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Kinome profiling of sugar signaling in plants using multiple platforms

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Although the primary sequence of kinases shows substantial divergence between unrelated eukaryotes, variation in the motifs that are actually phosphorylated by eukaryotic kinases is much smaller. Hence arrays developed for kinome profiling of mammalian cells are useful for kinome profiling of plant tissues as well, facilitating the study of plant signal transduction. We recently employed the Pepscan kinomics chip to reveal the small GTPases in plant sucrose signaling. Here we show that employing a different peptide library (the Pepscan kinase chip) largely similar results are obtained, confirming these earlier data, but such a different library also contributes new insights into the molecular details mediating plant cell responses to a sugar stimulus. Thus when studying plant signal transduction employing peptide arrays, using multiple platforms both increases the confidence of results and provides additional information.

Key words: sucrose, arabidopsis, kinome profiling, kinase, MAPK, SnRK, casein kinase, tyrosine phosphorylation, CDC2, AGC kinase

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In our recently published paper[1] we analyze the changes in the plant kinome after sucrose feeding compared to control sorbitol feeding of Arabidopsis. We employed kinomics chips (Pepscan Presto, The Netherlands) containing 960 different kinase consensus peptides selected for their importance in mammalian signal transduction. In addition we used kinase chips (Pepscan Presto, The Netherlands), containing 1,152 peptides covering the majority of peptides available through Phosphobase (version 2.0). These chips contain peptides derived from phosphorylation events described in many kingdoms and are taken from animals, plants, fungi and even bacteria.[2,3] Full details as to the peptides spotted can be found at www.pepscanpresto.com. Results of the cluster analysis of three independent biological replicas of kinome profiling after treatment with water, sorbitol, sucrose and glucose are depicted in Figure 3A of our recent paper.[1] A further analysis of this set of experiments was not given, due to a lower than expected biological reproducibility. In contrast to the >0.8 correlation observed for the set described in our recent paper, correlations of below 0.5 were seen for the experimental set analyzed on the kinase1 chips. However, we feel that some observation made based on these kinome profiles contain valuable information, if only to suggest follow-up experiments.

Comparison of the kinome profiles of Arabidopsis treated with sucrose or sorbitol for 1 h revealed a set of 93 differentially phosphorylated consensus peptides, with the majority of 59 peptides showing reduced phosphorylation after sucrose treatment (**Table 1**).

Kinome Profiling

When the results obtained from the set of experiments analyzed on the Kinomics chip are compared to those of the Kinase1 chip similarities can be noted. Tyrosine kinase consensus peptides are consistently more phosphorylated after sucrose and phosphorylation levels of CDC2 consensus substrates is increasing. However, also differences are apparent; phosphorylation of Casein Kinase (CK) consensus peptides is consistently going down over this set of experiments, whereas in the set of experiments analyzed before in the Kinomics chips CK activity is going up. This might be due to a difference in experimental

Table 1. List of differentially phosphorylated consensus peptides

Consensus	Kinase	sor vs. suc t-test	P up/down
ESSYSYEEI		0.0003	down
PASPSPQRQ	Cdk5-p23	0.0014	down
PKRGSGKDG	AGC	0.0019	up
IREESPPHS		0.0019	down
RTPPPSG	MAPK	0.0020	down
PASQTPNKT	CDC2	0.0029	down
STNEYMDMK	PI3 kinase	0.0030	down
SEENSKKTV	CKI	0.0040	down
APTPGGRR		0.0043	down
RFTDTRKDE	CaM-III	0.0046	down
LSELSRRRI	ds-RNA	0.0062	up
PINGSPRTP	CDC2	0.0065	down
TEGQYELQP	Tyr-K	0.0068	up
KRAQISVRGL		0.0069	down
AKRISGKMA		0.0069	up
VVGGSLRGA	AGC	0.0071	down
KRPSNRAKA		0.0072	up
ERQKTQTKL	SnRK, MLCK	0.0073	down
EEGISQESS		0.0080	up
PVPEYINQS	EGFR (Tyr-K)	0.0082	down
FGHNTIDAV		0.0082	down
ARVFSVLRE	CaM-II	0.0085	down
SNDDSDDDD	CKII	0.0085	down
GGVDYKNIH	Tyr-K	0.0094	up
SRSRSRSRS		0.0103	up
SPSLSRHSS	GSK3	0.0107	down
RAKRSGSV		0.0120	down
RRASLG	AGC1/2	0.0128	down
GRASSHSSQ	S6K	0.0129	down
SGYISSLEY	CKII	0.0139	down
FFRRSKIAV	AGC	0.0140	up
STNDSPL	beta-ARK	0.0145	down
LRRASPG		0.0149	up
SAVASNMRD	GRK	0.0154	up
KRPSGRAKA		0.0160	up
KRSNSVDTS	AGC	0.0165	down
RQLRSPRRT	CDC2	0.0171	up
GRALSTRAQ	CDPK, PhK	0.0172	down
VSRTSAVPT	AGC	0.0173	down
TRKISQTAQ	AGC	0.0174	down
STTVSKTET		0.0180	down
ESPASDEAE		0.0184	up
LSYRGYSL	PhK	0.0185	down
DDINSYEAW		0.0186	up
PNVSYIASR		0.0191	down
KQPIYIVME	FES (Tyr-K)	0.0195	up
LVVASAGPT		0.0198	down

set-up. In these experiments plants were submerged during sugar incubation, whereas in the other set of experiments the sugar solution was added to the filter on which plants were grown in vitro. Many AGC-protein kinase consensus substrates are present on the Kinase1 chip. This family of kinases consists of many family members. It is reported that AGC2 phosphorylates several targets of which consensus sequences were described by Anthony et al.[4] According to the analysis described in this paper peptides RRRASVA and LRRLSTKYR should be considered AGC2 kinase consensus peptides. Both show reduced phosphorylation after sucrose. In addition, peptide RRASLG is the classical PKA substrate called kemptide that can also be used to measure AGC1/2 activity.[5] Its phosphorylation also goes down after sucrose. Judged from the many AGC consensus peptides that show increased phosphorylation levels, other members of the AGC kinase family are probably activated by sucrose.

Kinome Analysis Indicates MAPK Signaling

The enhanced phosphorylation of peptide TGFLTEYVA after sucrose treatment points to a role of a MAPK cascade in the signaling. Since MAPK pathways are identified in signaling of many compounds, ranging from salt, water, pathogens and hormones, involvement of MAPKs is sugar signaling is not surprising.[6] The TGFLTEYVA peptide contains the TEY signature, which includes the Thr (T) and Tyr (Y) residues phosphorylated in MAPKs.[7] Phosphorylation of these residues increases the activity of MAPKs. The TEY triplet is present in 12 of the 20 Arabidopsis MPKs, the others have TDY.[8] At the same time the peptide TGFLTEYVA is also a MAPKK consensus peptide, since it represents the Thr and Tyr phosphorylation sites in MAPK that are phosphorylated by the dual-specificity kinase MAPKK.[9] Since TGFLTEYVA shows higher phosphorylation upon sucrose feeding we presume that MAPKK activity, and with that MAPK activity, is increased by sucrose.

Also some MAPK consensus peptides can be found among the significantly

differentially phosphorylated peptides, APVASPAAP, SSSSSPKAE, RTPPPSG, are annotated as such. In contrast to our prediction, two out of three show lower phosphorylation after sucrose, this could be due to either differential activities of a subset of MAPKs, or phorphorylation of these substrates by other kinases.[10]

Based on these data it is difficult to access the importance of MAPK cascades in sucrose signaling, we performed another inhibitor assay using PD98059, an inhibitor of MAPKK. This inhibitor could repress sucrose induction of the fructosyltransferase promoter completely (**Fig. 1**), providing evidence that MAPK cascades are important in sucrose signaling. As MAPK cascades are the classical effectors of small GTPases, this observation would further support our hypothesis that such small GTPases are central orchestrators of the sugar response in plants.

Kinome Analysis Reveals SnRK Involvement in Sucrose and Glucose Signal-Transduction

SnRK is the plant homologue of AMPK; Peptide THVASVSDV is both AMPK and SnRK consensus peptide and its phosphorylation is decreased after sucrose feeding. We searched for additional SnRK consensus peptides on the PepChip by using the peptides identified by Kleinow et al.[11] and found the peptides VRKRTLRRL, PRRDSTEGF, ERQKTQTKL and DRLVSARSV to resemble SnRK consensus peptides. Phosphorylation of these peptides is lower upon sucrose addition compared to sorbitol (**Table 1**). The reduced phosphorylation of these peptides upon sugar feeding is in agreement with the negative role that SnRK and its yeast and mammalian homologous play in carbon signaling.

Both human AMPKα as well as Arabidopsis SnRK1.1 are able to complement a yeast SNF1 mutant.[12] Therefore it is proposed that all three kinases work in the same manner and react to similar activation signals, such as phosphorylation of a Thr residue in a conserved region of the protein. For that reason antibodies against the phosphorylated fragment SDGEFLRpTSCGSPNY of human AMPKα (Cell Signaling Technologies) were used to detect phosphorylated SnRK from Arabidopsis (**Fig. 2**). SnRK1.1, SnRK1.2 and SnRK1.3 harbor sequences which are highly homologous to the fragment used for antibody production, therefore the phosphorylated state of these kinases is most likely the detected by this antibody. Quantification of western blot analysis revealed that slightly less phosphorylated SnRK1 is present after sucrose and glucose compard to sorbitol (**Fig. 2**). In analogy to AMPK, less phosphorylation would mean lower SnRK activity, which is in agreement with the data we obtained from kinome profiling (**Table 1**). Although contrasting views are present,

Table 1. List of differentially phosphorylated consensus peptides

Consensus	Kinase	sor vs. suc t-test	P up/down
TGFLTEYVA	MAPKK	0.0198	up
TEDQYSLVE	Src	0.0212	up
SSSSSPKAE	MAPK	0.0213	up
EKAKSPVPK		0.0221	down
RRRASVA	AGC1/2	0.0221	down
APVASPAAP	MAPK	0.0225	down
LRRLSTKYR	AGC1/2	0.0234	down
EKHHSIDAQ		0.0256	down
VRKRTLRRL	SnRK, AGC	0.0266	down
DLPGTEDFV	GRK2	0.0277	down
LSEHSSPEE	CKII	0.0278	down
KREASLDNQ	AGC	0.0279	down
TKKQSFKQT	AGC	0.0280	up
VRLRSSVPG	autoP	0.0285	down
KRPSLRAKA		0.0293	up
PGPQSPGSP		0.0308	down
YSGHSMSDP		0.0309	up
ADGVYAASG	FES (Tyr-K)	0.0311	up
ENQASEEED	CKII	0.0317	down
TLASSFKRR	AGC	0.0324	up
TVKSSKGGP	AGC	0.0326	down
GVLRRASVA		0.0327	up
SPRKSPRKS	sperm-specific	0.0328	down
PRRDSTEGF	SnRK, AGC	0.0332	down
RRRRAASVA		0.0346	down
SRKDSLDDS	GRK	0.0371	down
ENPEYLGLD	Tyr-K	0.0380	down
KAKTTKKRP		0.0382	up
RRPSV		0.0392	down
QKAQTERKS	AGC	0.0401	down
AKAKTTKKR		0.0404	up
GSDVSFNEE	CKII	0.0409	down
DEPSTPYHS	GSK3	0.0409	down
SSRPSSNRS	CDPK, AGC	0.0411	up
GGRASDYKS	AGC	0.0413	up
YMAPYDNYV	Tyr-K	0.0420	up
LELSDDDD	CKII	0.0422	down
THVASVSDV	SnRK AMPK	0.0423	down
SMANSFVGT	PDK1	0.0427	down

Table 1. List of differentially phosphorylated consensus peptides

DLLTSPDVG	CDC2	0.0441	down
RGKSSSYSK	AGC	0.0441	up
SSSNTIRRP	AGC	0.0453	up
RRDSV		0.0457	down
TKAASEKKS		0.0469	up
DRLVSARSV	CDPK, SnRK, AGC	0.0480	down
RLSISTESQ	AMPK	0.0489	up

Arabidopsis seedlings were incubated in a solution of 100 mM sucrose or sorbitol for 1 hour after which extracts were made as described before (Plos One) and incubated on Kinase1 PepChips (1152 consensus peptides spotted twice per slide; Pepscan). The averaged phosphorylation intensities obtained from three independent experiments were analyzed using a Student's t-test. Indicated is whether phosphorylation of consensus peptides is higher (up) or lower (down) after sucrose treatment, compared to sorbitol. Kinase annotation according to Pepscan Presto (www.pepscanpresto.com), or our own analysis (see main text).

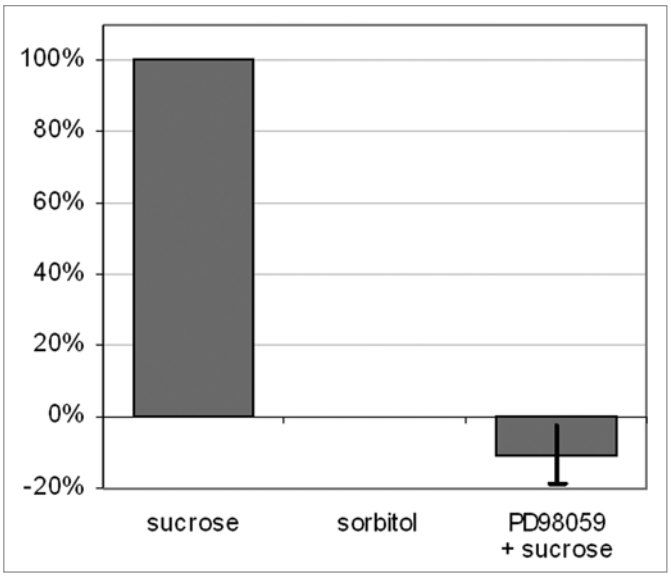

Figure 1. Role of MAP kinases in sucrose signaling. Activity of the MAPKK inhibitor PD98059 in sucrose-induced GUS activity as quantified using MUG as a substrate.

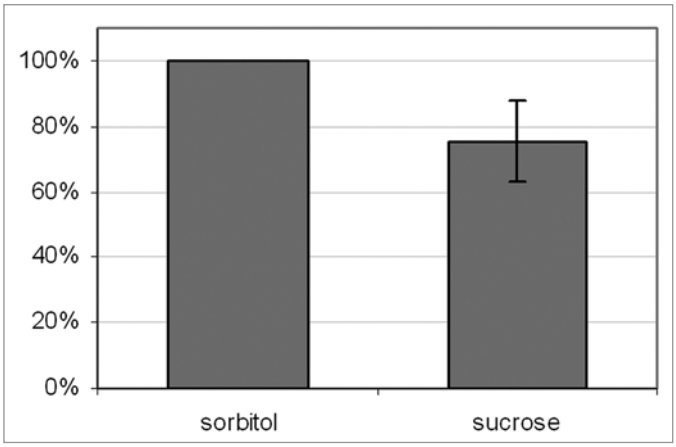

Figure 2. Role of AMPK homologues in sucrose signaling. Western blot analysis of putative SnRK phosphorylation, see main text for explanation on the antibody used.

inactivation of Arabidopsis SnRK1 upon sucrose was reported before.[13]

Conclusions

In view of limited tools available with respect to signal transduction research in plants, the advent of kinome profiling using peptide arrays seems useful, despite the absence of dedicated plant platforms. Nevertheless, it seems that changes of finding interesting phenomena increase by using multiple peptide libraries. With respect to sugar signaling it can be said that disregarding the exact platform used, data are still best explained by postulating a central role for small GTPases in the regulation of the molecular events evoked by a sucrose stimulus. As such GTPases are especially important in the regulation of cytoskeletal events,[13] it should prove interesting to investigate actin reorganization in response to sugar stimulus as well.

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