



Calcium-dependent protein kinases from *Arabidopsis* show substrate specificity differences in an analysis of 103 substrates

Amy Curran^{1†}, Ing-Feng Chang^{1,2†}, Chia-Lun Chang², Shilpi Garg¹, Rodriguez Milla Miguel^{1,3}, Yoshimi D. Barron¹, Ying Li¹, Shawn Romanowsky¹, John C. Cushman¹, Michael Gribskov⁴, Alice C. Harmon⁵ and Jeffrey F. Harper^{1*}

¹ Department of Biochemistry and Molecular Biology, University of Nevada, Reno, NV, USA

² Institute of Plant Biology, National Taiwan University, Taipei, Taiwan

³ Departamento de Biología de Plantas, Centro de Investigaciones Biológicas, Madrid, Spain

⁴ Department of Biological Sciences, Purdue University, West Lafayette, IN, USA

⁵ Department of Biology, University of Florida, Gainesville, FL, USA

Edited by:

Rodrigo Gutierrez, Pontificia Universidad Católica de Chile, Chile

Reviewed by:

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Ian Wallace, University of California, USA

*Correspondence:

Jeffrey F. Harper, Biochemistry Department, University of Nevada, Reno MS330, Howard Building, Reno, NV 89557, USA.

e-mail: jfharper@unr.edu

[†]Amy Curran and Ing-Feng Chang have contributed equally to this work.

The identification of substrates represents a critical challenge for understanding any protein kinase-based signal transduction pathway. In *Arabidopsis*, there are more than 1000 different protein kinases, 34 of which belong to a family of Ca²⁺-dependent protein kinases (CPKs). While CPKs are implicated in regulating diverse aspects of plant biology, from ion transport to transcription, relatively little is known about isoform-specific differences in substrate specificity, or the number of phosphorylation targets. Here, *in vitro* kinase assays were used to compare phosphorylation targets of four CPKs from *Arabidopsis* (CPK1, 10, 16, and 34). Significant differences in substrate specificity for each kinase were revealed by assays using 103 different substrates. For example CPK16 phosphorylated Serine 109 in a peptide from the stress-regulated protein, Di19-2 with $K_M \sim 70 \mu\text{M}$, but this site was not phosphorylated significantly by CPKs 1, 10, or 34. In contrast, CPKs 1, 10, and 34 phosphorylated 93 other peptide substrates not recognized by CPK16. Examples of substrate specificity differences among all four CPKs were verified by kinetic analyses. To test the correlation between *in vivo* phosphorylation events and *in vitro* kinase activities, assays were performed with 274 synthetic peptides that contained phosphorylation sites previously mapped in proteins isolated from plants (*in vivo*-mapped sites). Of these, 74 (27%) were found to be phosphorylated by at least one of the four CPKs tested. This 27% success rate validates a robust strategy for linking the activities of specific kinases, such as CPKs, to the thousands of *in planta* phosphorylation sites that are being uncovered by emerging technologies.

Keywords: calcium-dependent protein kinase, phosphorylation, substrate specificity, phospho-proteomics

INTRODUCTION

Ca²⁺ is an important second messenger in plant cells, and has been linked to the perception of and response to biotic and abiotic stimuli, as well as regulation of many aspects of growth and development (Lecourieux et al., 2006; Ma and Berkowitz, 2007; Kosuta et al., 2008; Song et al., 2008; Bertorello and Zhu, 2009; Cardenas, 2009; Chehab et al., 2009; Kim et al., 2009; Luan et al., 2009; Mazars et al., 2009; McAinsh and Pittman, 2009; Dodd et al., 2010; Hey et al., 2010). In *Arabidopsis*, Ca²⁺ signals are potentially decoded by 64 different protein kinases belonging to the CPK/SnRK superfamily (Ca²⁺-dependent protein kinases/sucrose non-fermenting related kinases; Hrabak et al., 2003; Harper et al., 2004). Of these, 34 are CPKs and 8 are CRKs (CPK-related kinases). CPKs in flowering plants can be further divided into 12 distinct subfamilies that show conservation between rice and *Arabidopsis*. The CPKs have a unique structure in which a calmodulin-like regulatory domain is present at the C-terminal end of the kinase (Harper et al., 1991; Harper

and Harmon, 2005; Wernimont et al., 2010). CRKs differ in having a degenerate calmodulin-like domain containing substitutions expected to disrupt the Ca²⁺ binding EF hands (Harper et al., 2004). Nevertheless, at least some CRKs can be activated by Ca²⁺ through a bimolecular interaction with calmodulin (Jeong et al., 2007).

Although CPKs have not been found in animals, they appear to be widespread in flowering plants, algae, and certain apicomplexan protists (Harper and Harmon, 2005). In plants, CPKs and related kinases have been implicated in multiple aspects of growth and development (Klimecka and Muszynska, 2007), for example, pollen tube growth (Yoon et al., 2006; Myers et al., 2009); root development (Ivashuta et al., 2005); defense responses (Ludwig et al., 2005; Freymark et al., 2007; Arimura and Maffei, 2010; Boudsocq et al., 2010; Coca and San Segundo, 2010; Kanchiswamy et al., 2010); regulation of reactive oxygen species (ROS) production (Kobayashi et al., 2007); symbiotic interactions (Gargantini et al., 2006; Gleason et al., 2006; Oldroyd and Downie, 2006; Tirichine et al., 2006; Marsh et al., 2007; Tsai et al., 2007; Yano et al., 2008; Hayashi et al., 2010); guard cell turgor (Mori et al., 2006; Geiger et al., 2010; Xu et al., 2010; Zou et al., 2010; Munemasa et al., 2011); osmotic, drought, and salt stress

Abbreviations: CaM, calmodulin; CPK, calcium-dependent protein kinase; ps, plasmid stock number; ss, seed stock number.

(Ma and Wu, 2007; Zhu et al., 2007; Mehlmer et al., 2010; Witte et al., 2010; Asano et al., 2011; Franz et al., 2011); and regulation through hormones such as ABA (Choi et al., 2005) and GA (McCubbin et al., 2004; Yuan et al., 2007; Ishida et al., 2008). In apicomplexans that cause malaria and toxoplasmosis, CPKs are essential for development, making them attractive drug targets (Kato et al., 2008; Billker et al., 2009; Dvorin et al., 2010; Lourido et al., 2010).

Despite being implicated in so many diverse aspects of plant and protozoan biology, relatively little is known about the downstream substrates regulated by CPKs. CPKs are expected to be multifunctional, with many target substrates. This speculation is supported by analogy to CaM-dependent kinases in animals (CaMKs), as well as an expanding list of candidate substrates tested by *in vitro* kinase reactions (e.g., Harper et al., 2004; Klimecka and Muszynska, 2007; Kanchiswamy et al., 2010).

While most candidate substrates for CPKs have been identified through *in vitro* kinase reactions, there are several for which there is evidence of *in vivo* phosphorylation or a kinase-substrate interaction, including the following. A tobacco CDPK1 has been implicated in regulating the phospho-dependent binding between a 14-3-3 protein and a transcription factor (RSG) involved in mediating GA signaling (Ishida et al., 2008; Ito et al., 2010). Isoforms AtCPK21 and 23 have been implicated in binding and activating a guard cell anion channel SLAC1 (Geiger et al., 2010). For AtCPK32, evidence supports an interaction with an ABA-induced transcription factor (ABF4) that can alter ABA-triggered responses (Choi et al., 2005). A soybean CPK has been linked to the regulation of a serine acetyltransferase in response to oxidative stress (Liu et al., 2006). Nitrate reductase (NR) activity can be regulated by CPK phosphorylation, which promotes a phospho-dependent binding interaction with 14-3-3 proteins (Bachmann et al., 1996; Douglas et al., 1997, 1998; Lambeck et al., 2010).

Multiple strategies have been used to help identify substrates for CPKs and other kinases in plants (Peck, 2006; de la Fuente van Bentem et al., 2008; Kersten et al., 2009; Uno et al., 2009; Huang et al., 2010; Mehlmer et al., 2010). While a “one protein at a time” strategy is still relevant, proteome-wide surveys employing mass spectrometry have rapidly expanded the list of mapped phosphorylation sites in plant proteomes since 2003 (Nuhse et al., 2003). An important aspect of these surveys is their potential to identify phosphorylation sites occurring *in vivo* (*in vivo*-mapped sites). However, correlating these data with the specific kinases responsible for each phosphorylation event remains a challenge. To this end, custom-designed peptide libraries provide a strategy to rapidly test large numbers of *in vivo*-mapped phosphorylation sites for their potential to be phosphorylated by a specific kinase.

Here we present results from three complementary strategies used to help identify CPK substrates. The first approach was a survey of candidate proteins using *in vitro* kinase reactions, followed by phosphorylation site mapping using mass spectrometry. The second approach was to test 274 *in vivo*-mapped phosphorylation sites (Peck, 2006; de la Fuente van Bentem et al., 2008; Kersten et al., 2009; Uno et al., 2009; Huang et al., 2010; Mehlmer et al., 2010) for their potential to be phosphorylated *in vitro* by a particular CPK. The third approach was to test sites that were predicted based on consensus phosphorylation site information. For example, 206 of the predicted sites tested here were derived from proteins with

evidence for a 14-3-3 interaction (Chang et al., 2009). Potential 14-3-3 binding sites were considered logical candidates for CPK substrates because a consensus site proposed for CPK phosphorylation overlaps with many phospho-sites implicated in 14-3-3 binding interactions (Johnson et al., 2010). Because most 14-3-3 interactions are thought to be mediated by phosphorylation, this focus was postulated to enrich for potential CPK sites of biological relevance.

Using peptides derived from both *in vivo* mapping and bioinformatic prediction strategies, we identified 89 new peptide substrates that were recognized by at least one of four CPKs tested (isoforms 1, 10, 16, and 34). These new peptide substrates, in addition to 13 reference peptides, revealed significant differences in substrate specificity for each of the four CPKs. Isoform CPK16 recognized the fewest substrates (nine), whereas CPK34 recognized the most (97 total, 31 of which were isoform-specific). This observed diversity of substrate specificity helps explain the origin of more than seven different CPK consensus sites that have been proposed from different studies based on different isoforms (Harper et al., 2004). This survey also supports a model for CPK signaling networks in which substrate specificity contributes to the isoform-specific functions of different CPKs.

RESULTS

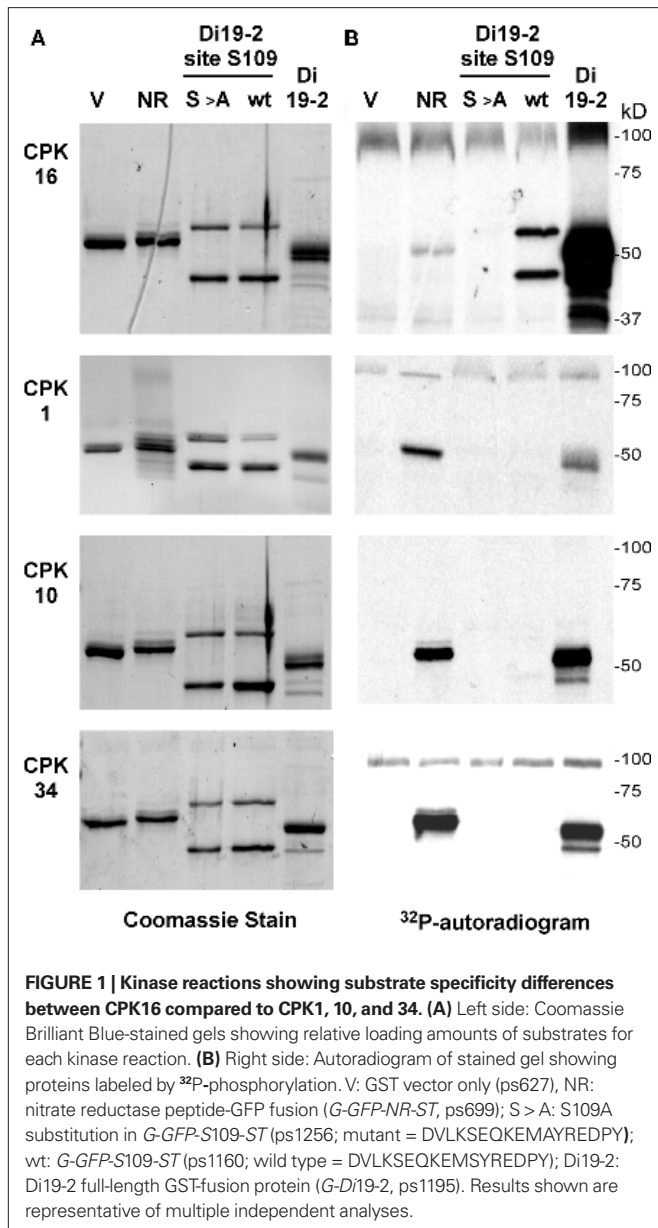
DI19-2 IS A GOOD SUBSTRATE FOR CPK16, BUT NOT CPK1, 10, AND 34

To search for potential CPK16 substrates, we initiated a survey of protein and peptide substrates using *in vitro* kinase assays. One group of proteins tested included members of a family of drought-induced proteins (Di19s; Milla et al., 2006; Rodriguez Milla et al., 2006; data not shown). Some Di19s had previously been found to interact with CPKs in a yeast two-hybrid screen (Rodriguez Milla et al., 2006). Isoform Di19-2 was chosen for further analysis because it was strongly phosphorylated by CPK16 (**Figure 1**). In contrast, assays of Di19-2 with three other CPKs (1, 10, and 34) showed more than 10-fold lower levels of phospho-labeling.

All four CPKs were further tested in parallel reactions with a fusion protein containing a known CPK phosphorylation site found in NR. In this case, CPK16 showed weaker substrate labeling compared with the other three CPKs. In contrast, the phosphorylation of substrate Di19-2 site S109 was more than 10-fold stronger for CPK16 than for the other three kinases (in **Figure 1**, compare lane NR with Di19-2 site S109 wt; discussed in more detail below). This inverse relationship of relative labeling efficiencies indicated that CPK16 had a distinct substrate preference compared to CPKs 1, 10, and 34.

To identify CPK16 phosphorylation sites in Di19-2, a fusion protein was phosphorylated, digested by trypsin or Glu-C, and analyzed by mass spectrometry. Residue S109 was identified as a phosphorylation site (data not shown). This site was subsequently confirmed using a fusion protein substrate that displayed the region from D99 to Y115 (described below). CPK16 also phosphorylated additional sites in Di19-2, as indicated by the observation that phosphorylation was still detected in a full-length Di19-2 fusion protein in which the S109 site was substituted with an alanine (S109A, data not shown).

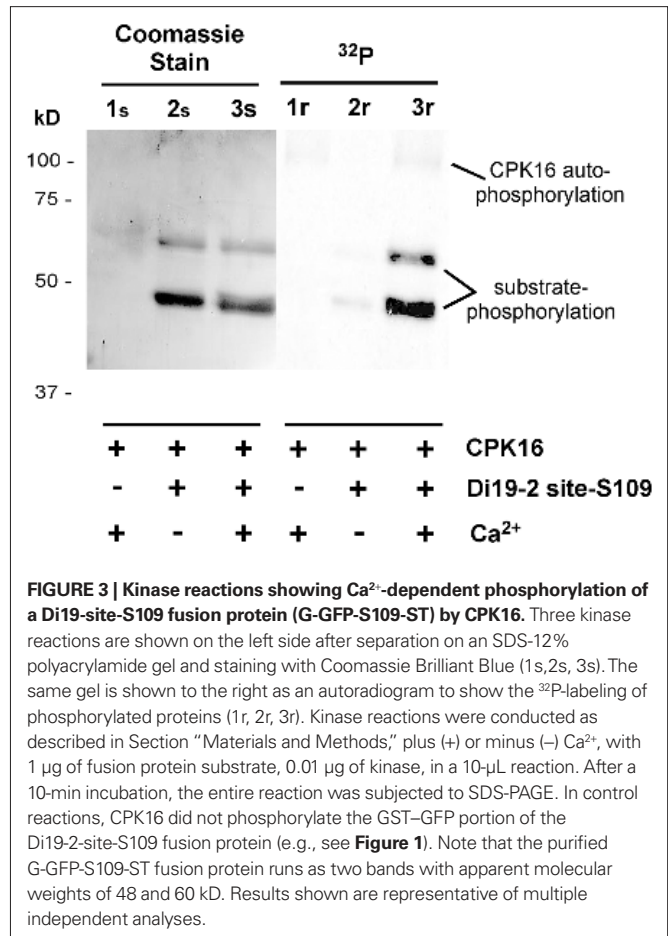
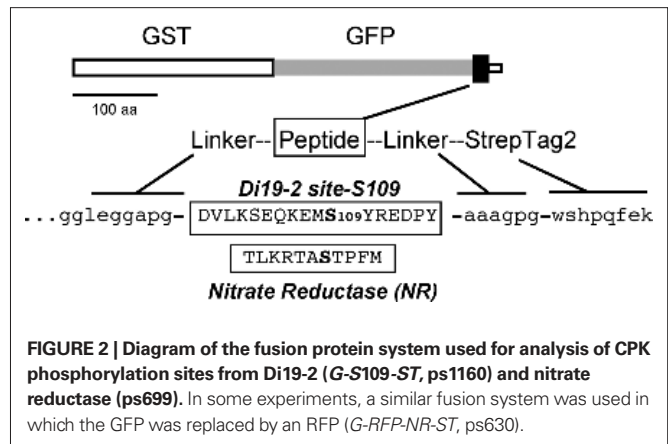
To conduct a more detailed analysis on the S109 site, a fusion protein (G-S109-ST2) was engineered that displayed a fragment from D99 to Y115 (DVLKS₁₀₃EQKEMS₁₀₉YREDPY), flanked



by two purification tags – an upstream GST–GFP (Glutathione S-Transferase–Green Fluorescence Protein), and a downstream Strep-2 tag (Figure 2). For comparison, a parallel fusion protein was created with an established CPK phosphorylation site from NR (Figure 2, NR sequence).

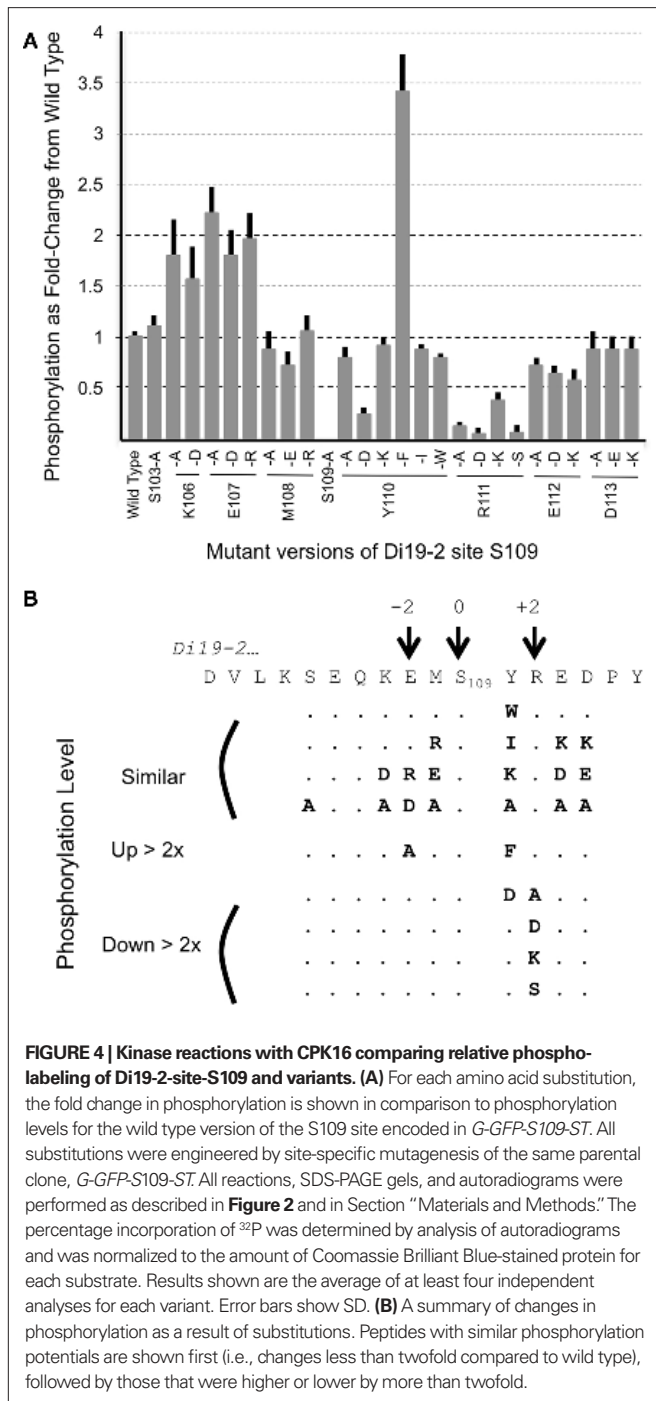
As expected for a CPK substrate, phosphorylation of the G-S109-ST2 fusion protein was stimulated by Ca^{2+} (Figure 3). This fusion protein substrate was consistently purified as mixture of two proteins (approximately 60 and 30 kD; Figure 3). The larger protein migrated at the predicted size for the engineered GST–GFP-peptide fusion. The smaller fragment was assumed to be a product of proteolysis. As shown below, both fragments were found to have a single CPK16 phosphorylation site.

To confirm that CPK16 phosphorylates S109 (and not nearby S103), the fusion protein was re-engineered with two separate substitutions and tested for phosphorylation by CPK16. First, a substitution



of S109 to A (S > A) was shown to eliminate any detectable labeling, consistent with S109 serving as the target phosphorylation site (Figure 4, and see Figure 1 to compare lane S > A to wt). Second, a substitution of S103 to A (a potential site upstream of S109) failed to result in any decrease in phosphorylation, confirming that S103 was not needed for substrate labeling. These results confirmed that fusion protein G-S109-ST2 has a single CPK16 phosphorylation site at S109.

To further define the residues surrounding S109 that were important for CPK16 phosphorylation, 26 additional site-specific substitutions were introduced and tested for their effect on



phosphorylation (Figure 4). Two positions appeared very sensitive to changes. At position +1 (Y110), a more than two-fold decrease in phosphorylation was observed by the substitution of a Y to D (acidic), but not a Y to K (basic). This indicates that a negative charge in this location is detrimental. However, a Y to F substitution showed a greater than three-fold improvement in phosphorylation. Because alternative hydrophobic substitutions of W or I failed to show similar improvements, the Y to F substitutions identifies a specific hydrophobic residue that is highly preferred at this location. At position +2 (R111; Figure 4), a more than two-fold decrease

in phosphorylation was observed with all four substitutions. This included a conservative substitution of R to K. In total, the analysis of these 26 substitutions supports a classification of site S109 as a specific variation of a CPK "simple-2" consensus site (Harper and Harmon, 2005), which was defined as (S/T) × (Basic). The variation here can be described as (S/T; F preferred, D detrimental; R preferred over K).

The G-S109-ST2 fusion protein was further tested for phosphorylation by three other CPKs (isoforms 1, 10, and 34; Figure 1, lane Di19-2 site S109 wt). All three failed to provide significant phosphorylation. In side-by-side controls, all three CPKs were confirmed to be as active as CPK16, as indicated by their relatively stronger phosphorylation of a fusion protein harboring a site from NR (see lane NR in Figure 1). The failure of CPK1, 10, and 34 to phosphorylate S109 is consistent with the original observation that the full-length Di19-2 was a poor substrate for these three CPK isoforms.

The K_M for the phosphorylation of S109 by CPK16 was determined to be approximately 70 μ M, when assayed in the context of the G-S109-ST2 fusion shown in Figure 2. Surprisingly however, no significant phosphorylation was obtained using a synthetic 15-amino acid peptide containing the S109 site at the center of the peptide. It is not clear if the synthetic peptide failed because of issues with secondary structure, solubility, or the inclusion of a norleucine (NorLeu) substitution at residue M108 (NorLeu was used in synthetic peptides to replace methionines with a non-oxidizable side chain).

PEPTIDE SUBSTRATE SURVEY

To conduct a more extensive survey for potential CPK substrates, we used four CPKs and compared their relative ability to phosphorylate 713 peptides, of which 179 represented experimental variations from the core list of 534 peptides (Table S1 in Supplementary Material). This library of synthetic peptides was generated based on five criteria: (1) A subset of 274 peptides were based on *in vivo* phosphorylation sites mapped in proteins isolated from plant extracts (Nuhse et al., 2003, 2004, 2007; Peck, 2006; Benschop et al., 2007; and Harper, unpublished); (2) 206 peptides were based on predicted 14-3-3 phospho-dependent binding sites in proteins for which evidence had previously been obtained for an *in vivo* interaction with a 14-3-3 (Chang et al., 2009); (3) 14 peptides were based on mapped sites from *in vitro* phosphorylation reactions with CPKs; (4) 15 peptides were designed based on bioinformatic predictions of potential CPK consensus phosphorylation sites showing strong conservation between rice and *Arabidopsis* (Harper, unpublished); and (5) the remainder were reference peptides corresponding to phosphorylation sites established for a variety of plant and animal kinases.

The relative phospho-labeling of peptides was compared for each CPK using a scintillation proximity assay (SPA). Of the 534 peptides tested, 102 ("hits") were found to show significant labeling by at least one of the four CPKs tested (Table 1). Of these, 13 were reference peptides, 74 peptides contained phosphorylation sites with evidence for *in vivo* phosphorylation, and the remaining 15 were predicted sites.

Note that the SPA assays were used here as a survey tool. As it was not practical to conduct the SPA assays under Michaelis-Menten conditions, these assays do not represent a rigorous test of

Table 1 | One hundred two peptides phosphorylated by at least one CPK in this study.

Peptide	Peptide sequence	AGI#	CPK1	CPK10	CPK16	CPK34	Description
REFERENCE PEPTIDES							
ach-393	PLARTLSVAGLPGKK	n/a	6	14	–	29	Syntide-2 (PMID-3028265)
ach-400	KRFSFKKSFKLSGFSFKK	n/a	10	8	4	28	MARCKS (PMID-2473066)
ach-375 ^g	PIKLDKM ^g PSFTM ^g DH	n/a	7	–	–	24	GmSAT site S367 (PMID-16854983)
ach-376	PIKLDKMPSFTMDH	n/a	5	–	–	–	GmSAT site S367 (PMID-16854983)
ach-385	RPFWARTESGIFRTV	AT2G46700	5	–	–	22	CRK3-site S352 (PMID-16758442)
ach-387	PGGNLQRQGSLLTLP	AT3G19290	4	8	–	21	ABF4 (PMID-16299177)
ach-407	KRREILSRRPSYR	n/a	5	5	–	19	Crebtide (PMID-7798217)
ach-388	RMRRGV ^s *CHGR	n/a	4	8	–	19	SPS, Suc P Syn, site S424 (PMID-9232876)
ach-402	KKKVSRSGLYRSPSPENL	n/a	–	–	–	13	CHK1/2 (PMID-9278511)
jfh-225	GAPTLKRTASTPFMA	n/a	7	5	–	13	Nitrate Reductase (based on PMID-8721752)
ach-405	AKRPQRATSNVFS	n/a	–	–	–	13	MLCK-Sk (PMID-2873140)
ach-401	RRRLSSLRA	n/a	–	–	–	9	S6 subunit 40S ribosome (PMID-6479343)
ach-406	KKRAARATSNVFA	n/a	–	–	–	9	MLCK-SM (PMID-3838312)
PEPTIDES BASED ON <i>IN VIVO</i> MAPPED SITES							
jfh-466	VNVYRRSV[S]*FGGIYN	AT2G01980	17	13	–	38	SOS1 Na ⁺ /H ⁺ antiporter
jfh-752 ^{sh}	LRELTKSA(S)*FLRAVS	AT5G37810	28	7	–	35	Aquaporin NIP4.1, pollen paralog of jfh-541
jfh-541	LREITKSG[S]*FLKTVR	AT4G19030	8	–	–	18	Aquaporin NIP1.1, compare to jfh-752
jfh-444	LAKIKKTQ[S]*QIFKYA	AT1G53390	9	–	–	32	ABC transporter
jfh-738 ⁿ	QPLLQRSL(S)*SPSPRA	AT3G11690	23	27	–	31	ABC transporter, pollen paralog of jfh-529
jfh-529 ^a	SPITQRSI[S]IESPRQ	AT3G62700	–	–	–	12	ABC transporter MRP10, compare to jfh-738
jfh-515 ^a	VKIISRFI[S]QRSFRR	AT3G27390	20	24	10	31	Expressed protein
jfh-729	RIKLLKFG(S)PS*ARLK	AT5G05350	11	9	–	31	SAG20-related, pollen paralog of jfh-504
jfh-504	RFQLLKFG(S)*PSAKFK	AT3G10980	15	7	–	26	SAG20, Senescence-associated, compare to jfh-729
jfh-590	NRTLTRNR[S]*LVDVQR	AT5G58350	22	33	–	30	WNK-4 protein kinase
jfh-453	RQLCLRAD[S]*FKGISD	AT1G71710	18	6	–	30	Inositol polyphosphate 5-phosphatase
jfh-527	RPFWARSE[S]GIFRAV	AT3G56760	8	4	–	29	CRK7, CPK-related kinase
jfh-456	RATLKRVS[S]*FEALQP	AT1G74910	11	–	–	29	ADP-glucose pyrophosphorylase
jfh-496	DWKLNRNT[S]LLVLP	AT3G05200	14	7	14	27	ATL6, zinc finger protein
jfh-475	QKALARVK(S)*MVQYPE	AT2G22300	16	5	–	26	CaM-binding protein, AtSR1, compare to jfh-665
jfh-665 ^h	QKALTRVK(S)*MVQYPE	AT5G09410	22	23	–	19	CaM-binding protein, pollen paralog of jfh-475
jfh-477	ENAFSRST[S]*FKDEIE	AT2G26910	13	9	–	26	PDR-4 ABC transporter
jfh-412	KRKVLRLN[S]*DLQKL	AT1G03080	16	–	–	26	Kinase interacting protein
jfh-439	RPFWARTE[S]GIFRAV	AT2G41140	6	–	–	26	CRK1, CPK-related kinase
jfh-531	RRVLERLD[S]*DLQKLE	AT4G02710	7	–	–	25	Kinase Interacting Protein
jfh-520	RPFYGRTE[S]AIFRCV	AT3G49370	8	4	–	24	CRK6, CPK-related kinase
jfh-452	QLLREI[S]*RRGLVANA	AT1G64100	8	12	–	23	Pentatricopeptide (PPR) repeat- protein
jfh-548	FGMPKRSR[S]*FGDVNE	AT4G32285	7	–	–	23	Clathrin assembly protein-related
jfh-495	DRWLFKRTPI[S]FLWRS	AT3G05200	16	–	10	22	ATL6, zinc finger protein
jfh-512	DYRFVRSPI[S]*LSLASL	AT3G25500	9	16	–	22	Formin-like protein AHF1
jfh-542	FGTRLSRSI[S]*EANIA	AT4G23640	6	5	–	22	TRH1, K transporter
jfh-494	DRWAFLRNA[S]*FLWRN	AT3G05200	11	6	–	21	ATL6, zinc finger protein
jfh-532	QGVTRVN[S]*LVQLPRF	AT4G03415	–	8	5	20	PP2C, protein phosphatase
jfh-579 ^p	VRRFDRTS[S]*AAIHAL	AT5G47910	6	8	–	20	RBOH-D NADPH oxidase
jfh-533	RSSLSRAS[S]*SVSTLY	AT4G03550	14	5	–	20	Glucan synthase component
jfh-497	VELMRRVV[S]*FQNPRV	AT3G06450	7	–	–	20	Anion exchanger
jfh-550	HVSRLARAS[S]*TEVRY	AT4G35060	–	–	–	20	Unknown
jfh-505	RTLDRLI[S]*TGESERF	AT3G11820	–	–	–	20	Syntaxin
jfh-454	DVKHLARSC[S]SDSVR	AT1G73080	6	4	–	19	PEPR1, LRR receptor kinase
jfh-425 ^c	KHSLKRKV[S]INPSFIA	AT1G19870	4	4	4	18	CaM-binding protein
jfh-414	SPRLVKSL[S]ASSFLI	AT1G07110	8	4	–	18	Fructose-6-phosphate 2-kinase

(Continued)

Table 1 | Continued

Peptide	Peptide sequence	AGI#	CPK1	CPK10	CPK16	CPK34	Description
jfh-524	EGNVKRAN[S]*LVRSQA	AT3G52400	–	–	–	18	Syntaxin
jfh-435	ASGPTRSS[S]FSATRS	AT1G34300	4	–	–	17	Lectin protein kinase related
jfh-416	LSKLTRQL[S]*IHDNRA	AT1G10290	14	40	–	16	ADL-6 Dynamin-like protein
jfh-552	SAIKALG[S]*FR[S]NATN	AT4G35100	10	–	–	15	PIP2.7, Aquaporin
jfh-583	KTKMNKSR[S]*LYDLQE	AT5G49760	–	5	–	15	LRR protein Kinase
jfh-573 ^a	PCLTKSD[S]GINGVDF	AT5G40390	10	–	–	15	Raffinose synthase related
jfh-572	KPRLPRIN[S]*AESMEL	AT5G20280	4	–	–	13	Sucrose-phosphate synthase
jfh-592	VAPLARAS[S]FEVKYT	AT5G66110	–	–	–	13	Metal ion binding protein
jfh-526	AGAIKALG[S]FRSQPH	AT3G54820	9	4	–	11	PIP2.5, Aquaporin
jfh-422 ^d	PGAPSRSG[S]*FAGTAQ	AT1G16860	4	–	–	11	Merozoite surface protein-related
jfh-530	EERYFRAD[T]*LDFSKW	AT3G63260	–	–	–	11	MRK1, protein kinase
jfh-440	YASIRSLP[S]PRNVLI	AT1G51260	–	–	–	11	Acyltransferase
jfh-724 ^b	RGGLERKF[S]*EQNIGA	AT3G59290	–	–	–	10	Unknown, pollen homolog of jfh-487
jfh-487	RGGLQRKF[S]*EQNIGA	AT2G43160	–	–	–	7	Unknown, compare to jfh-487
jfh-413	EGVTRVN[S]*LVQLPRF	AT1G03590	–	–	4	10	PP2C, protein phosphatase
jfh-458 ^d	NAPIQRSS[S]*TLLKMR	AT1G78610	5	–	–	10	Mechanosensitive ion channel
jfh-429	GLHKLRCN[S]*TAEINP	AT1G23080	–	–	–	10	Auxin efflux carrier component
jfh-266	DSRILSQML[S]*QKLRP	AT5G47910	–	–	–	10	RBOH-D NADPH oxidase
jfh-149	RTAFLSQY[S]*FYKSL	AT5G44240	5	–	–	9	ALA2, P-type Lipid Flippase
jfh-415	RERYQKQ S [S]ILL SKLT	AT1G10290	5	–	–	9	ADL6, Dynamin-like protein
jfh-490	EQKMSSIE[S]*FKQSSL	AT2G47000	4	–	–	9	ABC transporter
jfh-544	SRRRLSRPG[S]GVSFG	AT4G25970	–	–	–	9	Phosphatidylserine Decarboxylase 2
jfh-545	FQRLERRQ[S]*VEWVP	AT4G27060	–	–	–	9	Microtubule-associated protein
jfh-442	NRKLEKMA[S]*IDVHLR	AT1G53310	–	–	–	9	PEP carboxylase
jfh-445	PPPIDRNK [S]*FDEEDS	AT1G53730	5	–	–	8	LRR protein kinase
jfh-575	PREMGRNL[S]*GKAETM	AT5G41950	4	–	–	8	Expressed protein
jfh-578	RMGDSRIL[S]*QMLSQK	AT5G47910	–	–	–	8	RBOH-D NADPH oxidase
jfh-503	SGSITRLA[S]*IDSFDS	AT3G10540	–	–	–	8	IP3-Dependent protein kinase related
jfh-491	MSIE[S]FKQSSLRKSS	AT2G47000	–	–	–	8	ABC transporter
jfh-424	SRERFKSL[S]IINLMKD	AT1G18890	–	–	–	8	CPK10, Ca-dependent protein kinase
jfh-587 ^a	VSANAKSE[S]PKEQSP	AT5G56460	–	–	–	8	Protein kinase-related
jfh-443	ADGGGRSL[S]*LQERQR	AT1G53350	–	–	–	7	Disease resistance protein
jfh-427 ^a	YDGYNRSR[S]DLGSDL	AT1G20110	4	–	–	6	Zinc finger family protein
jfh-455 ^a	IVYRASLG[S]GKVVAV	AT1G73080	–	–	–	6	Receptor-like protein kinase
jfh-423 ^f	SVSMKKTN[S]GPLSKH	AT1G16860	–	–	–	5	Merozoite surface protein related
jfh-244	IRRDR[S]VDKND[S]INGD	AT3G55940	–	4	–	–	Phospholipase C
jfh-447	FSSGSRRTQ[S]VNDDE	AT1G59870	–	4	–	–	PDR8, ABC transporter
jfh-124	NNTLKKSVSPPFMNT	AT1G77760	4	–	–	–	Nitrate reductase
PEPTIDES BASED ON PREDICTED SITES							
jfh-319	RSLFKGSRSPSLKPP	AT3G63380	5	12	–	33	ACA12, P-type calcium pump
jfh-296	ASRELRVFSRRPSP	AT5G47910	4	18	–	27	RBOH-D, NADPH oxidase
jfh-097	RYRRMERTSSMPRAS	AT2G32400	10	17	8	24	GLR 3.7, glutamate receptor
jfh-317	SDFLKRLLS*SGMNP	AT5G37500	9	13	–	23	GORK, K-channel
jfh-476	RENFVRLDS*MDSRYS	AT2G23980	10	–	–	22	CNGC6, cyclic nucleotide gated channel
jfh-294	KFVRLDS*MDSRYSQT	AT4G30560	6	–	–	21	CNGC9, cyclic nucleotide gated channel
jfh-287 ^c	PMVITRSESSRLRS	AT1G15990	9	11	–	18	CNGC7, cyclic nucleotide gated channel
jfh-624	YKRRKLAKELS*LHEA	AT5G14870	6	–	–	17	CNGC18, cyclic nucleotide gated channel
jfh-071	SGGHS*RRYSDPAQNG	AT1G59610	–	–	6	12	ADL-3, Dynamin-like protein
jfh-142	AAKAITRLQSLPSGS	AT1G09570	–	–	–	11	Phytochrome A
jfh-307	KAPAHLGS*SRDYNVD	AT2G44100	–	–	–	10	RAB-GDP dissociation inhibitor
jfh-625	KLAKELSLHESSGY	AT5G14870	–	–	–	9	CNGC18, cyclic nucleotide gated channel

(Continued)

Table 1 | Continued

Peptide	Peptide sequence	AGI#	CPK1	CPK10	CPK16	CPK34	Description
jfh-767	NDKLPRVSSSDSMEA	AT1G52240	–	–	–	7	ROP-GEF11, guanyl-nucleotide exchange factor
jfh-035	RNMERSVSSSPSSSS	AT3G01610	–	–	–	7	Cell cycle control protein
jfh-131	AFATTRQRTCPRASG	AT5G05340	–	4	–	–	Peroxidase

^aCPK16 did not phosphorylate the *in vivo* mapped site [in brackets] when the other 2 serines were mutated to alanine.

^bCPK10 only phosphorylated the wild type peptide.

^cCPK1 and CPK10 only phosphorylated the wild type peptide.

^dCPK 1 only phosphorylated the wild type peptide.

^eOnly the wild type peptide was phosphorylated by CPK 34 (and others when indicated).

^f*In vivo* mapped site [] is not phosphorylated by CPK34 when all other serine and threonines are mutated to alanine.

^g*In ach-375*, M was not substituted with Nleu.

^h*In vivo* evidence for phosphorylation of pollen paralogs based on related protein from vegetative tissue.

Relative phosphorylation levels are shown as measured by SPA assays. Values represent the fold-increase over “no peptide” controls for each CPK (average of at least two independent replicates). Peptides are sorted according to degree of phosphorylation by CPK34, except in six cases where a comparison to a closely related peptide is shown immediately after the more highly phosphorylated example. A dash “–” indicates that the fold increase was “not significant.” Serine/threonine residues in brackets [S/T] indicate evidence exists for *in vivo* phosphorylation (see **Table S1** in Supplementary Material). Residues in parentheses () correspond to sites predicted based on *in vivo* mapping evidence from a homolog (see **Table S1** in Supplementary Material). Underlined and asterisk labeled residues were shown to be phosphorylated by CPK34 (and others when indicated) by either peptide mapping or when that residue represents the only serine or threonine residue in the peptide. Underlined residues without asterisk represent the candidate sites based on available evidence. For sequences from *Arabidopsis* proteins, the AGI# identifies the corresponding gene. Origins of reference peptides are indicated by a PubMed Identification. Origins of all other peptides can be found in **Table S1** in Supplementary Material.

a peptide’s kinetic properties as a substrate. Thus, a relatively small change in cut-off thresholds could change a particular substrate’s designation as a positive or negative. For this reason, selected peptides were chosen for follow-up assays to verify examples of substrate specificity (discussed below). In contrast to the SPA assays, the standard filter-based assays were done under Michaelis–Menten conditions. These assays (**Tables 4 and 5**) confirmed that the SPA assay conditions used here provided a reasonable indicator of a peptide’s relative strength as a CPK substrate.

Of the 102 peptide substrate hits in the SPA assay, the largest subset (33) were phosphorylated by three CPKs: isoforms 1, 10, and 34. However, each of these CPKs phosphorylated a distinct subset of shared and isoform-specific peptides, supporting a conclusion that each isoform has unique substrate specificity. CPK34 had the largest number of isoform-specific substrates (31), whereas CPKs 10 and 1 had only 3 and 2 substrates, respectively.

CPK16 phosphorylated the fewest synthetic peptides (nine). The most strongly labeled peptides included two peptides from a ubiquitin ligase (ATL6, AT3G05200, peptides jfh-495 and -496) and a protein of unknown function (AT3G27390, peptide jfh-515), all three of which contained a putative CPK site identified through mapping surveys of *in vivo* phosphorylated proteins. Another was jfh-097, a peptide containing a predicted 14-3-3 binding site from a glutamate receptor 3.7 (AT2G32400), previously identified as a 14-3-3 interactor (Chang et al., 2009). The only reference peptide to show significant labeling was the non-plant peptide sequence MARCKS (ach-400).

Of the nine synthetic peptide substrates for CPK16, all were phosphorylated by at least one of the other three CPKs tested (i.e., substrates were not CPK16-specific). However, because each of these peptides contain multiple serines, it was not clear if CPK16 recognized the same or a different phosphorylation site. In one case (peptide jfh-097), Edman degradation was used to confirm that the same serine could be phosphorylated by CPK16 and CPK34 [in the tryptic fragment, RTS(pS)MP]. This site matched a “simple-1” consensus motif, indicating that CPK16 is not restricted to a “simple-2” consensus site (like the S109 site of Di19-2, **Figure 4**).

IDENTIFICATION OF PHOSPHO-SITES BY “PEPTIDE MAPPING” WITH ALANINE SUBSTITUTIONS

Because most of the 102 synthetic peptide hits contained multiple Ss or Ts, the specific phosphorylation site(s) could not be assigned from the initial survey reactions. For a subset of positive hits, additional mutant peptides were synthesized in which selected S or T residues were replaced with alanines. Using this approach, 43 phospho-sites were mapped to a specific S or T (indicated by an asterisk in **Table 1**). An additional 12 peptides contained only a single S or T, in a total of 55 peptides in which specific phospho-sites were established. Of these, only one site did not conform to either a “simple-1” consensus site [basic-X-X-(S/T)] or a “simple-2” site [(S/T)-X-basic]. The exception was jfh-149 (RTAFLSQY[S]*FYKSL), which was derived from an *in vivo*-mapped phospho-site in a lipid flippase, ALA2 (Harper lab, unpublished). However, the low frequency of non-consensus sites does not necessarily indicate that CPKs are more likely to conform to these simple consensus sites, as nearly all the peptides tested here were selected because they already contained a proposed CPK consensus site.

This “mutant-peptide” mapping strategy also corroborated significant differences in substrate specificity determinants for each of the CPKs tested. For six peptides with various alanine-substituted versions, differential effects on substrate recognition were observed among CPKs 1, 10, and 34 (**Table 2**). In eight cases, recognition by two of the three isoforms was lost, and in four cases recognition was lost by only one isoform. For example, a mutant version of jfh-579 (i.e., jfh-688) revealed a disruption of phosphorylation by CPK10, but not by CPK1 and CPK34; and a mutant version of jfh-458 (i.e., jfh-723) provided a disruption of phosphorylation by CPK1, but not by CPK34.

Table 3 shows SPA results for variations on peptide jfh-515, which was recognized by all four CPKs tested, including CPK16. This peptide contained three Ss, the central one identified as an *in vivo* phosphorylation site. By mutating the central S (jfh-734), we found that at least one of the flanking Ss was still labeled by all four CPKs. However, when both flanking S residues were changed to

alanine (leaving only the central mapped pS; peptide jfh-735), phosphorylation occurred with CPKs, 1, 10, and 34, but not with CPK16. The failure of CPK16 to recognize this double alanine-substituted version is consistent with it having distinct substrate specificity.

EXAMPLES OF KINETIC DIFFERENCES BETWEEN CPKs 16, 1, 10, AND 34

To confirm the qualitative differences observed here for substrate specificity among the four CPKs tested, we compared reactions with selected substrates and kinases for differences in K_M s, k_{cat} s, and catalytic efficiencies under standard Michaelis–Menten conditions.

In analyses using three peptides recognized by CPK16 and 34 (jfh-097, ach-400, and jfh-515), significant differences were observed in multiple kinetic parameters (Table 4). For each

substrate, reactions with CPK16 showed significantly weaker K_M s (as much as 40-fold) compared to CPK34. However, CPK16 reactions showed higher turnover rates (k_{cat}), resulting in catalytic efficiencies for CPK34 and CPK16 that were within five-fold of each other. For CPK16, the best catalytic efficiency was observed with the ach-400 peptide (MARKS), which was approximately five-fold greater than with CPK34.

Similar kinetic differences were observed in reactions comparing two peptides recognized by CPKs 1, 10, and 34 (ach-393/syntide-2 and jfh-597; Table 5). The peptide jfh-597 was derived from a K⁺ channel, GORK (jfh-317), and is modified with an alanine substitution that leaves a single S residue corresponding to the location of a phospho-S in a potential 14-3-3 binding site. Consistent with the

Table 2 | Substrates from “peptide mapping” of phosphorylation sites revealing substrate specificity differences among CPK1, 10, and 34.

Peptide#	Phosphorylation by			Peptide sequence	Description
	CPK1	CPK10	CPK34		
jfh-752	28	7	35	LRELTKSA (S) FLRAVS	Pollen homolog to NIP4.1, aquaporin
jfh-753	–	–	9	LRELTKSA (a) FLRAVS	Mutant of jfh-752
jfh-754	18	–	33	LRELAKaA (S) FLRAVa	Mutant of jfh-752
jfh-579	6	–8	20	VRRFDRTS (S) AAIHAL	RBOH-D NADPH oxidase
jfh-688	6	–	22	VRRFDRAa (S) AAIHAL	Mutant of jfh-579
jfh-689	–	–	–	VRRFDRTS [a] AAIHAL	Mutant of jfh-579
jfh-287	9	–	18	PMVITRSE (S) SSRLRS	CNGC7, cyclic nucleotide gated channel
jfh-636	–	–	6	PMVIaRaE (a) a S RLRa	Mutant of jfh-287
jfh-637	–	–	6	PMVIaRaE (a) S aRLRa	Mutant of jfh-287
jfh-638	–	–	13	PMVIaRaE (S) aaRLRa	Mutant of jfh-287
jfh-639	–	–	–	PMVIaR S E (a) aaRLRa	Mutant of jfh-287
jfh-640	–	–	–	PMVI T RaE (a) aaRLRa	Mutant of jfh-287
jfh-425	4	4	18	KHSLKRKV [S] NPSFIA	CaM-binding Protein
jfh-704	–	–	12	KH S LKRKV [a] NPSFIA	Mutant of jfh-425
jfh-705	–	–	15	KHaLKRKV [S] NP a FIA	Mutant of jfh-425
jfh-422	4	–	11	PGAPSRSG [S] FAGTAQ	Merozoite surface protein-related
jfh-697	–	–	–	PGAP S RSG [a] FAGTAQ	Mutant of jfh-422
jfh-698	–	–	6	PGAPaRaG [S] FAGaAQ	Mutant of jfh-422
jfh-458	5	–	10	NAPIQRSS [S] TLLKMR	Mechanosensitive ion channel
jfh-722	–	–	–	NAPIQRSS [a] T LLKMR	Mutant of jfh-458
jfh-723	–	–	10	NAPIQRaA [S] aLLKMR	Mutant of jfh-458

Relative phosphorylation levels are shown as determined by a standard SPA assay. Values shown represent the average of at least two independent assays. Position of predicted or in vivo mapped sites are designated by () or [] respectively. Underlined residues in mutant peptides show potential phosphorylation sites. S or Ts with evidence of phosphorylation are shown in bold. Small case “a” indicates an S to A substitution.

Table 3 | “Peptide mapping” of phosphorylation sites in substrate jfh-515 provides evidence for a substrate specificity difference between CPK16 and CPKs 1, 10, and 34.

Peptide#	Phosphorylation by				Peptide sequence	Description
	CPK16	CPK1	CPK10	CPK34		
jfh-515	10	20	24	31	VKII S RIF [S] QR S FRR	Expressed protein
jfh-734	17	21	14	31	VKII S RIF [a] QR S FRR	Mutant of jfh-515
jfh-735	–	16	18	29	VKIIaRIF [S] QRaFRR	Mutant of jfh-515

Relative phosphorylation levels are shown as determined by a standard SPA assay. Values shown represent the average of at least two independent assays. The in vivo mapped phosphorylated S is denoted as [S] in bold. Potential phosphorylation sites are underlined. Small case “a” indicates an S to A substitution.

Table 4 | CPK16 and 34 show kinetic differences in the phosphorylation of three shared peptide substrates.

Peptide #	Kinase			Catalytic efficiency
	CPK	K_M (μM)	k_{cat}	
jfh-97	16	152, 94	3734, 2677	25, 29
	34	26, 28	502, 718	19, 26
ach-400	16	108, 119	1795, 1429	17, 12
	34	6.0, 6.6	398, 541	66, 82
jfh-515	16	208, 173	5114, 4854	25, 28
	34	5.3, 5.4	223, 231	41, 43

The k_{cat} is represented as nmol phosphate incorporated/min/mg CPK. Catalytic efficiency is k_{cat}/K_M , expressed as nmol phosphate incorporated/min/mg enzyme/ μM substrate. Results are shown from two independent reactions performed using a filter assay method.

Table 5 | CPK1, 10, and 34 show kinetic differences in the phosphorylation of two shared peptide substrates.

Peptide #	Kinase			Catalytic efficiency
	CPK	K_M (μM)	k_{cat}	
ach-393	1	49, 60	362, 482	7.5, 8.0
	10	238, 244	252, 217	1.1, 0.9
	34	65, 76	155, 225	2.4, 3.0
jfh-597	1	84, 86	5790, 5900	69.0, 69.0
	10	35, 37	1264, 1639	36.3, 43.9
	34	8.5, 8.9	475, 614	55.9, 68.9

Substrates are syntide-2 (ach-393) and a mutant version of the GORK peptide (jfh-597, aDFLKRLLSaGMNPN, with only a single phosphorylation site "S"). Values for k_{cat} are represented as nmol phosphate incorporated/min/mg enzyme. Catalytic efficiency is k_{cat}/K_M , expressed as nmol phosphate incorporated/min/mg enzyme/ μM substrate. Results are shown from two independent reactions using a filter assay method.

notion that each of these CPKs has different substrate specificities, each peptide/kinase reaction displayed diverse kinetic parameters, with as much as a 10-fold difference in K_M s and k_{cat} s, and a two- to eight-fold range in catalytic efficiencies.

DISCUSSION

SUBSTRATE SPECIFICITY DIFFERENCES FOR CPKs 1, 10, 16, AND 34

CPK isoforms 1, 10, 16, and 34 represent 4 of the 12 different subgroups of CPKs found in flowering plants (Harper et al., 2004). Three lines of evidence presented here support a model in which CPKs from different subgroups are likely to have functionally distinct substrate specificities. First, each of the four CPKs recognized unique subsets of substrates (see the 102 peptides in **Table 1** and protein Di19-2 in **Figure 1**). Second, alanine substitutions in seven selected peptides uncovered different substrate recognition determinants for each of the four kinases (**Tables 2 and 3**). Finally, kinetic analyses using five selected peptides showed more than 10-fold differences in K_M s and k_{cat} s for each of the four different CPKs (**Tables 4 and 5**).

The most distinct substrate specificity was observed for CPK16 (**Table 1** and **Figure 1**). CPK16 is a representative of the most divergent subgroup of CPKs in *Arabidopsis*. In this subgroup,

the kinase domains are more similar to a family of CPK-related kinases (CRKs) than to the core group of 31 other *Arabidopsis* CPKs (Harper et al., 2004). Nevertheless, CPK16 displays Ca^{2+} -stimulated activity (**Figure 3**) and contains a typical calmodulin-like regulatory domain – the structural feature that initially defined the CPK family.

CPK16's unique substrate specificity was revealed initially by analysis of a Di19-2 fusion protein. This fusion protein was strongly phosphorylated by CPK16, but more weakly by CPK1, 10, or 34 (**Figure 1**). A fusion protein G-S109-ST2 was engineered to display just one of the CPK16 sites present in the Di19-2 protein. This substrate had a K_M of 70 μM in reactions with CPK16, but failed to show any significant phosphorylation by the other three CPKs. In contrast, CPK16 only weakly recognized a site from NR, which was strongly phosphorylated by the other three kinases (**Figure 1**). This comparison showed that CPK16 had a substrate preference contrary to that of CPKs 1, 10, and 34. In another recent study, NR was found to be a good substrate for AtCPK isoforms 17 and 2, but not for CPKs 16, 24, or 29 (Lambeck et al., 2010). This supports the expectation that CPK16 and other CPKs will show significant differences in substrate specificity.

The extent to which the substrate specificity of CPK16 differed was further demonstrated here by a survey of 534 unique peptide substrates (**Table 1**). From the 102 synthetic peptide hits that were identified as *in vitro* substrates for at least one of the four CPKs, only nine showed significant phosphorylation by CPK16. By comparison, the next lowest number of substrates identified, 41, was found for CPK10. While all nine synthetic peptide substrates for CPK16 were also recognized by CPK34, the presence of multiple S/Ts in each peptide leaves open the possibility that each enzyme might still recognize other sites within a given peptide.

Substrate specificity differences were also revealed by comparison of CPK1, 10, and 34. In the survey of peptide substrates using an SPA assay (**Table 1**), CPK34 had the highest number of isoform-specific substrates (32), whereas CPK10 had only three. While CPK1 showed only two isoform-specific substrates, its 64 positive substrate hits were a unique mixture of those recognized by the other three isoforms. In follow-up assays on five peptides, a comparison of kinetic parameters with each of the CPKs illustrated reactions showing greater than 10-fold differences in K_M s and k_{cat} s (**Tables 4 and 5**). This indicates that each of these four enzymes have significant differences in substrate recognition and turnover (i.e., substrate specificity).

THE MOLECULAR BASIS OF SUBSTRATE SPECIFICITY DIFFERENCES

The structural basis for CPK substrate recognition is not yet clear. Multiple features of the kinase might contribute to specificity, from various amino acid residues associated with the substrate binding groove in the kinase domain, to various features associated with the N- and C-terminal domains interacting with substrates. For NtCDPK1, the variable N-terminal domain can be transferred to AtCPK9, thereby altering the substrate specificity of CPK9 to match that of CDPK1 (Ito et al., 2010). The crystal structures of apicomplexan CPKs suggest a possible interaction of the C-terminal regulatory domain with a "latch" in the N-terminal domain (Wernimont et al., 2010, 2011). All of the intramolecular interactions that occur during activation of a CPK might also influence substrate recognition and turnover kinetics.

It is also important to emphasize that the kinase assays here were based on recombinant proteins with N- and C-terminal modifications. Although each recombinant kinase had a similar fusion protein design, their respective enzyme kinetics might differ from their native enzyme counterparts under *in vivo* conditions.

BIOLOGICAL RELEVANCE OF SUBSTRATE SPECIFICITY DIFFERENCES

The isoform-specific functions of CPKs are potentially influenced by features that control their subcellular location, protein-complex interactions, lipid and Ca²⁺ activation kinetics, and substrate specificity. This study was aimed at evaluating the potential influence of substrate specificity.

The extent to which CPKs in *Arabidopsis* are functionally interchangeable has yet to be determined. However, a consideration of pollen-expressed CPKs provides examples of both redundant and non-redundant activities (Yoon et al., 2006; Myers et al., 2009). For example, a double disruption of *Arabidopsis* CPK17 and 34 results in a nearly sterile phenotype with pollen tubes showing deficiencies in growth rate, growth potential, and tropism toward ovules (Myers et al., 2009). Because single disruptions in either CPK17 or 34 alone did not show any detectable phenotypes, this pair of CPKs provides an example of functional redundancy. Nevertheless, expression in pollen of 10 other CPKs is not able to compensate for the double knockout of CPK17 and 34. Specifically, one such exogenous kinase, CPK16, is also highly expressed in pollen and is targeted to a similar plasma membrane location (Dammann et al., 2003) as CPK17 and 34 (Myers et al., 2009), but is not able to restore the double loss of function.

While multiple features are expected to make different CPKs functionally distinct, results here suggest that substrate specificity differences could make a significant contribution. *In vivo* support for this expectation comes from a recent study on NtCDPK1 in which *in vitro* phosphorylation of a substrate (transcription factor RSG) was shown to be dependent on the variable N-terminal domain of the kinase (Ito et al., 2010). When the N-terminal domain of NtCDPK1 was substituted for the analogous region in a different CPK (AtCPK9), the chimeric kinase displayed *in vitro* and *in vivo* activities similar to that of NtCDPK1.

DI19-2, A CPK16-SPECIFIC SUBSTRATE

Of the candidate phosphorylation sites identified here for CPK16, the S109 site in Di19-2 was the most extensively analyzed. Although this site overlaps with both simple-1 and simple-2 consensus sites for CPKs (Harper and Harmon, 2005), it was not recognized by three other isoforms, CPK1, 10, and 34 – emphasizing that features beyond a simple consensus site are clearly important for substrate recognition by different CPKs.

Di19-2 belongs to a family of seven related proteins in *Arabidopsis*, structurally defined by the presence of two atypical zinc finger domains. The Di19 name derives from a founding member whose mRNA expression was observed to be drought-induced. While the biological function of Di19-2 is not known, evidence indicates that its mRNA expression can be induced by a salt stress (but not drought stress; Rodriguez Milla et al., 2006). Public domain expression profiling results indicate that it is expressed in all major plant tissue types, including pollen, suggesting a function of potential importance to a subset of stress tolerance pathways in most cell types. Di19-2 is thought to be present in both nuclear and cytosolic

locations, as indicated by DsRED2 tagging experiments (Milla et al., 2006). Because CPK16 appears to be located at the plasma membrane, the phosphorylation of cytosolic Di19-2 might provide a pathway to relay Ca²⁺ signals from the plasma membrane to the nucleus. The observed 70 μM K_M for the phosphorylation of the S109 site by CPK16 is consistent with substrate affinities expected for biologically relevant phospho-regulation (Harper et al., 2004). Interestingly, Di19-2 is not recognized as a good substrate by at least five other CPKs isoforms, including 1, 10, 34 (Figure 1), 3, and 11 (Milla et al., 2006). In contrast, the other five members of the Di19 family in *Arabidopsis* were shown to be good substrates for multiple CPK isoforms. Thus, a potential CPK-mediated phospho-regulation of Di19-2 may require a CPK16-like substrate specificity.

MODULATION OF PHOSPHORYLATION BY METHIONINE OXIDATION

The Huber lab recently documented an example of a CPK phosphorylation site in NR (similar to peptide jfh-225) in which phosphorylation is blocked by the oxidation of a flanking methionine (e.g., P + 4 position; Hardin et al., 2009). Because thioether groups of methionines can be oxidized to sulfoxide groups by ROS such as H₂O₂, this modification (termed MetSO) provides a mechanism to transform an ROS signal into an on–off switch for a specific phospho-signaling pathway. Because plant cells have enzymes that can reduce MetSO back to Met, this regulatory switch is potentially reversible.

Of the 102 synthetic peptide substrates identified here for CPKs, 24 have methionines near the phosphorylation site. However, it is important to note that with one exception, each of the synthetic peptides used here was designed with a norleucine substitution of methionines to prevent MetSO formation. This might have altered recognition of certain sites by specific CPKs. For example, while CPK34 failed to phosphorylate a site in a peptide containing two norleucine substitutions for its methionines (GmSAT S367 ach-376), it showed strong phosphorylation of the same sequence synthesized with both methionines left unsubstituted (ach-375; Table 1). This failure of CPK34 to recognize the norleucine-substituted peptide is in contrast to CPK1, which retained strong phosphorylation of both peptides. Further analysis will be required to determine whether various CPKs will show isoform-specific differences in either increased or decreased substrate recognition as a result of MetSO-site modification.

CPK CONSENSUS SITES?

The use of consensus sites to predict kinase phosphorylation sites is sometimes useful, but often inaccurate (Que et al., 2010). Here we provide evidence for CPKs that predictions are usually wrong when based on consensus sites alone. Nearly all of the 534 peptides designed and tested in this study contained a simple-1 CPK consensus site (basic-X-X-S/T). Surprisingly, more than 80% of these peptides failed to show significant phosphorylation. While we cannot rule out that some peptides failed to be phosphorylated because of poor solubility or lack of appropriate secondary structure, analysis of selected peptides with alanine substitutions corroborate that recognition of a simple-1 consensus site can be strongly influenced by flanking residues (e.g., Tables 2 and 3), as previously indicated (Huang and Huber, 2001; Hernandez Sebastia et al., 2004).

The differences in substrate specificity found here provide a partial explanation for the origins of more than seven different consensus sites for CPKs (Harper and Harmon, 2005). The

development of a single meaningful consensus site for all CPKs does not appear possible, given that 4 of the 12 subgroups tested showed clear differences in substrate specificity. Thus, the evolution of bioinformatic tools for predicting potential CPK phosphorylation sites will need to incorporate a range of isoform-specific differences.

THE FRONTIER: LINKING THOUSANDS OF KINASES TO THEIR TARGETS AND BIOLOGICAL FUNCTIONS

CPKs have been proposed as multifunctional kinases, translating Ca^{2+} signals into the phospho-regulation of many different substrates (Harper et al., 2004; Klimecka and Muszynska, 2007). This paradigm is strongly supported here by the identification of 103 different substrates for four different CPKs (102 unique synthetic peptides, and residue S109 in Di19-2; **Table 1** and **Figure 1**).

In this study, 274 peptides were designed, each with an *in vivo*-mapped phosphorylation site that matched a predicted CPK consensus site. Of these, 74 (27%) were found to be phosphorylated by one of the four CPKs tested. Because CPKs have been shown to be present in most cellular locations (Dammann et al., 2003), a corroboration between *in vivo* and *in vitro* phosphorylation provides a reasonable basis for postulating that a particular *in vivo* phosphorylation event could have been mediated by a CPK. For example, phospho-sites identified in **Table 1** support specific hypotheses implicating CPK signaling pathways in regulating diverse aspects of cellular physiology, including auxin efflux (AT3G54820), ethylene response (AT4G23640), mechanosensitive ion channels (AT1G23080), and vesicle trafficking (AT1G10290).

In *Arabidopsis*, phospho-proteomics is providing a rapidly expanding list of phosphorylation sites (Heazlewood et al., 2008; Durek et al., 2010) that have been mapped in proteins isolated from plant tissues, and therefore represent *in vivo* phosphorylation events (de la Fuente van Bentem et al., 2008; Kersten et al., 2009; Uno et al., 2009; Kyono et al., 2010; Nakagami et al., 2010; Bi et al., 2011; see database¹). In an analysis of sites reported by (Sugiyama et al., 2008), more than 10% include a “simple-1” CPK consensus site (Harper, unpublished analysis).

In our study using 274 *in vivo*-mapped sites that contained a simple-1 motif (excluding reference peptides), we observed a 27% success rate in finding a CPK that recognized the same peptide in an *in vitro* kinase reaction. By contrast, we observed only a 6% success rate for peptides that were designed without any direct support from *in vivo* mapping data. However, given a 27% success rate as a precedent, it is reasonable to project that more than 200 additional CPK substrates might be confirmed through the application of this strategy to the nearly 8000 mapped phospho-sites listed in PhosPhAt (Heazlewood et al., 2008). Because the list of *in vivo*-mapped phosphorylation sites is certain to expand, we project that more than 300 phosphorylation sites will soon emerge as potential points of Ca^{2+} -dependent phospho-regulation.

The expanding list of CPK substrates is now shifting the scientific frontier from a focus on simply identifying potential CPK phosphorylation sites, to the larger question of understanding the biological functions associated with hundreds of Ca^{2+} -triggered changes in phosphorylation.

MATERIALS AND METHODS

KINASE EXPRESSION CLONES

The four kinases used in this study were AtCPK1 (At5g04870), AtCPK10 (At1g18890), AtCPK16 (At2g17890), and AtCPK34 (At5g19360). Sequences for all previously unpublished clones are provided in **Table S2** in Supplementary Material. For production of fusion proteins in *E. coli*, all clones were made as cDNAs subcloned into a pGEX expression vector with a GST N-terminal affinity tag, and modified to include a C-terminal 6x His tag (Binder et al., 1994). All expression vectors harbored an ampicillin resistance gene. Clones *G-CPK34-6H* (ps897) and *G-CPK1-6H* (ps553, also called *AK1-6H*) were previously described (Vitart et al., 2000a,b; Myers et al., 2009). For *G-AtCPK16-6H* (ps449) and *G-CPK10-6H* (ps1066), cDNA was PCR-amplified from a SuperScript *Arabidopsis* cDNA Library (Life Technologies, Carlsbad, CA, USA) with primers that included restriction sites suitable for subcloning.

SUBSTRATE EXPRESSION CLONES

G-Di19-2 (ps1195) encodes a full-length *AtDi19-2* (At1g02750) cDNA cloned in a *pGEX4T-1* vector to provide an N-terminal GST affinity tag (Rodriguez Milla et al., 2006).

G-GFP-ST (ps627) is a parental vector used for substrate clones described below. It is modified from a *pGEX* backbone that encodes a N-terminal GST affinity tag, and includes a GFP, a polylinker with *AscI* and *NotI* cloning sites, and a C-terminal Strep-II affinity tag.

G-GFP-S109-ST (ps1160) encodes a short sequence of Di19-2 surrounding the S109 phosphorylation site (the amino acid sequence for the S109 site shown in **Figure 2**). This construct was modified from ps627 by insertion of a DNA fragment from Di19-2 into the *AscI/NotI* site. Using ps1160, variations were made to produce the mutations shown in **Figure 4**.

G-GFP-NR-ST (ps699) is identical to ps1160 except that it has an insert that replaces the Di19-2 S109 site (sequence between *AscI* and *NotI*) with a sequence from a NR (NR site shown in **Figure 2**). *G-RFP-NR-ST* (ps630) was used in some experiments to provide an alternative RFP-tagged (in place of GFP) version of the same NR substrate sequence encoded in ps699.

RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

All kinase fusion proteins were purified as affinity sandwiches using a 6x His tag at the C-terminal end and a GST tag at the N-terminal end (Harper et al., 1994). For substrate fusion proteins encoded by *G-Di19-2* S109 (including mutants) and *G-GFP-NR-ST*, each protein was affinity-purified by a GST tag at the N-terminal end, followed by a Strep-tag-2 (IBA Biotechnology) at the C-terminal end. The fusion protein encoded by *G-Di19-2* (ps1195) was purified using a GST affinity tag as described (Rodriguez et al., 2006). Purified proteins were stored frozen at -20°C in 100 mM Tris-HCl, pH 8.5, 0.1 mM EDTA, 10 mM glutathione, and 50% glycerol. The concentration of each sample was determined via the Bradford method (Bradford, 1976), and purity evaluated by separation using SDS-PAGE (10% polyacrylamide gel) and staining with Coomassie Brilliant Blue. All kinase fusions were estimated to be greater than 90% pure.

¹<http://phosphat.mpimp-golm.mpg.de/index.html>

SYNTHETIC PEPTIDE DESIGN AND PRODUCTION

Non-biotinylated syntide-2 was purchased from Bachem (Torrance, CA, USA). Other synthetic peptides were made by Sigma Genosys (St. Louis, MO, USA) with purities estimated at 50%. Peptides used in SPA assays were all made with an N-terminal biotin group. Unless specifically noted, a norleucine was substituted for every methionine. Peptides were designed based on sequences known or predicted to be CPK substrates, as described in the results section.

KINASE ACTIVITY GEL-BASED ASSAYS

In vitro kinase assays were performed and modified as previously described (Rodriguez Milla et al., 2006) and labeled proteins separated by SDS-PAGE. Briefly, 50 μ M ATP (spiked with 2.5 μ Ci gamma- 32 P (3000 Ci/mmol, MP Biomedicals, Solon, OH, USA) was added to start the kinase reaction (final volume 10 μ L) which consisted of 1 ng/ μ L purified CPK, 100 ng/ μ L fusion protein substrate, and standard kinase reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 1.1 mM CaCl₂). The reactions were incubated for 10 min at RT and stopped by adding 10 μ L 2X SDS sample buffer. Proteins were separated by 12% SDS-PAGE and the gel was air-dried between acetate sheets. The dried gel was processed using either a phosphorimager (Cyclone Storage Phosphor System, PerkinElmer, Boston, MA, USA) or exposure to X-ray film (GE Healthcare Biosciences, Piscataway, NJ, USA). Images were processed using Quantity One (Bio-Rad) to determine the relative intensity of labeling. Signals were normalized to the amount of protein as determined from Coomassie Brilliant Blue-stained gels after SDS-PAGE.

PHOSPHORYLATION OF SYNTHETIC PEPTIDES USING SCINTILLATION PROXIMITY ASSAY

Synthetic peptides with N-terminal biotin tags were resuspended in 50% acetonitrile to a final stock concentration of approximately 5 mM. Kinase reaction conditions consisted of 20 μ M peptide (or ddH₂O as a control), 10 μ M ATP, 0.0625 μ Ci 33 P[γ -ATP], 25 ng purified CPK, and 0.1 mg/mL BSA (Bovine Serum Albumin) in standard kinase buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1.1 mM CaCl₂, 1 mM EGTA) in a final reaction volume of 25 μ L. Reactions were started by addition of ATP. After 24 min incubation at RT, 10 μ L aliquots were diluted with 30 μ L dilution buffer (0.13% SDS, 13.3 mM EDTA) and 10 μ L of this dilution was added to 200 μ L pre-aliquoted stop solution (500 μ M ATP, 1% Triton x-100, 1 \times PBS pH 11.0, and 100 μ g SPA beads [streptavidin-coated, yttrium silicate (GE Healthcare)] in 96-well plates. Counts per minute (CPM) for each stopped reaction were determined by a microbeta scintillation counter (PerkinElmer) with a 5–750 window setting, as per manufacturer's recommendations. Those peptide reactions showing at least fourfold greater CPM than a no-peptide control (in an average of two to four independent reactions) were considered to represent significant levels of phosphorylation.

STANDARD FILTER-BASED ASSAY FOR KINASE ACTIVITY

Kinetic assays were performed essentially as described (Harper et al., 1994). Briefly, kinase reactions were started by adding ATP [final concentration of 50 μ M ATP plus 0.375 μ Ci 33 P[γ -ATP] (3000 Ci/mmol)] to a reaction that consisted of 1 ng/ μ L CPK-fusion protein, 0.1 mg/mL BSA, and varied peptide substrate concentrations (0, 5,

10, 20, 50, 80, 100, or 200 μ M) in a kinase reaction buffer (above). A typical reaction volume was 15 μ L. The reaction was allowed to proceed at RT for 12 min and was stopped by spotting an 8- μ L aliquot onto P81 phosphocellulose chromatography paper (GE Healthcare Biosciences, Piscataway, NJ, USA). Filters were immediately immersed in 75 mM phosphoric acid (PA), washed 4 \times 5 min in 150 mL 75 mM PA, immersed in 95% EtOH for 1 min and air-dried. Dry filters were submerged in Ecoscint (Fisher, Pittsburgh, PA, USA) and 33 P counts were determined by a scintillation counter. To calculate activities in nmol P incorporated/min/mg enzyme, the total amount of 33 P label in reactions was determined by counting a 5- μ L aliquot of a sample reaction that was dried on filter paper and left unwashed. A Lineweaver–Burk method was used to determine K_m s. Results shown are from two independent protein purifications for each CPK, with each preparation assayed at least twice.

PHOSPHO-SITE MAPPING

Phosphorylation site mapping was performed as previously described (Rodriguez Milla et al., 2006). Briefly, phosphorylated protein was dried at room temperature in a speed-vac, resuspended in a trypsin solution containing 0.4 μ g trypsin (Promega, Madison, WI, USA) and 1 mM DTT in 25 mM ammonium bicarbonate, pH 8.0, and digested for 16 h at 37°. The digested peptides were dried and then redissolved in 0.1% (v/v) trifluoroacetic acid (TFA). Peptides were desalted using a reverse phase Zip-Tip C18 column (Millipore, Billerica, MA, USA) pre-equilibrated with 100% (v/v) acetonitrile (ACN) and washed three times with 0.1% (v/v) TFA. Peptides were loaded on the Zip-Tip by five cycles of aspiration and dispensing. The C18 column was washed with 0.1% (v/v) TFA three times and peptides were eluted using a solution of 50% (v/v) ACN and 0.1% (v/v) TFA. The eluate was dried with a speed-vac.

Peptides were analyzed by a neutral loss mass spectrometry method in the UNR Proteomics Core Facility (Schroeder et al., 2004). A reverse phase C18 column (Michrom) was used to separate peptides. The program was set to perform MS/MS/MS whenever it detected neutral loss of 32.6, 38.6, 49, 58, 65, and 98 D. The m/z values of the precursor, y and b ions were retrieved after the scans. Database searching was performed to identify the phosphorylated peptides. For Ion-Trap MS/MS data, m/z values of the precursor, y and b ions were searched against the NCBI database by use of the TURBOSEQUENT algorithm (Thermo Scientific) or MASCOT algorithm² (Perkins et al., 1999). All MS/MS spectrum were manually inspected to assure the accuracy of the identification.

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²<http://www.matrixscience.com>

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/plant_physiology/10.3389/fpls.2011.00036/abstract/

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Table S1 | Peptides tested in SPA assays. Each peptide was made with norleucine substitutions for methionines (except ach-375), and a biotin tag on the N-terminal end.

Table S2 | Sequences of plasmids used in this study.

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