201	LTMLLLLSKYMKMSIPVVDLGVAKIENTITQSGVIHMLAECAGLLWFEDWGKTLSEVGLPTMSKDHIITKTYEDEPVLQAFKLMRRKRIGGIPVIERNSE	300
4CFE:E	139	FDAVSSLIRNKIHRLPVIDPESGNTLYILTHKRILKFLKLFITEFPKPEFMSKSL EE--LQIGTYANIAMVRTTTPVYVALGIFVQHRVSALPVVDEKGR	236
KING1	301	KPVGNISLRDVQFLTAPEIYHDYRSITTKNFLVSVREHLEKCGDTSAPIMSGVIACTKNHTLKEILMLDAEKTHRIYVVDVDFGNLEGLITLRDIIRL	400
4CFE:E	237	--VVDIYSKFDVINLAAEKTYN-----NLDVSVTKALQH----RSHYFEGVLKCYLHETLETIINRLVEAEVHRLVVVDENDVVKGI VSLSDILQAL	322
KING1	401	VHEPSGYFGDFDGMPLPENYRV	424
4CFE:E	323	VLT-----	325

Fig. 2 Suppl. The hybrid sequence and structural alignment of KING1.

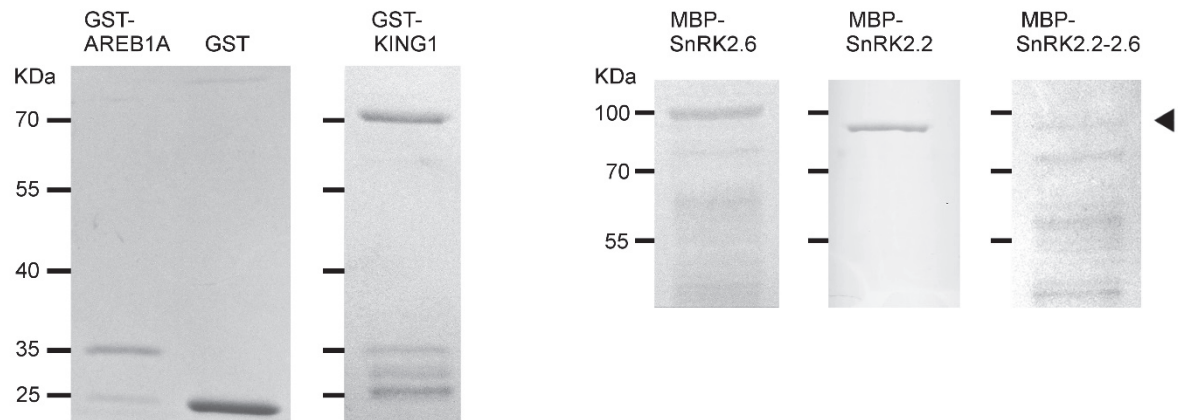


Fig. 3 Suppl. Purified proteins used for *in vitro* kinase assays. Proteins were run in SDS-PAGE gels and stained with Coomassie blue. The predicted size is indicated by a *black arrowhead*.

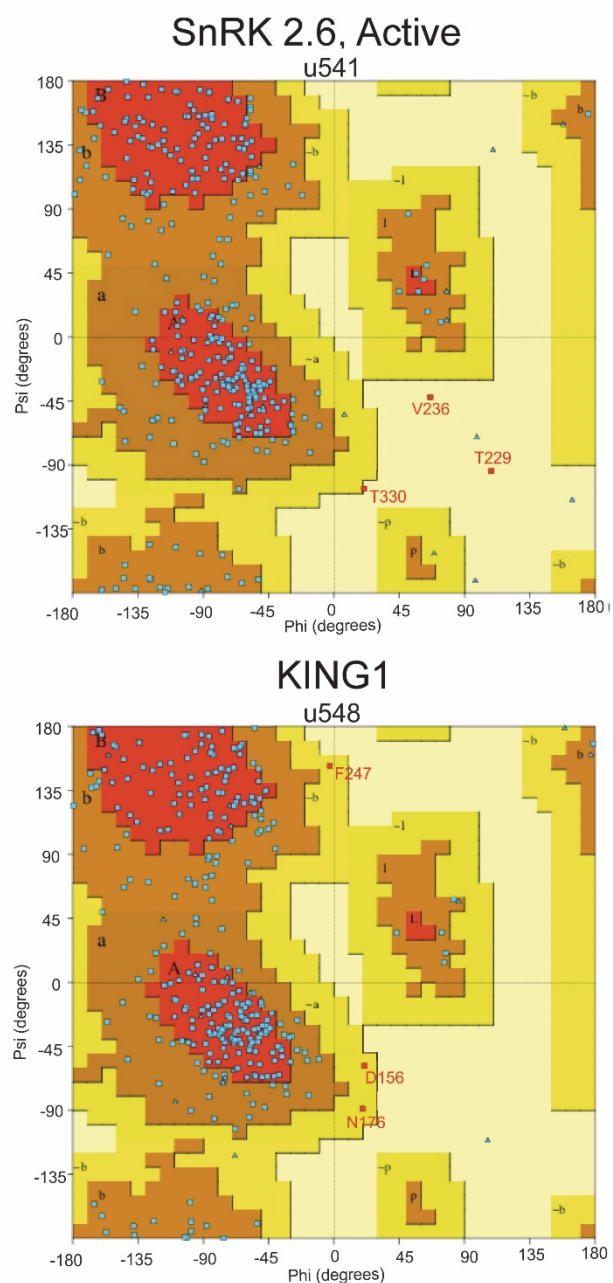


Fig. 4 Suppl. Ramachandran plots demonstrating results of the structural validation of models with only three amino acids with bad geometries in each structure.