Supplementary Experimental Procedures

Tandem MS/MS- In-gel digestion was performed with the Progest system (Genomic Solution) according to a standard trypsin protocol. Gel pieces were washed twice by successive separate baths of 10% acetic acid, 40% ethanol, and acetonitrile (ACN). They were then washed twice with successive baths of 25mM NH_4CO_3 and ACN. Digestion was subsequently performed for 6 h at 37°C with 125 ng of modified trypsin (Promega) dissolved in 20% methanol and 20mM NH_4CO_3. The peptides were extracted successively with 2% trifluoroacetic acid (TFA) and 50% ACN and then with ACN. Peptide extracts were dried in a vacuum centrifuge and suspended in 20 µL of 0.05% TFA, 0.05% HCOOH, and 2% ACN.

HPLC was performed on an Ultimate LC system combined with a Famos autosampler and a Switchos II microcolumn switch system (Dionex). A 4µL sample was loaded at 5µL/min^{-1} on a pre-column cartridge (stationary phase: C18 PepMap 100, 5µm; column: 300µm i.d., 5mm; Dionex) and desalted with 0.05% TFA, 0.05% HCOOH, and 2% ACN. After 2.5 min, the pre-column cartridge was connected to the separating PepMap C18 column (stationary phase: C18 PepMap 100, 3µm; column: 75µm i.d., 150mm; Dionex). Buffers were 0.1% HCOOH, 3% ACN (A) and 0.1% HCOOH and 95% ACN (B). The peptide separation was achieved with a linear gradient from 5 to 30% B for 25 min at 200nL/min^{-1}. Including the regeneration step at 100% B and the equilibration step at 100% A, one run took 45 min.

Eluted peptides were analyzed on-line with a LTQ XL ion trap (Thermo Electron) using a nanoelectrospray interface. Ionization (1.5kV ionization potential) was performed with liquid junction and a non-coated capillary probe (10µm i.d.; New Objective). Peptide ions were analyzed using Xcalibur 2.07 with the following data-dependent acquisition steps: (1) full MS scan (mass-to-charge ratio (m/z) 400 to 1900, centroid mode), (2) MS/MS (qz = 0.25, activation time = 30ms, and collision energy = 35%; centroid mode) and (3) MS3 (qz = 0.25, activation time = 30ms, and collision energy = 35%; centroid mode). Step 2 was repeated for the two major ions detected in step 1. Step 3 was performed if a neutral loss (98 or 80 Da) was detected in step 2. Dynamic exclusion was set to 30 s.

A database search was performed with XTandem 2008.02.01 (http://www.thegpm.org/TANDEM/). Enzymatic cleavage was declared as a trypsin digestion with one possible miss-cleavage. Cys carboxyamidomethylation and Met oxidation were set to static and possible modifications, respectively. Precursor mass and fragment mass tolerance were 2.0 and 0.8, respectively. A refinement search was added with similar parameters except that possible Ser, Thr or Tyr phosphorylation (or dehydrated Ser and Thr for MS3 spectra) were searched with 5 accepted miss-cleavages. The *Escherichia Coli* proteome database (4347 entries, download on 30 October 2008, http://www.ebi.ac.uk/integr8/OrganismSelection.do?action=makeCurrent&proteomeId=18) and a contaminant database (trypsin, keratins, *etc*.) were used in complement of recombinant protein sequences. Only peptides with an E value smaller than 0.1 were reported.

Identified proteins were filtered according to: (1) A minimum of two different peptides required with an E value smaller than 0.05, (2) a protein E value smaller than 10^{-4}. In the case of identification of phosphorylation sites, similarity between the experimental and the theoretical MS/MS spectra was visually checked.

Quantification of phosphorylation sites were performed by integrating the extracted ion chromatogram of the phosphorylated peptide within [m/z - 0.5; m/z + 1.5]. Intensity was normalized relatively to the non-phosphorylated peptide. Processing included background subtraction, smoothing and peak detection using home-made tools.

For the time course experiment, quantifications were performed by Multiple Reaction Monitoring (MRM) with the following transition: TSCGSPNYAAPEVISGK (869.4 – 1245.7), T*SCGSPNYAAPEVISGK (909.4 – 860.4), VAPSETAMGDVLR (681.35 – 596.5), VAPSET*AMGDVLR (721.3 – 636.5), IGDFSVSQVFK (613.8 – 794.5), IGDFSVS*QVFK (653.8 – 605). Methionines were chemically oxidized using H2O2 as described in Hardin *et al* (1).

Supplementary Tables

Table S1. Oligonucleotides used in PCR experiments.

Supplementary Figures

Figure S1. **Identification of two phosphorylation sites on AtSnAK2 and one on AtSnRK1.1.**
MS/MS spectra of phosphorylated peptides are annotated according to b and y ions produced by collision-induced dissociation as presented on the ion table.

Figure S2. **Alignment of the two AtSnAK protein sequences with homologous upstream kinases of human and yeast and with other kinases.**
AtSnAK1 (Uniprot: Q5HZ38), AtSnAK2 (Uniprot: Q93V58), CaMKKβ (Uniprot: Q96RR4), LKB1 (Uniprot: Q15831), TAK1 (PDB: 2EVA), TOS3 (Uniprot: P43637), ELM1 (Uniprot: P32801), PAK1 truncated (1 to 654) (Uniprot: P38990), AtSnRK1.1 (Uniprot: P92958), SNF1 (PDB: 3HYH), ROCK1 (PDB: 2ESM) and MEK1 (PDB: 3E8M) were aligned using ClustalW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) and completed using ESPrit (http://espript.ibcp.fr/ESPript/ESPript/). The corresponding AtSnAK auto-phosphorylation region is underlined in green (AP loop) and of the AtSnAK feedback phosphorylation region is underlined in purple (FP loop). Phosphorylatable residues are boxed in blue in these two regions except for those shown to be auto-phosphorylated (green) or shown to be phosphorylated by another kinase (red).

Supplementary References

1. Hardin, S. C., Larue, C. T., Oh, M. H., Jain, V., and Huber, S. C. (2009) *Biochem J*

Table S1

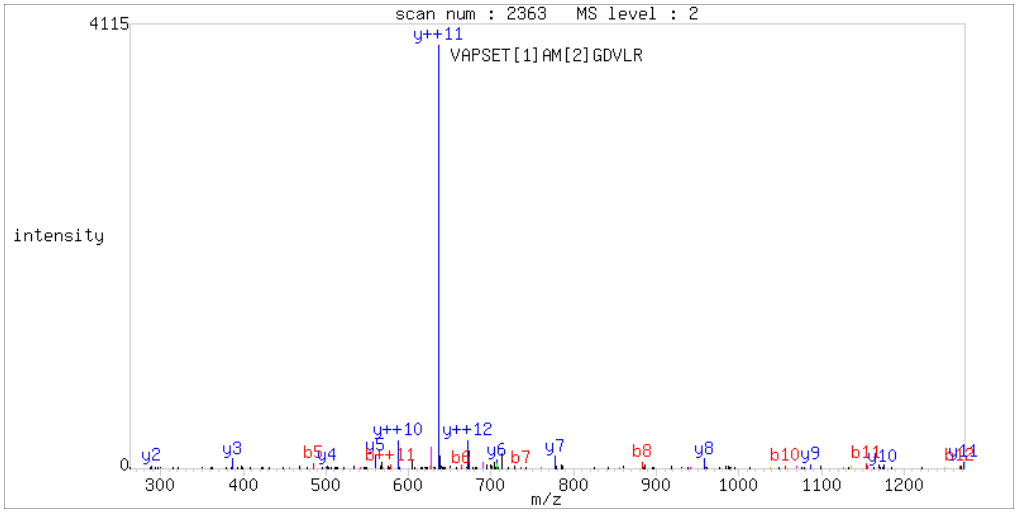
Gene	primer orientation	Sequence (5'-3')
AtSnRK1.1	Forward	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATGGATCAGGCAC-3’
	Reverse	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGGAGCTGAGCAAGAAA-3’
AtSnRK1.1-T175D	Midsense	5’-GGTCATTTTTTGAAGGACAGTTGTGGAAGTCC-3’
	Midantisense	5’-GGACTTCCACAACTGTCCTTCAAAAAATGACC-3’
AtSnRK1.2	Forward	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCATTCATCAAAT-3’
	Reverse	5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCACACGAAGCTCTGTAAG-3’
AtSnAK1	Forward	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTTCGTGATAGTTTTTTG-3’
	Reverse	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTAGTTAGGATCTGAGGT-3’
AtSnAK1-T153A	Midsense	5’-GGTGGCACCGTCAGAGGCTGCTATGAGTGATGTTC-3’
	Midantisense	5’-GAACATCACTCATAGCAGCCTCTGACGGTGCCACC-3'
AtSnAK1-T153D	Midsense	5’-GGTGGCACCGTCAGAGGATGCTATGAGTGATGTTC-3’
	Midantisense	5’-GAACATCACTCATAGCATCCTCTGACGGTGCCACC-3'
AtSnAK1-S260A	Midsense	5’-GGAGATTTCAGTGTCGCCCAAGTTTTCAAGGATG-3’
	Midantisense	5’-CATCCTTGAAAACTTGGGCGACACTGAAATCTCC-3’
AtSnAK1-S260D	Midsense	5’-GGAGATTTCAGTGTCGACCAAGTTTTCAAGGATG-3’
	Midantisense	5’-CATCCTTGAAAACTTGGTCGACACTGAAATCTCC-3’
AtSnAK1-S151A	Midsense	5’-GCTAAGGGTGGCACCGGCAGAGACTGCTATGAGTG-3’
	Midantisense	5’-CACTCATAGCAGTCTCTGCCGGTGCCACCCTTAGC-3’
AtSnAK1-S151D	Midsense	5’-GCTAAGGGTGGCACCGGACGAGACTGCTATGAGTG-3’
	Midantisense	5’-CACTCATAGCAGTCTCGTCCGGTGCCACCCTTAGC-3’
AtSnAK2	Forward	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGTTTTGTGATAGTTTTGCA-3’
	Reverse	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTTCAGCTATGGTTTTGATC-3’

GST-AtSnAK2_At3g45240, .VAPSET[1]AM[2]GDVLR., 2363

Protein : GST-AtSnAK2_At3g45240
Ms sample name : 2008_12_12_VIDAL_JEAN_338_2_44_AB2
Scan number : 2363
Peptide sequence : .VAPSET[1]AM[2]GDVLR.
Delta mass : 0.48
Charge : 2

modifications table

#	accession	name	diff mono
[1]	MOD:00696	phosphorylated residue	79.96
[2]	MOD:00719	oxidation to L-methionine sulfoxide	15.99



spectrum image

ion table

#	a	b	b++	b*	b*++	bO	bO++	Seq.[mods]	y	y++	y*	y*++	yO	yO++	#
1	72.08	100.08	50.54	83.05	42.03	82.07	41.54	V							13
2	143.12	171.12	86.06	154.09	77.55	153.11	77.06	A	1342.56	671.78	1325.53	663.27	1324.55	662.78	12
3	240.17	268.17	134.59	251.14	126.07	250.16	125.58	P	1271.52	636.26	1254.49	627.75	1253.51	627.26	11
4	327.2	355.2	178.1	338.17	169.59	337.19	169.1	S	1174.47	587.74	1157.44	579.22	1156.46	578.73	10
5	456.24	484.24	242.62	467.21	234.11	466.23	233.62	E	1087.44	544.22	1070.41	535.71	1069.43	535.22	9
6	637.25	665.25	333.13	648.22	324.61	647.24	324.12	T[1]	958.4	479.7	941.37	471.19	940.39	470.7	8
7	708.29	736.29	368.65	719.26	360.13	718.28	359.64	A	777.39	389.2	760.36	380.68	759.38	380.19	7
8	855.32	883.32	442.16	866.29	433.65	865.31	433.16	M[2]	706.35	353.68	689.32	345.16	688.34	344.67	6
9	912.34	940.34	470.67	923.31	462.16	922.33	461.67	G	559.32	280.16	542.29	271.65	541.31	271.16	5
10	1027.37	1055.37	528.19	1038.34	519.67	1037.36	519.18	D	502.3	251.65	485.27	243.14	484.29	242.65	4
11	1126.44	1154.44	577.72	1137.41	569.21	1136.43	568.72	V	387.27	194.14	370.24	185.62	369.26	185.13	3
12	1239.52	1267.52	634.26	1250.49	625.75	1249.51	625.26	L	288.2	144.6	271.17	136.09	270.19	135.6	2
13								R	175.12	88.06	158.09	79.55	157.11	79.06	1

GST-AKIN10_At3g01090, .T[1]SC[2]GSPNYAAPEVISGK., 2899

Protein : GST-AKIN10_At3g01090
Ms sample name : 2008_12_12_VIDAL_JEAN_338_2_69_AB1
Scan number : 2899
Peptide sequence : .T[1]SC[2]GSPNYAAPEVISGK.
Delta mass : 0.356
Charge : 2

modifications table

#	accession	name	diff mono
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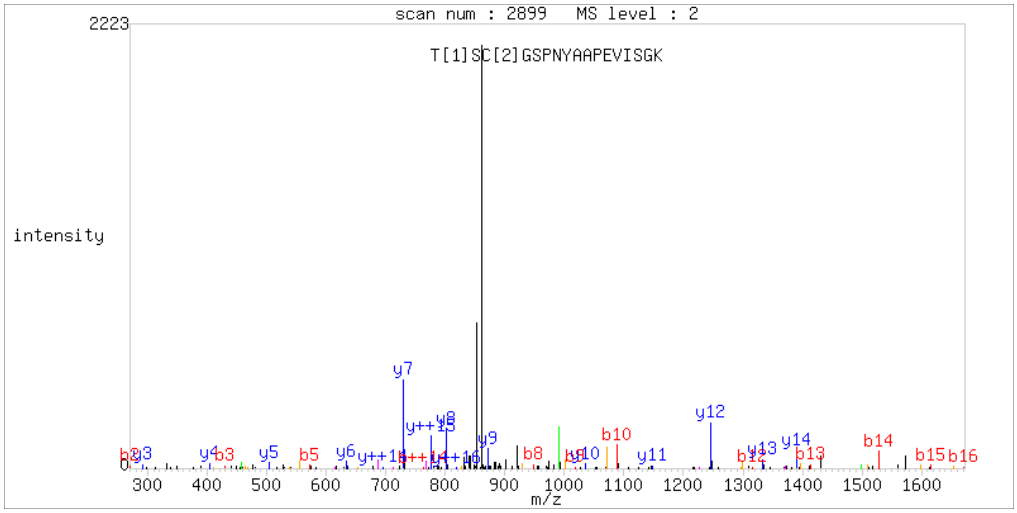
[2]	MOD:00397	iodoacetamide	57.04



spectrum image

ion table

#	a	b	b++	b*	b*++	bO	bO++	Seq.[mods]	y	y++	y*	y*++	yO	yO++	#
1	154.02	182.02	91.51	164.99	83	164.01	82.51	T[1]							17
2	241.05	269.05	135.03	252.02	126.51	251.04	126.02	S	1636.76	818.88	1619.73	810.37	1618.75	809.88	16
3	401.1	429.1	215.05	412.07	206.54	411.09	206.05	C[2]	1549.73	775.37	1532.7	766.85	1531.72	766.36	15
4	458.12	486.12	243.56	469.09	235.05	468.11	234.56	G	1389.68	695.34	1372.65	686.83	1371.67	686.34	14
5	545.15	573.15	287.08	556.12	278.56	555.14	278.07	S	1332.66	666.83	1315.63	658.32	1314.65	657.83	13
6	642.2	670.2	335.6	653.17	327.09	652.19	326.6	P	1245.63	623.32	1228.6	614.8	1227.62	614.31	12
7	756.24	784.24	392.62	767.21	384.11	766.23	383.62	N	1148.58	574.79	1131.55	566.28	1130.57	565.79	11
8	919.3	947.3	474.15	930.27	465.64	929.29	465.15	Y	1034.54	517.77	1017.51	509.26	1016.53	508.77	10
9	990.34	1018.34	509.67	1001.31	501.16	1000.33	500.67	A	871.48	436.24	854.45	427.73	853.47	427.24	9
10	1061.38	1089.38	545.19	1072.35	536.68	1071.37	536.19	A	800.44	400.72	783.41	392.21	782.43	391.72	8
11	1158.43	1186.43	593.72	1169.4	585.2	1168.42	584.71	P	729.4	365.2	712.37	356.69	711.39	356.2	7
12	1287.47	1315.47	658.24	1298.44	649.72	1297.46	649.23	E	632.35	316.68	615.32	308.16	614.34	307.67	6
13	1386.54	1414.54	707.77	1397.51	699.26	1396.53	698.77	V	503.31	252.16	486.28	243.64	485.3	243.15	5
14	1499.62	1527.62	764.31	1510.59	755.8	1509.61	755.31	I	404.24	202.62	387.21	194.11	386.23	193.62	4
15	1586.65	1614.65	807.83	1597.62	799.31	1596.64	798.82	S	291.16	146.08	274.13	137.57	273.15	137.08	3
16	1643.67	1671.67	836.34	1654.64	827.82	1653.66	827.33	G	204.13	102.57	187.1	94.05	186.12	93.56	2
17								K	147.11	74.06	130.08	65.54	129.1	65.05	1

GST-AtSnAK2_At3g45240, .IGDFSVS[1]QVFKDDDDQLR., 4510

Protein : GST-AtSnAK2_At3g45240
Ms sample name : 2008_12_12_VIDAL_JEAN_338_2_44_AB2
Scan number : 4510
Peptide sequence : .IGDFSVS[1]QVFKDDDDQLR.
Delta mass : 1.652
Charge : 3

modifications table

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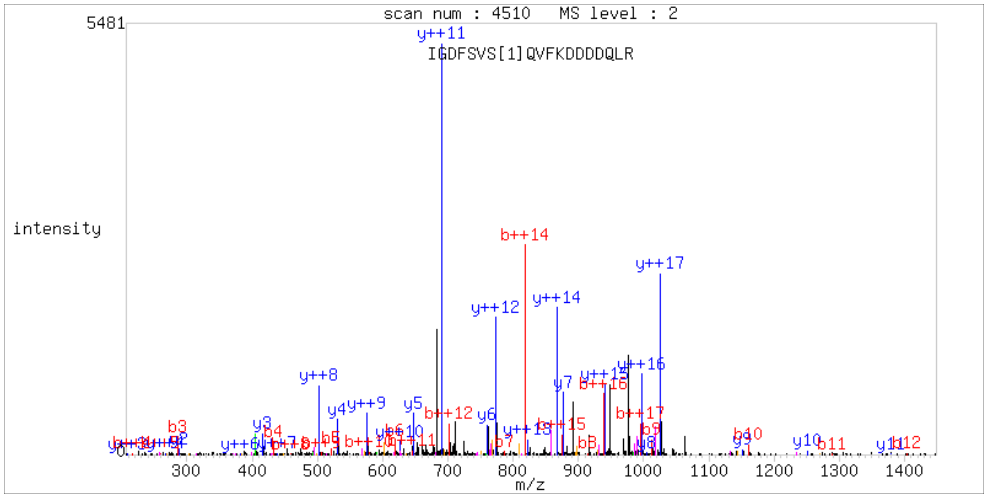
[1]	MOD:00696	phosphorylated residue	79.96

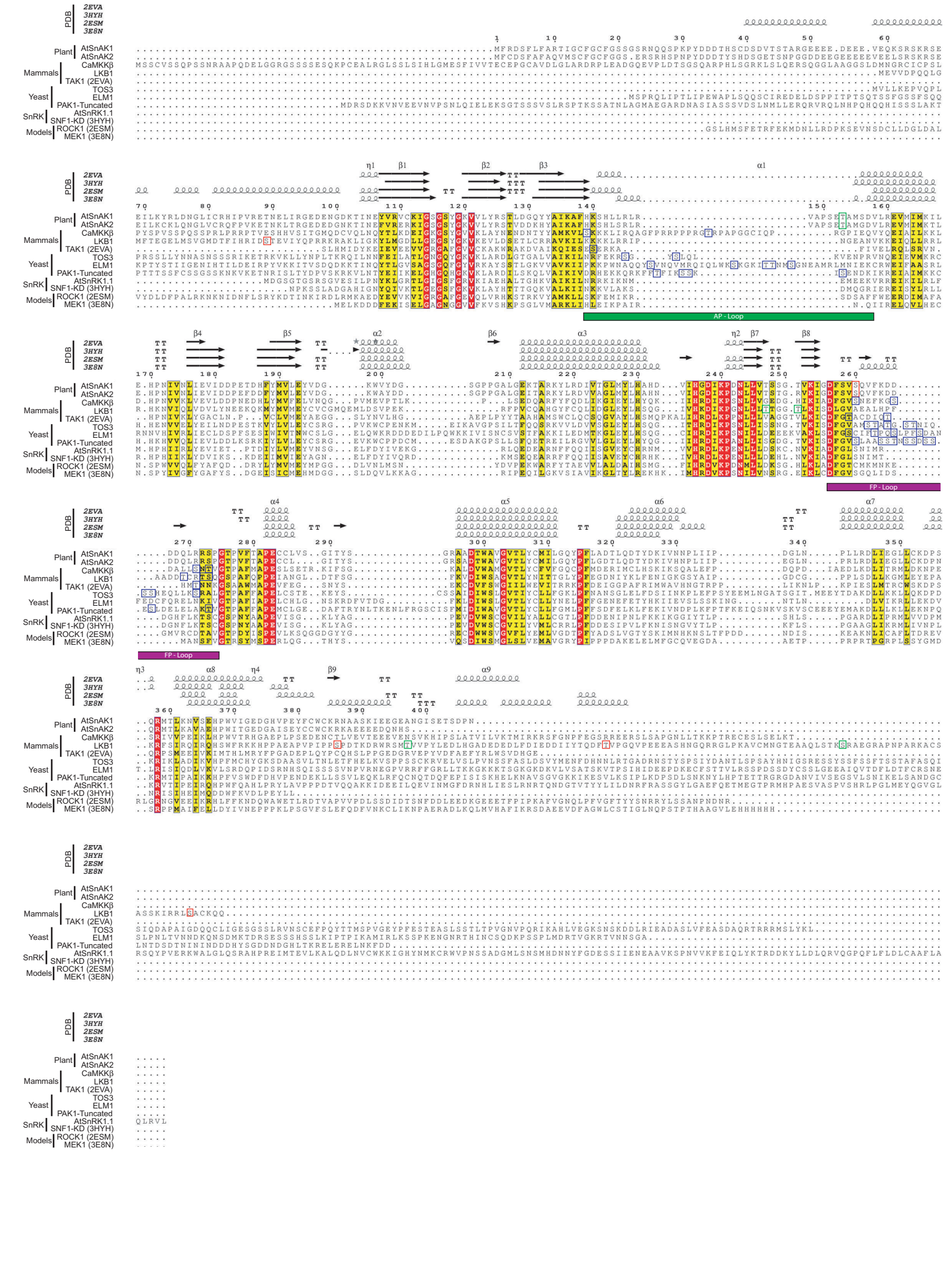


spectrum image

ion table

#	a	b	b++	b*	b*++	bO	bO++	Seq.[mods]	y	y++	y*	y*++	yO	yO++	#
1	86.09	114.09	57.55	97.06	49.03	96.08	48.54	I							18
2	143.11	171.11	86.06	154.08	77.54	153.1	77.05	G	2050.88	1025.94	2033.85	1017.43	2032.87	1016.94	17
3	258.14	286.14	143.57	269.11	135.06	268.13	134.57	D	1993.86	997.43	1976.83	988.92	1975.85	988.43	16
4	405.21	433.21	217.11	416.18	208.59	415.2	208.1	F	1878.83	939.92	1861.8	931.4	1860.82	930.91	15
5	492.24	520.24	260.62	503.21	252.11	502.23	251.62	S	1731.76	866.38	1714.73	857.87	1713.75	857.38	14
6	591.31	619.31	310.16	602.28	301.64	601.3	301.15	V	1644.73	822.87	1627.7	814.35	1626.72	813.86	13
7	758.3	786.3	393.65	769.27	385.14	768.29	384.65	S[1]	1545.66	773.33	1528.63	764.82	1527.65	764.33	12
8	886.36	914.36	457.68	897.33	449.17	896.35	448.68	Q	1378.67	689.84	1361.64	681.32	1360.66	680.83	11
9	985.43	1013.43	507.22	996.4	498.7	995.42	498.21	V	1250.61	625.81	1233.58	617.29	1232.6	616.8	10
10	1132.5	1160.5	580.75	1143.47	572.24	1142.49	571.75	F	1151.54	576.27	1134.51	567.76	1133.53	567.27	9
11	1260.59	1288.59	644.8	1271.56	636.28	1270.58	635.79	K	1004.47	502.74	987.44	494.22	986.46	493.73	8
12	1375.62	1403.62	702.31	1386.59	693.8	1385.61	693.31	D	876.38	438.69	859.35	430.18	858.37	429.69	7
13	1490.65	1518.65	759.83	1501.62	751.31	1500.64	750.82	D	761.35	381.18	744.32	372.66	743.34	372.17	6
14	1605.68	1633.68	817.34	1616.65	808.83	1615.67	808.34	D	646.32	323.66	629.29	315.15	628.31	314.66	5
15	1720.71	1748.71	874.86	1731.68	866.34	1730.7	865.85	D	531.29	266.15	514.26	257.63	513.28	257.14	4
16	1848.77	1876.77	938.89	1859.74	930.37	1858.76	929.88	Q	416.26	208.63	399.23	200.12	398.25	199.63	3
17	1961.85	1989.85	995.43	1972.82	986.91	1971.84	986.42	L	288.2	144.6	271.17	136.09	270.19	135.6	2
18								R	175.12	88.06	158.09	79.55	157.11	79.06	1

Figure S2.


PDB
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3HYH
2ESM
3E8N
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AtSnAK2
Mammals
CaMKKβ
LKB1
TAK1 (2EVA)
Yeast
TOS3
ELM1
PAK1-Tuncated
SnRK
AtSnAK1.1
SNF1-KD (3HYH)
Models
ROCK1 (2ESM)
MEK1 (3E8N)
AP - Loop
FP - Loop
FP - Loop