Chapter 6

Use of Phospho-Site Substitutions to Analyze the Biological Relevance of Phosphorylation Events in Regulatory Networks

Nico Dissmeyer and Arp Schnittger

Abstract

Biological information is often transmitted by phosphorylation cascades. However, the biological relevance of specific phosphorylation events is often difficult to determine. An invaluable tool to study the effect of kinases and/or phosphatases is the use of phospho- and dephospho-mimetic substitutions in the respective target proteins. Here, we present a generally applicable procedure of how to design, set-up, and carry out phosphorylation modulation experiments and subsequent monitoring of protein activities, taking cyclin-dependent kinases (CDKs) as a case study. CDKs are key regulators of cell cycle progression in all eukaryotic cells. Consequently, CDKs are controlled at many levels and phosphorylation of CDKs themselves is used to regulate their kinase activity. We describe in detail complementation experiments of a mutant in CDKA;1, the major cell cycle kinase in *Arabidopsis*, with phosphorylation-site variants of CDKA;1. CDKA;1 versions were generated either by mimicking a phosphorylated amino acid by replacing the respective residue with a negatively charged amino acid, e.g., aspartate or glutamate, or by mutating it to a non-phoshorylatable amino acid, such as alanine, valine, or phenylalanine. The genetic complementation studies were accompanied by the isolation of these kinase variants from plant extract and subsequent kinase assays to determine changes in their activity levels. This work allowed us to judge the importance of posttranslational regulation of CDKA;1 in plants and has shown that the molecular mechanistics of CDK function are apparently conserved across the kingdoms. However, the regulatory wiring of CDKs is strikingly different between plants, animals, and yeast.

Key words: Cyclin-dependent kinase, CDKA;1, Cdk1, Suc1, Cks, Plant cell cycle, Kinase assay, Phosphorylation, Phospho-mimicry, Phospho-mimetic, Phospho-site, Substitution

1. Introduction

Modulation of protein activities through phosphorylation and de-phosphorylation is a recurrent theme in most if not all regulatory cascades. Typically, this modulation is repeated such as protein

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A phosphorylates protein B, which becomes activated by this and subsequently phosphorylates protein C (a well-studied example are MAP kinase cascades, see also Chapters 2, 4, 7, 8, 9, and 14). The consecutive change in protein activities serves many purposes. Among others, it allows information to be transmitted from one compartment, e.g., from the cell membrane to the nucleus. In addition, the initial information becomes amplified since one upstream kinase might activate several downstream kinases. Moreover, especially long cascades allow the integration of several information pathways and different inputs can be compared with each other, resulting in a fine-tuned response. These cascades in turn might be coupled to threshold levels of protein activities, resulting in very sensitive biological switches. A paradigm for such a wiring of phosphorylation cascades is cell cycle regulation in which extrinsic (environmental) cues such as temperature or light are integrated with intrinsic (developmental) information such as tissue or organ cues.

1.1. Regulation of CDK Activity During Cell Cycle Progression by Phosphorylation

The prototypical mitotic cell cycle in plants and in other eukaryotes is composed of four phases: DNA synthesis or S phase when replication events take place, M phase or mitosis when chromosomes are separated and cells subsequently divide, and gap phases G1 (after M) and G2 (after S), in which most of the cell-physiological processes take place. The progression through the mitotic cell cycle is regulated by the protein kinase activity of a heterodimeric complex formed by a substrate-specific cyclin protein and a catalytically active cyclin-dependent kinase (Cdk) subunit. The small 34-kDa serine/threonine protein kinase in yeast designated p34, Cdc2^{+} (*Schizosaccharomyces pombe*), and CDC28p (*Saccharomyces cerevisiae*), or the homologous cyclin-dependent kinase 1 (Cdk1) in humans is a key regulator of the eukaryotic cell cycle, and controls entry into S- and M-phase (for a general review, see (1)). Similarly, the *Arabidopsis* homolog of Cdk1, designated CDKA;1, was found to be involved in the regulation of both cell cycle checkpoints (2, 3). Studies in yeast and human cell cultures have demonstrated that monomeric CDKs have no detectable activity and need to bind their cyclin cofactors (1). Different cyclins can form complexes with CDKs that are implicated at specific cell cycle phases such as, for instance, B-type cyclins for mitosis. CDK activity is also negatively regulated by cofactors, called CDK inhibitors (CKIs) that are – like cyclins – themselves under strict transcriptional and proteolytic control. Similar to cyclin families, there exist especially in plants large families of CKIs (4).

Besides the binding of positive or negative cofactors, CDK-cyclin activity has been found to be strongly modulated by phosphorylation at two different sites in the protein, the so-called T- and P-loop (1). Phosphorylation in the T-loop at a highly

conserved threonine residue around position 160 (depending on the homolog, for instance in *Arabidopsis* position 161) is required for kinase activity and was recently shown to be essential in *Arabidopsis*, too (5, 6). T-loop phosphorylation is catalyzed by CDK-activating kinases (CAKs) (7). In *Arabidopsis*, at least four putative CAKs can be found, called CDKD1-D3 and CDKF, that can all phosphorylate CDKA;1 in vitro (8). However, which kinase(s) are functioning as CAKs in vivo is not clear yet.

In contrast to T-loop phosphorylation, phosphorylation of the conserved residues Thr14 and/or Tyr15 (or the homologous position) in the P-loop inhibits CDK activity in animals and yeast. Phosphorylation of the P-loop is executed by WEE1-, MYT1-, or MIK1-like tyrosine or dual-specificity kinases in animals and yeast. Phosphorylation of the P-loop is reverted by CDC25-like phosphatases and the wiring of positive (CDC25) with negative (WEE1, MYT1, and MIK1) regulators gives rise to oscillating levels of CDK activity that drive cell cycle progression (9). In addition, negative phosphorylation is crucial for cell cycle arrest upon DNA damage to allow sufficient time for repair.

Whether *Arabidopsis* CDKA;1 is also subject to P-loop phosphorylation control was not clear since the identified CDC25 candidate gene from *Arabidopsis* does not contain the hallmark of CDC25 phosphatases, i.e., a very large N-terminal regulatory region (10, 11). Moreover, the CDC25 candidate was found to have arsenate reductase activity, questioning further an involvement in cell cycle regulation (10, 12, 13). A straight-forward approach was, therefore, to test the importance of phospho- and dephosphorylation events in the P-loop of CDKA;1 by phosphomimetic mutants.

1.2. Application of Phospho-Site Mutations

As phospho-site mutations, the charged residues aspartate and glutamate are used to mimic phosphorylation, whereas a constitutive dephosphorylation is mimicked by Ala, Val, or Phe substitutions. To pay attention to overall sterical considerations and to reduce the effect of unspecific effects, one might consider replacing Ser with Ala and Asp, Thr with the slightly larger Val and Glu, and the much larger phospho-acceptor Tyr with Phe. However, there are also cases where substitution of any hydroxy group-containing amino acid with any charged amino acid or any small non-phosphorylatable amino acid was conclusive. Examples of the use of phosphorylation-site mutants in the cell cycle field are listed in Table 1.

Examples of phospho-site substitutions in *Arabidopsis* outside the cell cycle field are experiments on nitrate reductase, transcription factors, oxidases, receptors, and kinases. A selection is listed in Table 2.

Table 1
Effects of phospho-site mutations in CDK homologs

Homolog	Mutation[a]	Effect	References
Substitutions of the P-loop			
SpCDC2	T14F15	Advances cells prematurely into mitosis	[14]
GgCDC2	A14	No significant changes	[15]
GgCDC2	F15	No significant changes	[15]
GgCDC2	A14F15	Much higher in vitro kinase activity than in wild-type; premature entry into mitosis in transfected HeLa cells	[15]
GgCDC2	T14E15 or E14Y15	Dominant negative cell cycle arrest when overexpressed	[16]
GgCDC2	D14F15 or A14D15	Dominant negative	[16]
GgCDC2	A14Y15 or T14F15	Rescue of temperature-sensitive cdc mutant	[16]
GgCDC2	A14F15	Rescue of temperature-sensitive cdc mutant; advanced and premature mitosis; loss of coordination between cell cycle events; cells fail to respond normally to checkpoint controls at S-phase and mitosis; increased levels of histone H1 kinase activity	[16]
GgCDC2	A14T15 or T15	Dominant negative cell cycle arrest; possibly production of nonfunctional kinase	[16]
Substitutions of the T-loop			
HsCDC2	V161	Inhibits association with A- and B-type cyclins; causes a lethal phenotype in the *S. pombe*	[17]
HsCDC2	E161	Potentially mimicking phosphorylation, causes loss of coordination of mitosis and multiple cytokinesis	[17]
GgCDC2	A161 or D161	Nonfunctional; dominant cell cycle arrest	[16]
GgCDC2	E161	Nonfunctional; gave rise to slowly growing cells showing abnormalities in septation and cell separation; might retain partially active	[16]
SpCDC2	A167	No in vitro kinase activity	[18]
AtCDKA;1	D161	Generation of a weak allele that can partially rescue *cdka;1* mutant plant. The same construct is dominantly negative in *S. Pombe*	[5, 6]
AtCDKA;1	V161	Nonfunctional; construct behaves dominantly negative in *S. pombe*	[5, 6]
Triple mutants of both the P- and T-loop			
GgCDC2	A14F15D161	Dominant cell cycle arrest	[16]

[a]The wild-type residues of the phosphor-acceptor sites are Thr14; Tyr15; Thr161 (Hs and Gg)/Thr167 (Sp). *Sp Schizosaccharomyces pombe* (fission yeast), *Gg Gallus gallus* (chicken), *Hs Homo sapiens* (human), *At Arabidopsis thaliana*

Table 2
Examples for phospho-site substitutions in the plant field

Gene	Substitution	Effect	References
In plants[a]			
ABSCISIC ACID RESPONSIVE ELEMENTS BINDING FACTOR/ PROTERN 2 (AREB1)	S26A T31A S86A S94A T135A	Results in an autophosphorylated, transcriptionally active form of the bZIP-type transcription factor	[19]
ARABIDOPSIS K TRANSPORTER 2 (AKT2)	S210A/E/N S329A/E/N	These substitutions in the K^+-channel interfere both with function and gating modes of channel. S-to-N substitution mimics a phosphorylated serine in a mutant of AKT2	[20]
ARABIDOPSIS NITRATE REDUCTASE 2 (NR2)	S534D/A	Block both 14-3-3-inhibitory interactions	[21]
ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2)	D80E	Creates a dominant-active form of ARR2 that causes plants to become partially independent of the exogenously applied phytohormones ethylene	[22]
ARABIDOPSIS RESPONSE REGULATOR 7 (ARR7)	D85E/N	Mutant ARR7 alleles mimicking the active, phosphorylated form, cause the formation of aberrant shoot apical meristems. Ubiquitous overexpression of the dominant-negative form D85N did not cause any morphological defects	[23]
BASIC LEUCINE ZIPPER 63 (BZIP63)	S11A/D S15A/D S19A/E	Phosphomimetic substitutions of some of these serines strongly interfere with the DNA binding of the prototypical transcription factor bZIP63. Decreases binding to the corresponding promoter elements. Double and triple mutants tested	[24]
BRASSINOSTEROID INSENSITIVE 1 (BRI1; JM and CT domains)	T872A	Increases its kinase activity by over tenfold, due to an increased V_{max} and reduced K_m	[25]
	S286D	Abolishes BAK1 activity in vitro and in vivo, although S286A has little effect	[26]
	S838D T842D T846D S858D	Enhances the phosphorylation of a BRI1 synthetic peptide substrate in the absence of BRI1-ASSOCIATED KINASE 1 (BAK1)	[26]

(continued)

Table 2 (continued)

Gene	Substitution	Effect	References
BRASSINOSTEROID INSENSITIVE 2 (BIN2)	Y200F	Phosphorylation at this site is required for kinase activity	[27]
ELONGATED HYPOCOTYL 5 (HY5)	S36A	Modulation of suspected CKll site leads to stronger interaction with target promoters of this bZIP transcription factor	[28]
	S15A/D	Aspartate substitutions strongly interfere with the DNA binding of the prototypical bZIP HY5. Results in a considerable decrease in binding to the promoter elements	[24]
3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE-1 (PDK1)	T176A T211A	Partial loss of autophosphorylation and downstream phosphorylation activity	[29]
	S288E S290E	Results in an increase in the basal autophosphorylation level	[30]
RESPIRATORY BURST OXIDASE HOMOLOG F (RBOHF)	S174A	Results in an approximately 40% reduction of the phosphorylation of the NADPH oxidase by the ABA-activated SnRK2 protein kinase OPEN STOMATA 1 (OST1)	[31]
ROOT HAIR DEFECTIVE 2 (RHD2)	S318A S322A	Causes more than 50% reduction in ROS production of the NADPH oxidase	[32]
TOBACCO MOSAIC VIRUS RESISTANCE GENE N (N)	Y12F	The tobacco protein “N” alters its hypersensitive response (HR)-inducing activity of the plant resistance® gene (Toll interleukin-1 receptor homology region (TIR)-nucleotide-binding site-LRR). Y12S alters the activity but to a lesser extent compared to the Y12F substitution	[33]
	T223R/N	Results in a dominant inhibitory function	[34–36]
TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 33 (TOC33)	S181A/D/E	All transgenes were able to complement mutants for the GTPase transit peptide receptor of the chloroplast outer envelope membrane efficiently. S181A lacks phosphorylation	[37–39]

Plant pathogens			
AvrPto	S149A	No phosphorylation of the *Pseudomonas syringae* pv tomato protein by *Arabidopsis* leaf protein extracts. Multiple Ser substitutions (S147, S149, and S153) generate a phosphorylation-null protein	[40]
	S258A	Loss of virulence and the diminution of avirulence activity. S258D has activities like wild type	[41]
coat protein precursor (pre-CP)	S66A/D S68A/D S72A/D	Delays symptom development and affects the infectivity of the *Cauliflower mosaic virus* (CaMV)	[42]
P30	S212A T214A S218G S226G S227G S231G S247G S258A/D T261A/D S265A/D S267A	Changes in single-strand nucleic acid-binding activity, correct protein folding, and cell wall anchoring of the *tobacco mosaic virus* (TMV) nucleic acid-binding protein	[43]

[a] if not stated otherwise, the plant species is Arabidopsis thaliana *Arabidopsis thaliana*

1.3. Generation of Phospho-Site Mutations in CDKA;1 and Analysis

To test the importance of CDKA;1 phosphorylation, the following mutants were generated: Thr14, Tyr15→Asp14, Glu 15 (short DE) and Thr14, Tyr15→Val14, Phe15 (short VF). The so-modified cDNAs were cloned behind a promoter fragment of CDKA;1 that was known to fully complement *cdka;1* mutants when driving the unmodified *CDKA;1* cDNA. Heterozygous *cdka;1* mutants were transformed with the individual constructs, and the T2 generation was screened for the appearance of homozygous *cdka;1* mutants that harbored the phospho-mutant construct.

A morphological analysis of these isolated plants revealed that DE could not fully rescue *cdka;1* mutants and only small and sterile plants were recovered (5, 10). Subsequent kinase assays showed that DE plants indeed displayed very little kinase activity in comparison to wild-type plants. These results were consistent with an inhibitory function of P-loop phosphorylation on CDK activity. In contrast, the dephosphomutant VF could fully rescue *cdka;1* mutants and in contrast to studies in animals and yeast, VF plants neither proceed faster through mitosis nor display hypersensitivity against genotoxic stress. Together with the observation that neither mutants in the CDC25 candidate gene in *Arabidopsis* displayed any alteration of the cell cycle nor plants overexpressing the CDC25 candidate showed any enhancement of cell proliferation, we conclude that phosphorylation of CDKA;1 does not play such a major role as CDK phosphorylation does in animals or yeast.

Many studies in the animal and yeast fields investigating CDK activities have employed a cell lysate system to produce active complexes in vitro mostly using bacterial, insect cell, or cell-free (rabbit reticulocyte or wheat germ lysate) expression systems. One advantage of using *E. coli* or cell-free expression systems besides simplicity is that far fewer contaminating protein kinases might be present than in eukaryotic cells. However, especially when it comes to multicompound protein kinase complexes that must be formed from individual binding partners and – like in the case of CDK/cyclin complexes – even activated by further cofactors, one might desire to use in vivo expression systems as far as possible to reduce contamination or unwanted protein modifications by the host organism. Although the recent literature reports in vivo and in vitro binding partners of *Arabidopsis* CDKA;1 in detail (44–47), the actual requirements of these protein complexes to promote cell cycle progression, e.g., the G2-M transition, are still unclear. Thus, one possibility was to try to complement the *cdka;1* mutant with the CDKA;1 variants and to pull down active complexes in their in vivo composition to further study them in kinase activity assays.

1.4. Purification via CKS-Affinity Binding

The core part of the protocol describes the purification and kinase assay of CDKA;1 variants complexed with their in vivo subunits. Different approaches can be followed in order to purify the desired CDK/cyclin complexes. For example, immunoprecipitation (IP)

is widely used to co-precipitate active complexes, but some domains of both the CDK and cyclin can be masked in active complexes and are, therefore, not accessible by the antibody. The powerful anti-PSTAIRE antibodies raised against the conserved PSTAIRE motif in the cyclin-binding domain can only recognize the unbound and inactive CDK subunit. Thus, they are solely useful for immunoblotting but cannot be used for co-IPs of active kinase complexes since they would precipitate monomeric CDK (48).

We use a p13^{Suc1}-affinity matrix for CDK/cyclin purification (see also Chapter 4). Suc1 stands for "suppressor of cdc2" from *S. pombe* and is a member of the so-called CKS (CDK subunit) subfamily of CDK adaptor proteins, see Fig. 1. The matrix is comprised of Suc1 protein (MW 13 kDa) that is either covalently cross-linked to or non-covalently associated with the agarose bead material and represents the method of choice for obtaining semi-purified mitotic CDK activity in a quantitative scale (52–54) (see Note 1). Suc1 and its Cks1 homologs from *S. cerevisiae*, human, or *Xenopus*, and CKS1 in *Arabidopsis* have all been found to bind to CDK complexes. These can be specifically precipitated with these beads and it was shown that CKS directly binds to the CDK polypeptide chain (Fig. 1; (49)).

Also, other proteins of the CKS family can be used as an affinity matrix. Besides *S. pombe* p13^{Suc1}, these include p9^{Cks1} from *Xenopus* and humans as well as *Arabidopsis* p10^{CKS1} and bind a number of CDK/cyclin complexes in cell lysates – notably CDK/cyclin B that is present at the G2/M transition – with high affinity. De Veylder et al. describe a method to precipitate different plant-specific CDKs selectively by using subsequent purification steps of

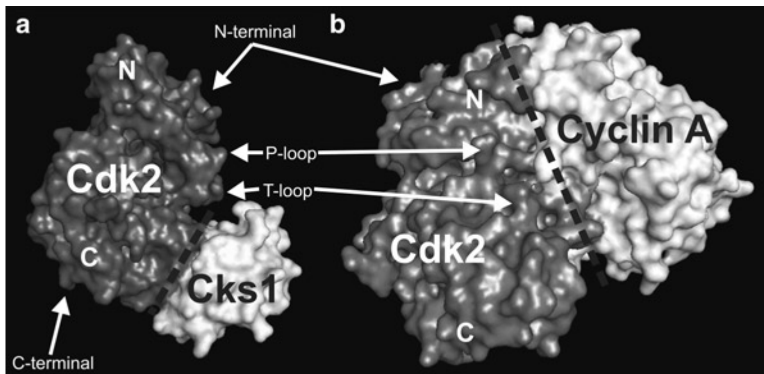

Fig. 1. Cks1 binds to the C-terminal part of Cdk2. A and B crystal structures of human Cdk2 bound to either the cell cycle regulatory protein Cks1 (**a**) or human Cyclin A (**b**) in a similar orientation. For better orientation, the subunits are separated by a dashed line and the N- and C-terminals of Cdk2 are indicated by *letters* (N, C). (**a**) Side view of the crystal structure of a human Cdk2-Cks1 kinase complex (PDB ID: 1BUH; [49]). Cks1 binds via four beta strands to the Cdk2 C-terminal lobe. The mode of binding suggests that Cks1 may target Cdk2 to other proteins during the cell cycle. (**b**) Structure of a complex of human Cdk2-Cyclin A (PDB ID: 1QMZ; [50]) to illustrate the site of Cks1-binding in a possible multimeric complex (close to the C-terminal (C) and below the Cdk2-Cyclin A complex in the figure). There is no crystallographic data available for a Cdk-Cyclin-Cks complex up to now. Visualized with PyMOL [51].

either p9 (human Cks1) or p10 (*Arabidopsis* CKS1) beads (55). Human p9^{Cks1} binds selectively to CDKA;1, whereas *Arabidopsis* p10^{CKS1} and *S. pombe* p13^{Suc1} bind to both A- and B1-type CDKs (5, 55, 56). The preparation of a CKS affinity medium is, besides the laborious protein expression and purification steps, quite straight forward (57) (see also Chapter 4 and Note 2).

In order to obtain highest saturation of the beads with mitotic CDK/cyclin complex, yeast or human cell cultures can be arrested in mitosis with nocodazole for maximum activity. Since this is impossible in our case where in planta material is to be assayed, we depend on the use of proliferative material with a high mitotic index. The optimal material is the shoot apical meristem including flower buds with its rapidly dividing tissues.

The methodology of affinity binding to directly agarose- or sepharose-coupled bait proteins can be transferred to other proteins by adapting the actual coupling (not described here) and assay protocols. However, most commonly, baits are either fused to entire helper proteins or parts such as glutathione S-transferase (GST), maltose-binding protein (MBP), avidin, calmodulin, thioredoxine, ubiquitin-binding domains, and lectins, or to small peptide tags such as Strep, tandem-affinity purification (TAP), hemagglutinin (HA), myc, FLAG, or His that indirectly lead to binding of the target protein to the bait bound to the matrix via these tags.

In this chapter, we describe an integrated series of subsequent protocols to test the activity of different CDKA;1 phospho-site variants isolated from the *cdka;1* mutant background. These kinases are purified using CKS beads and subjected to a phosphorylation reaction involving bovine histone H1 as an in vitro substrate together with radioactively labeled γ-ATP (see Note 3).

2. Materials

Most of the experiments do not necessitate special infrastructure. However, the plant cultivation and transformation steps have to be prepared and completed in either greenhouses or – better – climate chambers in a highly controllable environment. For the key part of the kinase assay, the phosphorylation reaction, a distinct laboratory is required where radioisotopes can be used under authorized conditions.

2.1. General Equipment and Infrastructure

Climatized greenhouse or growth chambers.

Fridge or cold room with tumbler.

Cooling microcentrifuge or regular microcentrifuge in cold room.

Gloves (due to the use of carcinogenic acrylamide and radioisotopes).

Safety goggles (due to the use of β-irradiating radioisotopes).

Radiation safety equipment (for further details, see Subheading 2.6).

1.5-mL microcentrifuge tubes, prechilled to 4°C.

Forceps, tweezers, and scissor (used for sample collection, radioisotope handling, or Western blotting).

Dewar container with swimmer, liquid nitrogen.

Clean, ethanol-rinsed plastic pistils (Roth or Eppendorf) or clean porcelain pestle and mortar (autoclave and bake, for example).

Ice bucket and wet ice.

Vortex mixer.

Graduated cylinders and containers for reagent preparation and storage.

Pipets accurately delivering 2.5, 20, 200, and 1,000 μL.

Soil mixture for *Arabidopsis* cultivation, selection, and propagation: mix, e.g., 8 bags of MiniTray® (70 L/bag, Balster Einheitserdewerk) and add 50 L water containing 800 mL Osmocote® Start (Scotts International BV) and 250 g BioMükk® (Sautter & Stepper, see Note 4).

2.2. Generation of Transgenic Plant Material

2.2.1. Cloning of the Expression Constructs

cDNA from *Arabidopsis thaliana* (L.) Heynh., ecotype Columbia-0 (Col-0).

Standard cloning equipment.

Site-specific oligonucleotides (see Table 3).

Proofreading polymerase such as Pfu (Fermentas).

ExoSAP-IT® (usb/Affymetrix).

Gateway® BP and LR kits (Invitrogen).

Gateway®-compatible Entry vector such as pDONR201 (Invitrogen).

DH5α® or equivalent cloning hosts (Invitrogen).

Binary Gateway®-compatible Destination vector such as pAM-PAT-ProCDKA;1 containing the endogenous promoter of CDKA;1 (3).

2.2.2. Plant Transformation and Selection of Transformants

Agrobacterium tumefaciens strain GV3101-pMP90RK, which is compatible with pAM-PAT binary Destination vector (58, 59), store in 25 μL aliquots at −80°C.

Luria–Bertani (LB) medium for *E. coli*.

Table 3
Examples for cloning and mutagenesis oligonucleotides used in CDKA;1 studies (5)

Name	5′-Sequence-3′
attB1 fwd	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAACAATGGATCAGTA CGAGAAAG
attB2 rev	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAAGGCATGCCTCCAAG
T161D fwd	GTCAGGACATTTGATCATGAGGTTGTTACT
T161D rev	AGTAACAACCTCATGATCAAATGTCCTGAC
T14D/Y15E fwd	ATTGGTGAAGGAGATGAGGGTGTGGTTTAT
T14D/Y15E rev	ATAAACCACACCCTCATCTCCTTCACCAAT
T14V/Y15F fwd 1	ATTGGTGAAGGAGTTTTCGGTGTGGTTTAT
T14V/Y15F rev 1	ATAAACCACACCGAAAACTCCTTCACCAAT
T14V/Y15F fwd 2	TGAGAAGATTGGTGAAGGAGTCTTCGGTGTGGTTTATAAGGCACG
T14V/Y15F rev 2	CGTGCCTTATAAACCACACCGAAGACTCCTTCACCAATCTTCTCA

YEB medium for *Agrobacterium*; for 1 L: each 5 g meat extract, tryptone/peptone, and sucrose; 1 g yeast extract; and 2 mL $MgSO_4$ 1 M; sterilize by autoclaving.

Carbenicillin 50 mg/mL, this semi-synthetic analog was replaced by ampicillin in our laboratory due to its increased thermo- and pH stability and resistance in the presence of ß-lactamase; store at −20°C.

Kanamycin 25 mg/mL, store at −20°C.

Rifampicin 50 mg/mL in DMSO, dissolves only if warmed up to 30–40°C; store at −20°C.

Sucrose (saccharose/household sugar works great).

Arabidopsis thaliana cdka;1 mutant seeds, line SALK_106809 (Nottingham *Arabidopsis* Stock Center, Nottingham, UK, cat. no. N606809).

Silvet L–77® surfactant (“Vac-In-Stuff”; OSi Specialties Inc. or GE Silicones, see Note 5).

Dismozon® pur (oxygen-active broad-spectrum disinfectant, Bode Chemie).

BASTA® (alternative names: phosphinothricin (PPT), glufosinate ammonium, 2-amino-4-(hydroxy-methyl-phosphinyl)-butanoic acid; Bayer Group, see Note 6).

Pressure sprayer to spread BASTA® solution (standard gardening quality, capacity of 10 L, e.g., from GLORIA or Birchmeier).

2.2.3. Genotyping

1.2 mL 96-well storage plates, polypropylene ("collection blocks", alphanumeric grid-referenced, U-shaped bottom; Thermo Scientific/ABgene, cat. no. AB-0564). Do not use polystyrene blocks, they break or burst easily.

Stainless steel beads (e.g., Brammer 3.5 mm, cat. no. KU.4 G28, or Mühlmeier 3.175 mm, cat. no. 75306).

Bead dispenser, 96-well, fits one 3.00–3.5-mm bead per well adapted to the collection blocks, can be made from Plexiglas®/ Perspex® by drilling 96 holes.

Collection microtube caps (polypropylene, 960 in strips of 8, perfectly matching onto the collection blocks, Qiagen, cat. no. 19566).

Sealing mats, 96-well, round (silicon, Thermo Scientific/ABgene, cat. no. AB-0674).

Bead mixer mill (Retsch MM 200 or Qiagen TissueLyser, cat. no. 69984).

Adapter set plates 2 × 96 for use with Qiagen collection microtubes, racked, matches perfectly with ABgene storage plates (Qiagen, cat. no. 69984).

45° Long-nosed pliers (from crafting or hardware store).

PCR plates, 96-well, cheapest quality.

Long pipette tips for up to 10 μL (e.g., Starlab TipOne pipette tip, 0.1–10 μL, extended length, cat. no. S1110–3000).

Multichannel pipette (Eppendorf or Thermo Labsystems).

Home-made Taq polymerase.

Allele-specific oligonucleotides (see Table 4).

Laboratory detergent.

Rubbing alcohol.

Eau de Javel (household bleach, e.g., from FLOREAL Haagen).

Extraction buffer for genomic plant DNA ("*sucrose solution/ buffer*" or "*Magic buffer*"). For 1 L: 50 mL 1 M Tris–Cl, pH 7.5 (for 50 mM), 60 mL 5 M NaCl (for 300 mM), and 100 g sucrose (for 300 mM), adjust to 1 L; sterilize by autoclaving.

10× PCR buffer ("*Direct buffer*" with gel-tracking dyes). For 1 L: 24.23 g Tris (for 200 mM), 37.275 g KCl (for 500 mM), and 4.07 g $MgCl_2$ hexahydrate (for 20 mM) to pH 8.7 with ~5 mL HCl. Add 1.5 g/L xylene cyanol FF (a blue, strongly quenching band runs at about 5 kb; Sigma cat. no. 95600) and 1.5 g/L Orange G (a yellow, weakly quenching band runs at approximately 100 b and an additional blue band that is only visible under UV runs directly at the migration border; Sigma cat. no. O3756); sterilize by autoclaving.

Table 4
Allele-specific genotyping oligonucleotides

Name	Target	5′-Sequence-3′
N034	CDKA;1 exon	CCAGATTCTCCGTGGAATTGCG
N048	CDKA;1 intron	CAGATCTCTTCCTGGTTATTCACA
N049	CDKA;1 intron	TGTACAAGCGAATAAAGACATTTGA
SALK-LB	Salk T-DNA, left border	GCGTGGACCGCTTGCTGCAACTCTCTCAGG

2.3. Phosphoprotein Extraction

Chemicals and reagents to be used in the following procedures should be of the best grade commercially available and all solutions prepared with sterile Milli-Q ultrapure water. All temperature-labile compounds must be added immediately before use and the completed buffer has to be kept on ice. Individual buffers and solutions should be autoclaved beforehand whenever possible. Most prepared solutions of the fine chemicals (such as inhibitors) used in this protocol should be stored in small aliquots at −20°C, unless otherwise indicated. Under these conditions, they are usually stable for at least a couple of months. All assay buffers should be prepared fresh on the day of assay and stored on ice. Storage of the completed solutions containing sensitive reagents and fine chemicals overnight at 4°C can lead to a significant loss of measurable kinase activity in the assay (60). Add the protease inhibitors immediately before use to prevent their degradation and avoid freeze–thawing of aliquoted stocks. In some cases, a 1:100 dilution of commercially available protease inhibitor cocktail (Sigma or Roche) is advisable. The mixtures include inhibitors of serine and cysteine proteases, which are the most prevalent protease classes in plant extracts (see Note 7).

2.3.1. Stock Solutions

500 mM tris(hydroxymethyl)aminomethane (Tris–Cl) at both pH 7.8 and pH 7.6.

150 mM magnesium chloride ($MgCl_2$).

2.5 M sodium chloride (NaCl).

50 mM ethylenediaminetetraacetic acid disodium salt (Na_2-EDTA, pH 8.0, see Note 8).

50 and 150 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt (Na_2-EGTA), pH 8.0.

5 and 50 mM dithiothreitol (DTT).

10% Igepal CA-630 or Nonidet P-40 (Sigma, the latter is not longer commercially available).

10% Tween 20.

2.3.2. Inhibitors

Use of inhibitors, see also Note 9.

50 mM sodium fluoride (NaF, Fluka; toxic).

10 mM sodium orthovanadate (Na_3VO_4, Sigma; toxic, must be activated, see Note 10).

150 mM p-nitrophenylphosphate disodium salt ($p\text{-}NO_2PhePO_4Na_2$).

500 mM β-glycerophosphate disodium salt.

100 mM phenylmethanesulfonyl fluoride (PMSF, Fluka, see Note 11).

100 mM benzamidine hydrochloride (Sigma).

1 mg/mL antipain dihyrochloride (Sigma).

1 mg/mL aprotinin from bovine lung (Fluka, store at −80°C).

1 mg/mL soybean trypsin inhibitor (STI, Fluka).

Extraction buffer: 25 mM Tris–Cl, pH 7.6, 85 mM NaCl, 15 mM $MgCl_2$, 2.5 mM NaF, 15 mM EGTA, 1 mM Na_3VO_4, 15 mM $p\text{-}NO_2PhePO_4$, 60 mM β-glycerophosphate, 5 mM DTT, 0.1% Tween 20, 0.1% Igepal, and a protease inhibitor cocktail composed of 1 mM PMSF or 10 μg/mL AEBSF, 10 μg/mL antipain, 10 μg/mL aprotinin, 10 μg/mL STI, and 0.1 mM benzamidine.

Optional protease inhibitors, to achieve a broader range of protease inhibition (see Note 9):

1 mg/mL chymostatin (Sigma, in DMSO).

1 mg/mL leupeptin hemisulfate (Fluka, store at −80°C).

1 mg/mL pepstatin A (Fluka, store at −80°C).

1 mg/mL 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma; store at −80°C). Some of these inhibitors can also be purchased in a mixture as follows:

Protease inhibitor cocktail (Sigma cat. no. P8340, or Roche Applied Science cat. no. 1836153).

2.4. Protein Concentration Determination

Protein Assay Dye Reagent Concentrate (Bio-Rad).

1 mg/mL bovine serum albumin (BSA).

Cuvettes with 1-cm path length matched to laboratory spectrophotometer.

Spectrophotometer set to 595 nm.

2.5. p13^{Suc1}-Sepharose Affinity Binding and Pull-Down

p13^{Suc1} agarose conjugate (GST-Suc1 fusion protein, non-covalently bound to agarose, do not freeze or vortex, store at 4°C; Upstate/Millipore, cat. no. 14–122).

1-mL syringes and 21-G needles, prechilled to 4°C.

End-over-end rotator or rotating wheel.

Bead or wash buffer: 2,000 μL/sample is required. Add 50 mM Tris–Cl, pH 7.6, 250 mM NaCl, 5 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 1 mM DTT, 0.1% Tween 20, 0.1% Igepal, 1 mM PMSF or 10 μg/mL AEBSF, 10 μg/mL antipain, 10 μg/mL aprotinin, 10 μg/mL STI, and 0.1 mM benzamidine. Store on ice.

Pre-kinase buffer: 500 μL/sample is required. Add 50 mM Tris–Cl, pH 7.8, 15 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, 1 mM PMSF or 10 μg/mL AEBSF, 10 μg/mL antipain, 10 μg/mL aprotinin, 10 μg/mL STI, and 0.1 mM benzamidine. Store on ice.

2.6. Phosphorylation Reaction

Heating block or thermo shaker that rises up to 95°C (see Note 12).

Needle to eventually punch holes into tube lids.

Screw-cap microcentrifuge tubes (optional).

Histone H1 (10 mg/mL in water, store aliquots at −80°C, Millipore/Upstate, cat. no. 14–155).

10 mM adenosine-5′-triphosphate, lithium salt (Li-ATP, store in small aliquots at −20°C, Roche Applied Science cat. no. 11140965001, see Note 13).

1 mM Li-ATP.

[γ-^{33}P]-ATP (or [γ-^{32}P]-ATP both at 10 μCi/μL, >6,000 Ci/mmol (220 TBq/mmol, see Note 14), Hartmann Analytic or Amersham), both stabilized and conveniently delivered and stored at +4°C instead of −20°C, preferentially colored (in red) for convenient monitoring of the radionuclide during the pipetting steps.

Kinase buffer: 31 μL/sample is required. Add per sample 11.75 μL water, 3.5 μL each of 500 mM Tris–Cl, pH 7.8 (for 50 mM), 150 mM $MgCl_2$ (for 15 mM), 50 mM EGTA (for 5 mM), 10 mM DTT (for 1 mM), and complement with 1.75 μL histone H1 (stock 10 mg/mL). Store on ice.

ATP mix: 5 μL/sample is required. Add per sample 4.1 μL water, 0.3 μL 1 mM Li-ATP (here 60 μM), and 0.6 μL = 6 μCi [γ-^{33}P]-ATP (here 200 nM, calculated from a fresh stock of 10 μCi/μL; if ^{32}P is preferred as tracer, use same volumes/amounts/activities of [γ-^{32}P]-ATP). The overall specific activity of both cold and

hot ATP in this mixture is 20 mCi/μmol with 0.3 nmol ATP or 6 μCi per 5 μL. Store on ice behind β-shielding in a Plexiglas®/Perspex® block in an ice bucket.

2.7. SDS-Polyacrylamide Gel Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) equipment and power supply, we preferentially use large gel systems such as a PerfectBlue® Twin M (PeqLab).

Magnetic stirrer.

30% Acrylamide mix (30% acrylamide/*N*,*N*′-methylenebisacrylamide solution, ratio 37.5:1 in water, see Note 15).

1.5 M Tris–Cl, pH 8.8 (for separating gel).

1 M Tris–Cl, pH 6.8 (for stacking gel).

10% Ammonium persulfate (APS, immediately freeze upon preparation in single-use aliquots, store at −20°C).

10% Sodium dodecyl sulfate (SDS, sodium lauryl sulfate).

N,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED, see Note 16).

Isopropanol (see Note 17).

Gel loading tips, extended length.

Protein gel running buffer 10×; for 1 L: 30.2 g Tris–HCl, 144.2 g glycine, and 10 g SDS in water.

Pre-stained molecular weight markers such as Precision Plus Protein Standard All Blue (Bio-Rad).

5× SDS sample buffer: 0.225 M Tris–Cl, pH 6.8, 50% glycerol, 5% SDS, 0.05% bromophenol blue, and 0.25 M DTT.

Laemmli sample buffer 2×: 4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris–Cl, pH 6.8, and 0.02% bromophenol blue (61).

Prepare gel volumes according to the sizes of your gel system, all percentages in (v/v), modified from ref. 62:

For a 12% separating gel, add 33% water, 40% acrylamide mix (30%), 25% 1.5 M Tris–Cl, pH 8.8, 1% SDS (10%) and APS (10%), and 0.04% TEMED.

For a 5% stacking gel, add 68% water, 17% acrylamide mix (30%), 12.5% 1.5 M Tris–Cl, pH 8.8, 1% SDS (10%) and APS (10%), and 0.1% TEMED.

2.8. Western Transfer of Radioactive Proteins

Tank blot apparatus (such as Tankblot SCIE-PLAS EB10).

Magnetic stirrer.

Power supply.

Filter paper (Whatman®).

Nitrocellulose membrane (Amersham).

Ice bucket and wet ice.

Rabbit anti-Cdc2 antibody (polyclonal α-PSTAIRE; Santa Cruz cat. no. sc-53).

Goat anti-rabbit HRP-conjugated antibody ImmunoPure peroxidase (Pierce, included in cat. no. 1858415).

Saran wrap or plastic disposal bags.

Enhanced chemiluminescent reagents for chemiluminescent imaging (ECL, SuperSignal West Femto, Pierce, cat. no. 1858415).

BioMax Light film for chemiluminescent imaging (Kodak).

Tris-buffered saline (TBS) 10×; for 1 L, solve 87.66 g NaCl and 12.11 g Tris–Cl in water; adjust to pH 7.5.

TBST; for 1 L, add 100 mL 10× TBS (for 150 mM NaCl and 10 mM Tris, pH 8.0) and 10 mL 10% Tween 20 (for 0.1% v/v) to water.

Towbin wet blot transfer buffer 10×; for 1 L, add 30.3 g Tris (for 25 mM), 144 g glycine (for 192 mM), and 100 mL 10% SDS (for 1%). For 1×, add 100 mL 10× buffer and 200 mL methanol to 1 L. Use ice cold.

2.9. Autoradiography

Autoradiography cassette.

Autoradiography film (GE Healthcare Hyperfilm MP or Kodak Bio-Max MR).

Film developing unit.

PhosphorImager scanner with screen (MolecularDynamics or Amersham).

3. Methods

The methods described below outline (1) the construction and transformation of expression plasmids harboring phospho-site variants of CDKA;1, (2) the extraction and purification of the CDK/cyclin complex from proliferating plant material, and (3) the phosphorylation reaction. The following procedure is a modification of the methods of De Veylder et al. (55) and Bögre et al. (63) and has been used successfully in our laboratory (5, 10).

Here, we also highlight several references presenting a wide variety of protocols for analyzing the cell cycle (64–66), giving more details for analyzing CDK activity (67, 68), and presenting excellent information on handling radioisotopes and safety precautions (69–71).

3.1. Overview

In our case of assaying for mutant variants of *Arabidopsis* CDKA;1, we prefer to run a procedure that includes a transfer of the radioactively labeled substrate protein onto nitrocellulose membrane via Western blotting before autoradiography (see Note 18). To obtain reliable results, the samples of choice must be prepared and lysed under the same conditions. It is always preferable to process the samples to be compared simultaneously and run with one assay master mix on one SDS-PAGE gel. Thus, all samples must be subjected to one and the same blotting and evaluation procedure. Otherwise, it will be difficult or even impossible to determine whether a change in radiolabel incorporation is due to the amino acid substitution or treatment, or if that came along with running on and blotting from a different gel, for example.

3.1.1. Time Considerations

If you are planning to run the experiment, especially the kinase assay, for the first time, it is highly recommended to start with a practice run and process no more than a small number of samples at a time. For example, wild-type *Arabidopsis* and one phosphomutant could be harvested and processed simultaneously in order to test the degree of reproducibility of the approach and, more importantly, to allow the experimenter to get used to the methodology. A typical time frame from start to finish for the whole protein procedure is approximately 5 days. It includes approximately 8–12 h for the main assay part, depending not only on the number of samples, but also on the skill level of the researcher and optional preparation that might speed up the entire procedure. Thus, it might be more convenient to harvest and deep-freeze the flowers 1 day before the actual kinase assay experiment and get an early start the next day. The main assay day should end right after stopping the kinase assay, by loading the samples onto the gel and running the gel overnight or best already by overnight Western blotting. The next days have little hands-on work and require antibody incubation and autoradiography steps.

3.2. Generation of Transgenic Plant Material

We applied classical PCR cloning strategies to obtain the mutant alleles of *Arabidopsis* CDKA;1. This protocol was based on subsequent two-step fusion PCR steps and is explained in the following (see Note 19).

3.2.1. Cloning of the Expression Constructs

In brief, primers containing the Gateway® attB1 and attB2 sites (Table 5) were used for flanking the 5′- and 3′-ends of each final fusion construct. The substitution was introduced according to the *Arabidopsis* codon usage (see Note 20) and a silent restriction site was incorporated to later on allow tracking of the mutated version in planta (see Note 21). First, the separate 5′- and 3′-parts of the overall construct containing the attB sites were amplified, and the PCR product cleaned from the remaining oligonucleotides by treating with ExoSAP-IT® (usb/Affymetrix) and fused in a second

Table 5
Gateway® attB-recombination recognition sites for BP cloning of PCR fragments

Name	5′-Sequence-3′
attB1	ggggACAAGTTTGTACAAAAAAGCAGGCTtc
attB2	ggggACCACTTTGTACAAGAAAGCTGGGTc
SelA	TCGCGTTAACGCTAGCATGGATCTC
SelB	GTAACATCAGAGATTTTGAGACAC

gggg extra base pairs; tc and c spacer base pairs; SelA and SelB are standard sequencing primers annealing at either the 5′- or 3′-attB-sites, respectively

round of PCR. The obtained fragments were cloned into pDONR201 vector using Gateway® BP clonase enzyme mix (Invitrogen) and the obtained plasmids analyzed via restriction digest and sequencing. A list of cloning and sequencing primers can be found in Table 5. Thereafter, the insert of the resulting entry vector was recombined into the respective plant transformation destination vector (in this case, pAM-PAT-ProCDKA;1; (3)) using Gateway® LR clonase enzyme mix (Invitrogen), giving rise to the binary plant expression vector. For further details on the procedures, see ref. 72.

The isolated expression vector was transformed into *Agrobacterium tumefaciens* GV3101-pMP90RK (59). Be aware of the high transformation efficiency of this strain and streak out the transformed clones thoroughly. We typically transform 25 μL of thawed *Agrobacterium* suspension with 0.2 μL vector (that can also just be "a little remainder" in a crystal tip), incubate it for 45 min at 28°C, plate 400 μL of YEB onto the selective plates, and inoculate this puddle with 1 or 2.5 μL of the actual transformation reaction.

Before plant transformation, the presence of the constructs in the *Agrobacterium* strain was verified by plasmid isolation and subsequent restriction digestion. Since often the expression vectors can only be isolated in low concentrations or purity, a clearer result can be obtained by back-transforming the plasmid isolated from *Agrobacterium* in *E. coli* followed by plasmid isolation and a repetition of the digests. Unless stated otherwise, all manipulations were performed using standard molecular methods (73).

3.2.2. Plant Transformation and Selection of Transformants

Heterozygous *cdka;1* mutant plants were transformed according to a modified "floral dip" method (74). In our hands, the best transformation results were obtained with healthy plants that are in full blossom, e.g., grown at 18°C until the first filled siliques appeared. Four days before plant transformation, 50 mL *Agrobacterium* preculture was inoculated in selective YEB medium (50 μg/mL each

of rifampicin/kanamycin/carbenicillin) by a single *Agrobacterium* colony and incubated for approximately 2 days at 28°C and 250 rpm in the shaking incubator until opaque. This pre-culture was used to inoculate the final 500 mL culture, which was then incubated again for approximately 2 days with shaking under the same conditions. Before transformation, 5% sucrose and 0.05% Silvett L-77 surfactant were added to the culture. Flowering parts of the plants were immersed in this solution as long as properly wetted, laid down in a plastic tray, and afterward covered with a lid to keep moist. The lid was removed on the following day and all contaminated waste was autoclaved or decontaminated according to good microbiological practice, for instance with Dismozon® pur (Bode Chemie). Typically, 4 weeks after transformation, the seeds are ripe and can be collected. The T1 generation was then sowed on large trays containing soil, stratified at 4°C in the dark for 4–5 days, and transferred to the greenhouse. To identify the transformants, plants were grown on soil for 10–15 days until the cotyledons have formed and then sprayed with a 0.001% BASTA® solution. The spraying was repeated twice in an interval of 2–4 days to select for the plants carrying the construct.

3.2.3. Genotyping

Allele-specific PCRs were carried out to determine the T-DNA insertion and for isolating hetero- and homozygous *cdka;1* transgenics using the primers SALK-LB (left border T-DNA primer for the *cdka;1* SALK line) and N034 or N049 (anneals within genomic wild-type CDKA;1). To identify homozygous knockout plants rescued by a ProCDKA;1:CDKA;1 construct, the primers N048 and N049 were used. The latter two are intron specific to avoid binding with the transgenes. Plant DNA material was prepared in a modified protocol according to ref. 75.

Prepare bead dispenser with 96 stainless steel beads.

Transfer beads into 96-well polypropylene collection block by turning the dispenser/collection block setup overhead. Label blocks with water-proof marker for permanent identification.

Before starting to twitch leaves, check for the correct orientation (A to H and 1 to 12) of the collection blocks! Use a 96-formatted collection plan referring to the sample setup in the block itself.

With fine forceps, collect one or more samples per individual plant and add to the bead in the tube. Try to restrict yourself to collect leaf material of an approximate maximum size of the larger upper diameter of a 1,000 μL pipet tip and use young, still dividing tissue only. Take a small leaf rather than parts of a bigger and older one.

Complement samples to be genotyped with controls (e.g., Col-0 and/or water).

After collection, close blocks with 96-well sealing mat and – if samples cannot be processed the same day – deep-freeze and store at –80°C.

Add 500 μL of extraction buffer for genomic plant DNA preparation ("*sucrose solution/buffer*" or "*Magic buffer*") and seal the wells with polypropylene collection microtube caps. If less material was collected, downscale the buffer accordingly.

Make sure that all the sample tubes are equally filled with sucrose buffer.

Shake blocks in the mixer mill at a frequency of about 25 Hz for 2 min in one orientation of the collection block. Make sure that the leaf suspensions are greenish and that there is only little unsmashed plant material left in the tubes.

If necessary, spin down, open strips and push unsmashed leaves back into the tube, close lids, and shake again for 1–2 min.

Briefly spin down collection blocks at 1,500 RCF to avoid cross-contamination via sample splashing during the opening procedure of the caps.

Grip blocks properly and carefully, rip off 8-well strips with pliers and discard.

Samples in the closed collection blocks (use silicone sealing mats) can be stored at –20°C for many months. The crude suspension can be kept at 4°C for about 1 week without any remarkable loss in sample quality. Caution: Do not invert collection blocks closed with the silicon mats because they do not completely seal the tubes.

For PCR, prepare a master mix with some volumes in excess (e.g., prepare for 25 μL but pipet just 23 μL into the PCR plates). For 25 μL PCR setup, we use 18.8 μL water, 2.5 μL 10× PCR buffer ("*Direct buffer*"), 0.45 μL 10 mM dNTP, 1 μL of each primer, and 0.5 μL Taq polymerase.

Check for equal filling of PCR plate tubes with master mix; adjust if necessary. Use clear pipet tips and a multichannel pipet (8- or 12-channel) for transferring 1.5 μL of the cleared template suspension directly from the spinned plant sucrose buffer preparation. In case only small volumes such as 100 μL have been used for tissue lysis, only extended tips that reach the bottom of the U-shaped bottom can be used.

Always check for equal filling of the tips. If unsure, repeat transfer with fresh tips. You can leave the filled sample tips in the PCR wells for the purpose of overview. In addition, after having transferred all DNA samples, this will help to pipet up and down once with a volume of 10 μL into the PCR plate. Discard tips. If necessary, briefly spin down PCR plates at 1,500 RCF.

Use a hot-start PCR setup with, for example, 35 cycles for genotyping such as the following:

95°C for 5:00 min

95°C for 0:30 min

58°C for 1:00 min × 35

72°C for 2:00 min

72°C for 10:00 min

15°C forever

Underlined temperatures should be cycled about 35×.

After the PCR, check for possible evaporated PCR product and repeat reaction for eligible sample(s).

To clean the steel beads, do not acid-wash beads. Used beads can be cleaned by soaking them for a couple of hours or overnight in a solution of laboratory detergent in warm water. Rinse several times with warm tap water, then deionized water, and optionally with rubbing alcohol. Dry the beads in an open tray at 40–70°C or fill them in bottles for autoclaving (see Note 22).

3.3. Phosphoprotein Extraction

In cells, CDKs exist as phosphoproteins themselves and the maintenance of their phosphorylation state is crucial for their kinase activity. Thus, it is essential to attempt to preserve the phosphate groups linked to a particular threonine in the activation loop by inhibiting protein phosphatases, and appropriate inhibitors must be added to the extraction buffer immediately before use. For our experiments, we use flowering, approximately 4–6 weeks old transgenic *Arabidopsis thaliana* plants (see Note 23). Most experiments require that exactly the same growth conditions are used for all tests done. In addition, the material should be frozen only once since multiple cycles of freezing and unfreezing tissue lower the quality of the material and it starts to degrade. The plant protein extracts should be used immediately for the assays until proper storage conditions (e.g., −70°C and liquid nitrogen) have been determined. It may not be possible to store the extract to be used for activity measurements at all. Ideally, samples should be assayed in multiple replicates and it is recommended to always load those samples on one single gel which should be actually compared because only in this case, all samples will be treated identically.

The lysed plant material should not be frozen and stored for another round of kinase assays; all CDKs of interest have to be analyzed in parallel on the same day. Storing the supernatant for further use as normal protein extract, for experiments other than those relying on the presence of phosphoproteins, is usually not a problem.

All protein manipulation steps are carried out on either wet ice or at 4°C; this is to preserve the activity of the kinase as close as possible to its original state. Therefore, always use prechilled microcentrifuge tubes for extract and bead handling.

Harvest plant flower material (see Note 24) and either snap-freeze in liquid nitrogen and store at –80°C, or immediately grind with a plastic pistil first and then in ice-cold extraction buffer on ice. Larger amounts can be ground in a mortar that is deep-frozen with liquid nitrogen. Use 2 mL buffer per g fresh weight. 100 mg fresh weight will give about 2 mg overall protein; thus, if 100 mg tissue is taken up in 200 μL buffer, one will end up with about 10 mg/mL overall protein.

Continue immediately with processing the suspension for the next steps and finally the in vitro kinase reaction (see Note 25).

Pellet the debris by centrifugation. Spin down the crude extract in a precooled microcentrifuge at maximum speed for 10–20 min at 4°C.

Transfer the supernatant containing the proteins to a fresh tube and re-spin for another 10 min.

Transfer the supernatant to a new chilled microcentrifuge tube on ice. Be careful not to include any of the pellet.

Discard the pellet and proceed with the supernatant.

3.4. Protein Concentration Determination

Use your favorite method to determine the overall protein content of the cleared supernatant and follow the manufacturer's instructions (see Note 26). A decent BSA calibration curve is received when a protein standard such as BSA (stock is 1 mg/mL) is added in steps of 0, 2, 4, 6, 8, 10, 12, 16, 20, 24 μL + 2 μL extraction buffer to each tube. The concentrations usually represent the concentrations present in the supernatants to be tested. The linear range of the assay for BSA is 0.2–0.9 mg/mL.

Prepare as many cuvettes as needed with protein assay components (800 μL water and 200 μL Bio-Rad Protein Assay solution).

Add 2 μL of each sample to the sample cuvettes.

Invert the cuvettes wearing gloves, make sure not to carry over any protein from one assay to the other by wiping the gloves with paper towels.

Incubate at room temperature for at least 5 min. Absorbance will increase over time; samples should be incubated at room temperature for no more than 1 h.

Measure the absorbance at 595 nm.

Plot the standard and the sample values to extrapolate the protein concentrations.

Adjust protein concentrations either to 1 mg/mL or to the lowest concentration within the sample series with extraction or bead buffer.

Use the supernatants to purify the CDK/cyclin complex using $p13^{Suc1}$-conjugated agarose beads (see Note 27).

3.5. p13^{Suc1}-Sepharose Affinity Binding and Pull-Down

Throughout this step, use prechilled microcentrifuge tubes and perform the actual pull-down incubation at 4°C either in a cold room or a fridge. Two problems occur when using commercially available affinity matrix. First, the bead material is expensive and second, it is very difficult to aliquot. The matrix sediments quickly and will cover the surface of all used pipet tips, soon coming along with a partial loss of material. We found that the best way is to take approximately 30 μL of the 50% (v/v) slurry per kinase reaction plus one backup volume extra that will be lost during the procedure. The amount contained in 30 μL pulls down enough complex material even from samples of poor concentration in larger volumes.

Spin down the beads at 400 RCF for 2 min at 4°C and wash two times with 500 μL ice-cold bead buffer.

Now, it is crucial to survey the applied volumes of actual bead material and those of added wash buffer. According to the protocol below, 15 μL of the actual solid agarose conjugate remaining *per reaction* that have to be taken up by 500 μL buffer overall.

Thoroughly aspirate the washes from the beads with a syringe equipped with a 27-G needle. Always keep the pellet on ice (see Note 28).

After the first two washes, the beads must be distributed evenly into the fresh and prechilled assay tubes. It might be appropriate to use either screw-cap microcentrifuge tube or safe-lock microcentrifuge tubes at this point because they will be used all the way until loading the SDS-PAGE gel. The use of these tubes can prevent further radioactive contamination; see Subheading 3.6.

Thus, take the respective volume of a well-mixed but not vortexed (!) bead suspension to each tube:

$$\frac{(30\mu\text{L bead slurry})\times(\text{no. of assays})\times 0.5+500\mu\text{L bead buffer}}{\text{no. of assays}}$$
$$=\text{volume to be aliquoted into each tube}$$

Make sure to flip the tube before every pipetting procedure. The beads precipitate fast and it is of utmost importance to have the same amount of beads in each and every assay tube. Different bead loading gives rise to incomparable results after the actual kinase assay due to different amounts of bound CDK/cyclin complex.

Per desired kinase reaction, equal amounts of total protein (we use roughly 150 μg total protein per assay) were added to the pellet of initially 30 μL of p13^{Suc1}-agarose 50% (v/v) bead slurry in a prechilled 1.5-mL microcentrifuge tube on ice (see Note 29).

Spin down the mixture at 400 RCF for 2 min at 4°C.

Wash the beads three times with 500 μL ice-cold bead buffer until the yellow-greenish color of the p-NO_2PhePO_4 and/or the chlorophyll has almost disappeared.

Wash once with 500 μL pre-kinase buffer (see Note 30).

After the final wash, thoroughly aspirate the buffer from the beads. Take care neither to aspirate the beads nor to dry out the pellet (see Note 28).

Keep the pellets permanently on ice.

3.6. Phosphorylation Reaction

At this point, the pellet consists of the purified CDK/cyclin complex non-covalently bound to the $p13^{Suc1}$ affinity matrix and is ready for the in vitro kinase reaction. The following steps involve radioisotopes and must be carried out behind a β-shield to protect the experimenter from the emitted radiation. ^{32}P was formerly the label of choice but nowadays it gets often substituted by ^{33}P, which has a lower emission energy and therefore displays a lower radiation hazard (69). Always wear safely goggles and the follow the safely rules for working with radioisotopes. Especially the hard β-irradiation of the ^{32}P isotope is known to harm both the cornea and the eye lens strongly (see Note 31).

In this part of the experiment, hot γ-ATP, histone H1, and the desired concentration of unlabeled ATP are subsequently added to the beads. Within the reaction, about 1 μM ATP is required to enhance phosphorylation of co-precipitated substrates and up to 100 μM to favor linearity of the kinase reaction and histone H1 phosphorylation. The overall kinase reaction takes place in a volume of 36 μL (including γ-ATP-labeling mix, see Note 32).

If the SDS-PAGE should be run at the same day as the kinase assay, prepare the acrylamide mixes and cast the gels during the incubation time.

Prepare the assay reaction by adding to each pellet 31 μL of the kinase buffer.

Start the reactions by adding 5 μL of the ATP mix.

From now on, the samples are radioactive and have to be treated with proper caution. Keep the tubes in a β-shielded container.

Make sure that the reaction mix is properly mixed. If ^{32}P is used, according to our experiences, the best way to achieve this is to use a pair of tweezers with a solid grip to take out the sample tubes from the Plexiglas®/Perspex® rack at the cylindrical part and mix the content by carefully flipping the reagent-containing bottom with a glass rod or a similar tool to avoid any unnecessary radiation exposure of the finger tips. However, it is important to manipulate the tube both safely and quickly when handling outside the shielding, so use tools that you are familiar with rather than risk a possible accident by losing control.

Incubating for 45 min at room temperature or 30°C (see Note 33).

Flip the tubes once in a while, e.g., every 10–15 min.

Vortex the samples briefly and then spin the tubes for 1 s to collect the mix at the bottom of the tube.

Terminate the reactions by adding 7.5 μL 6× SDS sample buffer and boiling for 5 min at 95°C or heating for 10 min at 85°C in a heating block. Heat the samples either in screw-cap microcentrifuge tubes or in regular safe-lock microcentrifuge tubes under a radionuclide fume hood to reduce the risk of contamination by vaporizing radioisotope.

Centrifuge at maximum speed for about 1 min. The samples are now ready for immediate loading onto the SDS-PAGE gel. Either keep the tubes on ice until ready to run the gel or alternatively, the samples can be frozen and stored overnight at −20°C.

3.7. SDS-Polyacrylamide Gel Electrophoresis

The SDS-PAGE also needs to be run behind a β-shield. We recommend the use of large gel sizes (16 or 20 cm width, such as PerfectBlue® Twin M, PeqLab) for optimal separation of the protein bands and a possible resolution of hypo- or hyperphosphorylated substrates. Employ neither thin nor small mini-gels. The large gel size conveniently accommodates the sample volume and at a thickness of 1.5 mm, the gel is also relatively robust for the subsequent handling. Phosphorylation or phospho-mimetic substitutions of CDK (e.g., on Thr161) may lead to an increased or reduced mobility of the protein on SDS-PAGE that can be detected as a band-shift on regular immunoblots.

Place the fully assembled SDS-PAGE gel apparatus onto the bench and rinse carefully all the gel pockets that should be used. It helps to run some 2× sample buffer on top of the pockets to visualize where improperly polymerized or clogging polyacrylamide is remaining.

Purge the pockets with a 1,000-μL or 200-μL tip full of running buffer and straighten the pockets with a needle or fine gel loading tip if necessary.

Load the molecular weight marker on both sides of the samples. Also load the wells that remain empty and are directly adjacent to the sample wells with SDS-PAGE sample buffer. We use 200-μL gel-loading pipet tips with extended length to load the samples onto a gel. This helps to avoid spillover but the thin lumen tends to get clogged easily from the agarose bead matrix or the gel itself.

Mount the β-shielding.

Load the samples very carefully into the gel since even a small spill into the upper buffer chamber can make the whole gel radioactive. Make sure that they have been centrifuged at full speed to pellet the agarose beads that otherwise will clog the pipet tips. Load only the supernatant, which contains the CDK variants, cyclins, histone H1 substrate, recombinant p13^{Suc1}:GST, and other soluble proteins precipitated on the beads. Importantly, the supernatant also contains the non-incorporated γ-ATP that represents the majority of the radioactivity actually involved in the entire experiment.

Carefully close the SDS-PAGE tank behind the β-screen.

Run the gel according to the manufacturer's instructions. For example, set the power supply initially to 100 V and then, after 15 min or entry of the dye front into the separating gel, increase the voltage to 150 V. Use cooling devices if possible (see Note 34).

Make sure that the clearly visible running front is kept inside and does not run out of the gel into the bottom reservoir because it contains almost all of the non-incorporated radioisotope. Thus, stop the run with the running front at least 1 cm away from the end of the gel. It will require much more effort to dispose the radioactive waste properly and to take care of the additional and stronger contamination. In a well-run experiment, the bottom reservoir will contain almost no measurable radioactivity.

Completely disconnect the gel unit from the power supply, disassemble the apparatus, quickly measure the activity of the buffers to obtain an estimate of it's radioactivity, and keep the glass plates containing the gel behind a β-shield.

Cut off the very radioactive dye front and the separating gel below and dispose it as soon as possible into the solid radioactive waste to clear your working area from the bulk of the radioisotope. If this procedure has not been accomplished properly, there will still be some non-incorporated radioisotope present in the gel, and the free γ-ATP can diffuse within the gel and cause a significant increase in the general background of the whole gel.

Cut between the stacking and the separating gel to remove and discard the stacking gel and cut the remaining part vertically if not all the pockets of the gel have been loaded to remove and discard the empty lanes.

Cut off a small piece from a corner to mark the orientation of the gel. All unnecessary gel goes directly into the solid radioactive waste.

Rinse the gel briefly with water in a metal or glass tray and discard the wash into the radioactive waste, if necessary (see Note 35).

3.8. Western Transfer of Radioactive Proteins

In the following part, the proteins contained in the kinase reactions are blotted to nitrocellulose, probed with a Cdc2 antibody (α-PSTAIRE), and detected via chemoluminescence. This allows the proper correlation of CDKA;1 abundance with incorporation of the label and thus to estimate the ratio of mutant CDKA;1 variant to its activity toward histone H1. These directions assume the use of a tank blot transfer system that can be cooled and gently run overnight.

For each blot, prepare six sheets of Whatman® filter paper and one sheet of nitrocellulose membrane of the appropriate size, slightly larger than the gel.

Fill the transfer tank three-fourth with ice-cold transfer buffer and add a stir bar.

In a clean, fat-free glass or metal tray, allow the foam sheets and filter papers to wet by capillary action and equilibrate the membrane (see Note 36).

Assemble the lower part of the blotting sandwich: start with the bottom of gel cassette that will face the anode (red, +), foam sheet, three filter papers, and membrane.

Make sure to keep all the layers moist and take precautions not to include air bubbles in the setup. Roll out the air in all the layers using a rinsed test tube or glass rod.

Carefully lay the separating gel on top of the nitrocellulose membrane. Douse with transfer buffer repeatedly.

Add remaining three sheets of filter paper and the foam, ensuring that no bubbles are trapped in the resulting sandwich.

Close transfer cassette and assemble transfer unit. Place the cassette into the transfer tank so that the nitrocellulose membrane is enclosed between the gel and the anode (red, +). It is vitally important to ensure that this orientation or the proteins will be lost!

This procedure is preferentially continued in the cold room at 4°C. If done at room temperature, flood the cooling water system of the tank blot and start circulation to maintain the temperature between 10 and 15°C. Start stirring regardless of how you decided to blot.

Activate the power supply and transfer at roughly 0.65 mA/cm^2 gel size (height × width × 0.65 = mA/gel). The transfer can be accomplished at either 80 mA overnight or 400 mA for 1–2 h at 4°C.

Once the transfer is complete, take the cassette out of the tank and disassemble carefully. Monitor the radioactivity of the transfer buffer and proceed accordingly.

Leave the gel in place on top of the membrane and mark the shape of the gel. The remaining radioactivity can now be found on

the membrane. However, both the gel and excess nitrocellulose are to be discarded in the solid radioactive waste.

Label the membrane at the rims with a pencil or water-proof pen: marker and other important positions and, optionally, also the description of the antibody to be used.

The success of transfer can be estimated by the appearance of the pre-stained molecular weight marker, and phosphorylated histone H1 can usually be detected on the dried membrane with a hand-held β-counter (see Note 37).

Rinse the membrane briefly twice in TBST.

Incubate the nitrocellulose in 10 mL blocking buffer (3% BSA/TBST) for 1 h at room temperature on a rocking platform.

Discard the buffer and rinse the membrane quickly in TBST prior to addition of the primary antibody dilution of rabbit α-PSTAIRE 1:1,000 in 3% BSA/TBST (Santa Cruz).

Incubate with primary antibody overnight at 4°C while rocking.

Remove the primary antibody and wash the membrane three times for 5 min each with 50 mL TBST (see Note 38).

Freshly prepare the secondary horseradish-conjugated antibody dilution of goat α-rabbit 1:5,000 in 3% BSA/TBST (Pierce) and add to the membrane for 1 h at room temperature on a rocking platform.

Discard the secondary antibody and wash the membrane three times for 10 min each with TBST.

After the final wash, briefly dry the membrane on filter paper and label if not done previously.

Line the X-ray film cassette with Saran wrap or a plastic bag and position the blot into a wrap or plastic pocket to separate the membrane to be soaked from the film.

Mix the ECL reagents (SuperSignal West Femto, Pierce) in a ratio of 1:1, immediately spread it over the membrane, rotate it by hand, and incubate at room temperature for 1 min to ensure even coverage.

Squeeze out excess liquid, blot with tissue paper, and move toward the dark room where the remaining steps are done under safe light conditions.

Expose the first film for a suitable exposure time, typically a few minutes, and determine optimal exposure time later on.

3.9. Autoradiography

Phosphorylated histone is detected after Western blot and immunostaining via autoradiography or densitometry using PhosphorImager scanning. Typically, activity is high enough to have strong H1 bands in a 10–16-h exposure, *see* Fig. 2 (see Note 39).

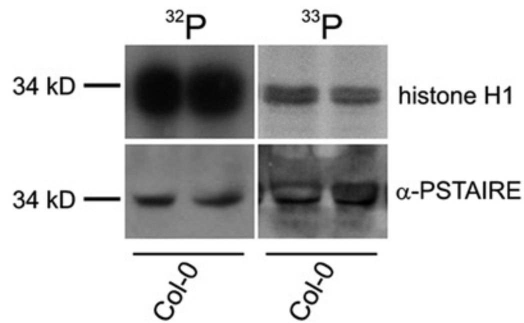

Fig. 2. Comparison of the phosphorus isotopes used in in vitro kinase assays. Two protein samples each of Col-0 flower material were subjected to p13Suc1-mediated pull down and further assayed in in vitro histone H1 kinase assays. On the left hand, [γ-^{32}P]-ATP was used in the kinase reaction, on the right hand [γ-^{33}P]-ATP. Using 32P, both films and screens can easily be overexposed by the high energy emission of β-particles. This can be avoided by using ^{33}P which – in the case of histone H1 as a substrate – gives the characteristic doublet band pattern of ohosphorylated H1. Equal loading was confirmed by immunoblotting of the same membrane used for kinase assay by α-PSTAIRE antibody.

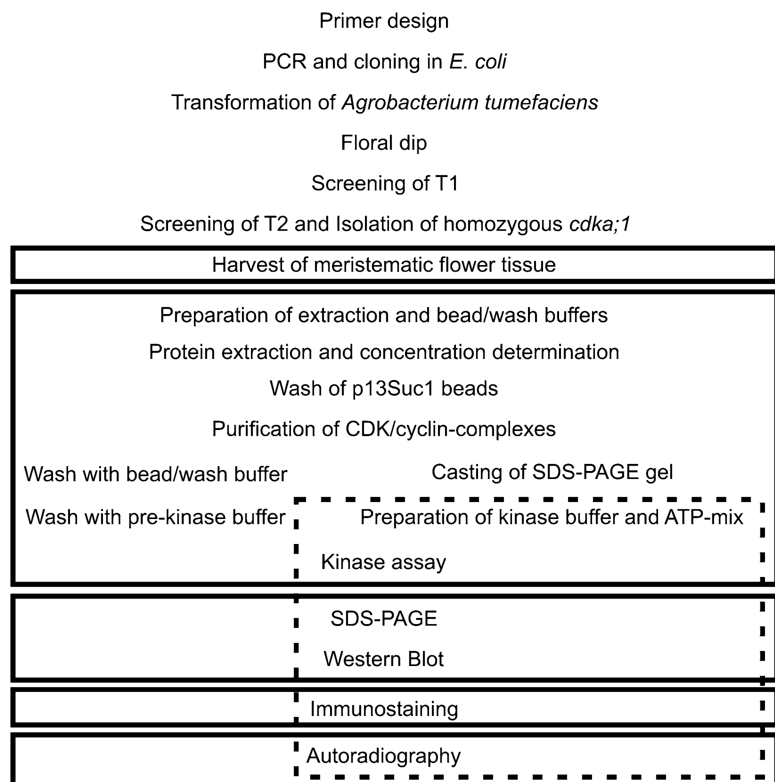

Fig. 3. Flowchart of the analysis of kinase activity in variants of CDKA;1. The steps of the entire procedure described in this chapter are listed in the chart with focus on the kinase assay part. Steps assembled within one black box should be done within one working day. The *dashed box* indicates the involvement of radioisotopes.

Put the sealed membrane into a film cassette with intensifying screen, assemble the film between gel and screen and transfer the entire cassette to a −80°C freezer during exposure of the film.

Alternatively, measure the amount of incorporated label using a PhosphorImager (see Note 40).

3.10. Troubleshooting

If the assay does not work, confirm that both the γ-ATP and the substrate protein have been added at the correct concentrations. It is also crucial to keep the phosphoprotein extract as source of the active kinase complex on ice before use and to always keep it on ice between experimental steps. The problem might be due to a poor storage of the labile enzyme, for example. It will always be better to use freshly harvested flower material. Further on, experimental errors can occur if a proper Western transfer failed. This can easily be checked by using the transfer efficiency of pre-stained markers or via the reversible Ponceau S staining.

4. Notes

1. Cyanogen bromide-activated agarose or sepharose matrix can be cross-linked with recombinant Suc1 in the laboratory or can be purchased (Millipore/Upstate). The matrix is simply used by incubating it in a cell or tissue extract, much as an IP would be done. If necessary, the activity can also be eluted from the beads using excess soluble Suc1 competitor. A Suc1 bead assay is much more sensitive and specific than an assay based on crude extract, but a disadvantage is that the definite composition of the CDK/cyclin complexes bound to it is not clear. Thus, there might be certain CDK/cyclin complex that fails to bind to the beads. In addition, there might be also kinases bound such as MAP kinases, possibly leading to a high background in the activity assay.
2. The binding capability of CDK catalytic-site mutants such as R126A, D127A/E, or R150A (all residues according to human Cdk2) to an affinity resin with a broader specificity such as ATP-agarose can be tremendously impaired, presumably due to alterations in the ATP-binding site (76). CKS affinity is not affected and can easily be double checked via Western blotting for CDK, which is also to be considered as mandatory to exclude unequal CDK loading.
3. One disadvantage of the kinase assay protocol included in this chapter is that it requires the use of a radioactive tracer isotope, either ^{33}P or ^{32}P, with the latter being a high-energy β-emitter. There are a few examples of kinase assays that do not require [γ-^{32}P]-ATP, but they tend to be more specialized

and generally not as widely applicable. However, we present here the use of ^{33}P as a safer alternative with reduced irradiation energy coming along with the very positive side effect of producing much sharper bands on the films of the final assay blots or gels. Furthermore, nonradioactive kinase assays could provide decent alternatives to the use of radioisotopes (KinaseGlo® and ProFluor®, Promega, or PolarScreenT CDK Assay Kit®, Invitrogen).

4. This is the soil composition commonly used at our plant facilities. BioMükk® is a non-poisonous, high-potency biological larvicide that selectively acts against mosquitoes (*Culicidae*), blackflies (*Simuliidae*), and fungus gnats (*Sciaridae*). It contains main protein compounds of *Bacillus thuringiensis var. israelensis* (Bti, 3,000 international units (ITU)/mg). BioMükk® can be replaced by 14 g Confidor® WG 70, which is a systemical insecticide and molluscicide and contains 700 g/kg Imidacloprid (Bayer CropScience).
5. Silvet L-77® is a silicone-polyether copolymer and a mixture of polyalkyleneoxide-modified heptamethyltrisiloxane (84%) and allyloxypolyethyleneglycol methyl ether (16%). It is an essential infiltration media ingredient for all *Agrobacterium*-based transformations of *Arabidopsis* and other plants. 1 mL is sufficient quantity to prepare 3.3 L of vacuum infiltration medium when used at the recommended rate of 0.033% (v/v). We use it, however, at a low concentration as 0.0002%, i.e., 200 ppm.
6. Phosphinothricin-*N*-acetyltransferase (PAT, from *Streptomyces viridochromogenes*) is a gene that confers resistance against the herbicide PPT, the active ingredient in the herbicides Basta, Rely, Finale, and Liberty. It is widely used as a selection marker enabling identification of transformed plant cells and as a source of resistance to the herbicide phosphinothricin for transgenic plants. This is especially due to its easy application, i.e., spraying of the small 1- to 3-week-old plantlets. PPT acts by inhibiting the plant glutamine synthetase, the only enzyme in plants that detoxifies ammonia by incorporating it into glutamine. Inhibition of this enzyme leads to an accumulation of ammonia in the plant tissues, which kills the plant within hours of application. PAT catalyzes the acetylation of the herbicide and thus detoxifies the chemical into an inactive compound.
7. Another well-intentioned advice is to always prepare sufficient amounts of "master mixes" of the reaction buffers, for example, the volume for one reaction in excess, also of the hot ATP mix. Prepare the mixes in a single Falcon or microcentrifuge tube for samples by multiplying the quantities for a single reaction by the total number of reactions plus 1 or 2. Then, the total volume of each reaction mixture can be added to each sample or tube, very conveniently reducing the risk of pipetting errors.

8. The chelators EDTA (Ca^{2+}, Zn^{2+}, Mg^{2+}) and EGTA (Ca^{2+}, Zn^{2+}) strongly inhibit metalloproteases and also both act as potent inhibitors of kinases that require divalent metal ions to be activated.
9. The Na_3VO_4 is both a potent and nonselective tyrosine phosphatase and kinase inhibitor. The inclusion of NaF (and optionally tetra-sodium pyrophosphate, $Na_4P_2O_7$) should effectively inhibit most serine/threonine phosphatases. NaF inhibits both the pyrimidine nucleoside monophosphate kinase and the protein phosphatase 2A (PP2A). To generally inhibit proteases, a cocktail of benzamidine, PMSF, pepstatin A, antipain, leupeptin, EDTA, and EGTA is found to be most effective. Benzamidine hydrochloride inhibits peptidases, Pepstatin A (Isovaleryl-L-Val-L-Val-statinyl-L-Ala-statin) specifically inhibits aspartic proteases and might precipitate from solutions. Aprotinin is also known as bovine pancreatic trypsin inhibitor (BPTI), trypsin kallikrein inhibitor (TKI), or Trasylol® (Bayer). Leupeptin (Ac-Leu-Leu-argininal) was isolated as a peptide antibiotic from *Streptomyces* and reversibly inhibits serine and cysteine proteases. Soybean trypsin inhibitor (STI) is a protein and thus cannot be dissolved in organic solvents and is heat sensitive. Both β-glycerophosphate and p-nitrophenylate (p-NO_2PhePO_4) compete as phosphatase substrates. Acting as potent inhibitors, they strongly stabilize the H1 kinase activity. The latter hydrolyzes to yellow p-nitrophenylate in basic pH ranges that absorbs at 405 nm and might interfere with certain spectrophotometric measurements. Sometimes we include 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (to replace the highly toxic PMSF), leupeptin, pepstatin, and/or chymostatin in the extraction buffer, although this will greatly increase the cost of the solution with relatively little gain in quality of the data.
10. Orthovanadate VO_4^{3-} is an analog of the phosphate ion PO_4^{3-}. Activation depolymerizes the vanadate, converting it to a more potent inhibitor of protein phosphotyrosyl phosphatases (77). Activation protocol: Prepare 200 mM sodium orthovanadate (MW 183.9, e.g. ,0.37 g to 10 mL solution) in water. Adjust pH to 10.0 with either 1 M NaOH or 1 M HCl, depending on the lot supplied by the manufacturer. At pH 10.0, the solution turns yellow. Boil the solution until it turns colorless, this might take up to 15 min. Allow the solution to cool to room temperature. Readjust pH to 10.0 and repeat the steps until the solution remains colorless and the pH stabilizes at 10.0. Store in aliquots at –20°C. Caution: adding DTT rapidly inactivates sodium orthovanadate and the inhibition of cystein proteases can also be reversed by reduced thioles such as DTT. Vanadate influences certain standard methods used for protein concentration

determination. Coomassie brilliant blue will be negatively affected, but Lowry, BCA, or Bradford still can be used (78, 79). Vanadate polymers that reform in aqueous solution turn the buffers used here into a yellowish color (see Note 22).

11. PMSF is highly toxic for mucous membranes, eyes, and skin. Every exposure has to be avoided. It is recommended to be replaced by the equally effective, less harmful but more expensive AEBSF. PMSF is a serine protease inhibitor and inhibits ATPase, alkaline, protein phosphotyrosyl, and other dual-specific phosphatases. It is soluble in water-free DMSO, methanol, ethanol, or best in isopropanol. Can be stored at 4°C or is even stable for months at room temperature. At −20°C, PMSF precipitates from isopropanol. PMSF is poorly soluble in water and since it has a short half-life in aqueous solutions, PMSF should be made fresh and added to all buffers shortly before they are used, and they should be used within 1 or 2 h. The concentrated PMSF should be added to a vigorously stirred solution to prevent the PMSF from precipitating.
12. Either use screw-cap microcentrifuge tubes when boiling the samples to reduce the risk of vaporizing radioisotope or work under a radionuclide fume hood, and punch holes into the lids to prevent that they open during heating and deliver droplets of their radioactive content in the working area.
13. Usually, ATP is purchased as a disodium salt (Na_2-ATP); however, most kinases require it to be complexed with Mg^{2+} (or Mn^{2+}) for being an efficient kinase substrate. If no Li-ATP, as suggested in this chapter, is available, a Mg^{2+}-ATP solution can easily be prepared from 20 mM $MgCl_2$ (or $MgSO_4$) and 20 mM Na_2A-TP stock solutions by mixing of equal volumes and adjusting the pH to 7.4 with 100 mM HCl or 100 mM NaOH. Mg^{2+}-ATP solution can be stored in small single-use aliquots at −20°C. Precipitation might occur if the pH is not properly adjusted.
14. γ-ATP is usually available at a variety of specific activities. A good specific activity to use is >3,000 Ci/mmol or even the highest one at approximately the double activity as mentioned in this protocol. The activity is normally provided at a concentration of 10 mCi/mL, ready to use. Here, we recommend using high specific activity to reduce the impact of "contamination" of cold ATP within the isotope lot itself.
15. Acrylamide is a neurotoxin and carcinogenic in the unpolymerized form (of which even the polymerized gel contains still traces of) and thus, has to be handled very carefully. Any waste has to be disposed off accordingly.
16. TEMED is best stored at room temperature in a desiccator. Buy small bottles as it may decline in quality after opening and thus, gels will take longer to polymerize.

17. To prevent bubbles at the border of the separating gel, it is overlaid with either isopropanol or water-saturated isobutanol. For the latter, shake equal volumes of water and isobutanol in a glass bottle and allow separation. Use the top layer. Store at room temperature.
18. If the experimenter desires the protocol to be altered from the way presented here in terms of including a cold Western blot for determining the amount of CDK in the samples, the pull-down material has to be split in two for Western blotting pull-down control and actual kinase assay reaction. We recommend to subject 150 μg total protein to the kinase assay and pull-down control if done as described in the main protocol, or if a separate cold blot is desired, then one could use 30 μg of extra total protein for this purpose.
19. Alternatively, site-directed or megaprime mutagenesis can be applied to receive the desired mutant variants. In our case, we tried the QuikChange Site-Directed Mutagenesis Kit, PfuTurbo and PfuUltra proofreading polymerases (all from Stratagene). These polymerases have sixfold or even a higher fidelity in DNA synthesis than regular Taq or Pfu enzymes. The error rate is also drastically reduced. The procedure utilizes a supercoiled donor vector with the insert of interest and two synthetic oligonucleotide primers containing the desired mutation(s) which were designed according to the manufacturer's instructions and had a minimal overlap of 15 base pairs from the actual site of mutagenesis. A small amount of starting template plasmid, the high fidelity of the DNA polymerase, and the low number of thermal cycles contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction. The method used here allowed using miniprep double-stranded DNA plasmids and this template can be directly subjected to the mutagenesis procedure. The procedure was used to generate single or multiple point mutations and multiple base pair exchanges both to switch amino acids and to introduce unique restriction sites for screening purposes. For single point mutations, 12 cycles were used and for single amino acid changes, 16 cycles. Proper amplification was verified by electrophoresis of several microliters of the PCR product on a 1% agarose gel. Following temperature cycling, the parental DNA template has to be removed of the PCR product by treatment with DpnI endonuclease (Fermentas). A prerequisite for the use of DpnI is that the template DNA must be isolated from *E. coli* strains such as DH5α that dam methylates its DNA. After digestion, the remaining nicked vector DNA containing the desired mutations was directly transformed into DH5α electrocompetent cells. It is not uncommon to observe very low numbers of

colonies, especially when generating large mutations. Most of the colonies that do appear will contain mutagenized plasmid. Due to the sometimes occurring leakiness of the DpnI digestions, the use of fusion PCRs instead of the site-directed method was often more efficient in our hands.

20. The *Arabidopsis* relative synonymous codon usage (RSCU) is the observed frequency divided by the expected frequency assuming random codon usage in the respective organism. Tables can be accessed at http://www.nal.usda.gov/pgdic/Probe/v4n1_2/table1a.html or http://www.kazusa.or.jp.

21. The silent mutation restriction enzyme scan (SILENT) is available at the Mobyle portal at http://bioweb.pasteur.fr/seqanal/interfaces/silent.html.

22. If nucleic acids are isolated from disrupted tissue as in our case, beads can also be soaked in a 1:10 dilution of *Eau de Javel* (store-bought household bleach, FLOREAL Haagen) for 5 min. This both cleans and sterilizes the beads and completely destroys contaminating nucleic acids (80).

23. The beginning of flowering strongly depends on the growth conditions applied in the greenhouse or the phytochambers and it is strongly recommended to always grow the different genotypes to be compared in their kinase activity at the same time under exactly the same conditions and to treat the crude material all the way equally. Besides that, when *Agrobacterium* transfer DNA (T-DNA) is transformed into *Arabidopsis*, it is randomly integrated into the genome. Thus, insertions occur by chance in hetero- or euchromatic regions, in single or multiple copies, in tandem, inverted repeats or truncated versions which can explain the consequent variability of expression. It is, therefore, recommended to test the expression level of several individual transgenic lines of the same construct, preferably by Western blotting, as it is necessary to both have high and comparable amounts of kinase to perform the activity assay.

    The binary vectors that we use for *Agrobacterium*-mediated transformation carry several selection markers, e.g., nptII/neo (neomycin phosphotransferase II gene from transposon Tn5, against Neomycin/Kanamycin), hph (Hygromycin-phosphotransferase from *E. coli*, destroys Hygromycin), sul2 (dihydropteroate synthase, degrades the sulfonamide antibiotic Sulfadiazine), or most conveniently, PAT (resistance against BASTA®). It is highly advisable to not treat differentially with these agents, e.g., spray a construct carrying thus resistant subpopulation of the individuals to be tested with the selective agents and keep other lines, e.g., the wild-type control or mutants without the specific transgene conferring the

resistance, only untreated (because they are sensitive). Different treatments of subpopulations will invariably result in differences in growth and development, and at least retard the treated population in terms of flowering start compared to the nontreated plants. This would give rise to a difference in harvesting date and quality of the flower material to be assayed.

24. Flower buds contain meristematic and thus highly dividing tissues, with high levels of CDK expression. Try to collect only closed flower buds using fine forceps. We found CDK/cyclin activity from flower material to be stable at −80°C and reliable assays were possible even up to 1 year after harvest.

25. Once the flower buds have been lysed by the addition of extraction buffer and grinding, the proteins will have to be processed all the way to the step where the kinase reaction products are loaded onto the SDS-PAGE gel. Flower buds can be preserved quite easily at −80°C and the next "storage format" or possibility to end the working day is only the radioactive kinase assay products from the stopped reaction. Before this, the experimenter has to deal with unstable complexed subunits and activatory phosphorylations that have to be preserved and must not be frozen in order to give reliable results. Thus, once the lysate is produced, it should not be stored but used immediately. Freezing and storing the cells in lysis buffer even for just 1 day can result in marked loss of kinase activity (81).

26. For the determination of the overall protein concentration in each sample, we routinely use the Protein Assay Kit from Bio-Rad that is based on the method of Bradford. Vanadate influences certain standard methods used for protein concentration determination (see Note 6). We usually add 800 μL water with 200 μL protein assay solution to each cuvette, add 2 μL of supernatant, and incubate for 5 min at room temperature. In case we expect unusually low yield (<1 mg/mL), we scale up the assay conditions in terms of transferred supernatant volume. The standard curve is established by using different amounts of BSA. To each BSA sample, we also add the same volume of extraction buffer (2 μL) that is contained in the unknowns; this compensates for the slight coloring from the inhibitors.

27. Preservation of CDK activity seems to be a very sensitive issue as often mentioned in animal or yeast protocols. Brooks et al. postulate that CDK activities in frozen lysates could remain measurable for 2–3 days after extraction when stored in liquid nitrogen. However, the degree of measurable activity deteriorates rapidly such that only 50–75% of original activity in freshly prepared lysates remains after 1 day of storage and 25–50% after 2 days (60).

28. During the wash steps of the bead material and the pull downs, it is crucial to not lose any of the pellet, but at the same time to wash it most efficiently. In order to remove the supernatant as completely as possible after each centrifugation step, we use syringe and fine needle (27 gauge/“27 G” or 0.4 mm in diameter and ¾ in. or 2 cm in length, gray color code; the higher the G or gauge number, the smaller the lumen of the needle) to aspirate the supernatant. The lumen of the needle tip is kept toward the wall of the tube and it must be prevented to insert the needle right into the pellet, because this will soon clog the very fine needles, whereas with needles of larger diameters, this procedure will lead to some loss of pellet and irreproducible results.
29. We routinely use this commercially available liquid suspension of recombinant $p13^{Suc1}$:GST fusion protein (source of Suc1 is *S. pombe*, MW of fusion protein is 39 kDa) that is non-covalently bound to glutathione-agarose. In these lots, 2.5 mg $p13^{Suc1}$:GST is bound to 250 μL of agarose beads provided as a 50% (v/v) gel slurry in PBS containing 50% glycerol and 0.05% sodium azide. Given the MW of 13 kDa of Suc1, 26 kDa of GST, and the stoicheometric binding of CKS to CDK/cyclin complex at a ratio of 1:1 (49), the content of one tube with its 2.5 mg binding partner could theoretically precipitate about 1.6 mg CDK. Since the association of the glutathione and the GST is not cross-linked and the bead material is quite sensitive per se, the material must not be vortexed.
30. If a separate pull-down control is desired, the pellet can *now* be splitted in two for Western blotting. At the same time, an optional additional pull-down step can be included: after centrifugation, fresh beads can be added to the supernatant and incubated for an additional time to increase the amount of extracted CDK/cyclin complex.
31. Working with ^{33}P and ^{32}P requires caution. This assay uses radioactively labeled γ-ATP that contains a covalently linked ^{32}P-orthophosphate group at the γ-position of the triphosphate. This phosphate is – in the case of ^{32}P – a strong emitter of β-particles of high energy which should be handled and disposed off according to the federal and institutional radiation protection procedures applicable to work with the respective radioactive isotope. Contact your institutional radiation safety representative for proper handling and disposal procedures. Some standard protocols about good laboratory practice with regard to working with ^{33}P and ^{32}P can be found in the appendix (literature: Radiation safety, (69–71)). Make sure to use Plexiglas®/Perspex® shields while in the vicinity of radiation and also take precaution for proper shielding for your lab mates. Never use plain lead with its high atom weight to

"protect" against β-irradiation emitted from ^{32}P because the high energy will be transferred further to Bremsstrahlung, which is as difficult to shield as γ-irradiation or X-rays and multiplies the risk of radiation exposure. Just use Plexiglas®/Perspex®, glass, or water, which are materials with a low atom weight and high shielding capacity toward β-particles.

In this experiment, either ^{33}P- or ^{32}P-labeled γ-ATP can be used. Most protocols rely on ^{32}P, maybe because "it has always been that way" and the use of ^{32}P is well established in the laboratory due to the lower cost compared to that of ^{33}P. However, we strongly recommend the use of ^{33}P for several reasons such as remarkably better experimental performance and radiation safety. ^{33}P offers clear advantages over ^{32}P with respect to the ease of handling because the maximum energy of the emitted β-radiation is between that one of 35 S (0.17 MeV) and ^{32}P (1.71 MeV) and does not require as much shielding as is needed for ^{32}P. The effects of the use of a "safer radionuclide" must not be underestimated because the experimenter will have less trouble with, e.g., handling and this can easily advantageously result in a higher confidence throughout performing the experiment. Thus, the replacement might turn out to be conductive for a more cool-headed working. The range of ^{32}P-β in air is up to 790 cm, and in water and Plexiglas® about 0.8 cm. ^{33}P-β reaches only 49 cm in air and shielding is not necessarily needed for activity amounts typically used in the laboratory (71). In fact, the β-radiation emitted from ^{33}P ($E_{max} = 0.25$ MeV) can barely penetrate through two pairs of gloves and the outer dead layer of skin, so the external exposure hazard associated with even mCi amounts of ^{33}P is minimal. ^{33}P preparations are accordingly only delivered in plain plastic containers instead of in the heavily lead-shielded ones for the transport of ^{32}P products. The best way to determine whether additional shielding is needed when using this isotope is to monitor the source using a β-sensitive radiation meter. If counts can be detected, add a layer of Plexiglas® as described for ^{32}P. Another great advantage of using ^{33}P is that gel bands visualized on autoradiographs of ^{33}P-labeled compounds are much sharper than bands labeled with ^{32}P because the low-energy β-radiation does not scatter as the high-energy β-radiation of ^{32}P. For 33P, a much higher specific activity can be achieved due to the longer half-life of ^{33}P (25 days compared to 14 days for ^{32}P), which also allows a broader time frame for experimental planning. Despite the slightly higher cost of ^{33}P, these distinct features make ^{33}P-labeled nucleotides very interesting for use in experiments where discrimination of closely spaced gel bands is important.

Good ranges for the final overall ATP concentration is up to 100 μM (including negligible amounts of the γ-ATP and at a specific activity of approximately. 1 μCi/μL reaction volume).

32. Controls are important to evaluate the results of your experiments. You should always include a no-substrate control, a no-enzyme source control, and a heat-denatured enzyme control in your assays. As a further test for the specificity of the kinase assay, a parallel set of controls can be inhibited with chemical inhibitors of the CDK/cyclin complex. Two of the most specific inhibitors are probably roscovitine (5 μM) and olomoucine (10 μM). They can be added to a separate reaction to indicate whether the bulk of the kinase activity is due to a CDK/cyclin complex.

33. The enzymatic activity assayed is the initial rate of the reaction when the substrates are not limiting. The number of moles of reaction product formed after a certain time (number of moles of phosphate transferred to H1 histone) is a measure of the initial reaction rate if the reaction is still linear at this time. The assay described here fulfils these conditions: neither ATP nor histone is limiting and the reaction is still linear even toward the end of the incubation time. It is often advantageous to perform kinase assays at 30°C rather than 37°C because the lower temperature makes it easier to stay within the linear range of the phosphotransfer reaction catalyzed by the kinase, thus providing more control over the assay. Most kinases have a K_m for ATP of 1–100 μM and if there is too much ATP in the reaction mixture, it will be difficult to measure phosphotransfer. An ATP concentration of 50–100 μM tends to work well; at this concentration, the enzyme should be working in the range of 50% of maximum, depending on its apparent K_m for ATP. Usually, the substrate concentration is high so that the enzyme is working at or close to V_{max}. The concentration of radioactive γ-ATP is negligible compared to the added cold ATP.

34. Some instruments allow cooling via water tubes, which might help to chill down the buffer tank and the gels during a long run. One can also run the gel overnight, e.g., at 35–50 V according to the apparatus of choice. In this case, in the next morning, the voltage can be increased again to complete the run, if required.

35. At this point, the SDS-PAGE gel can be processed in two different ways. We prefer the hot Western blot to correlate the histone H1 kinase activity directly with the abundance of – desirably equal – CDKA;1 loading monitored by immunoprobing with α-PSTAIRE antibody. However, if the experimenter decides to just check overall protein abundance and/or only incorporation of radiolabel, the gel can

be optionally stained with Coomassie Brilliant Blue (CBB). CBB staining solution is 50% methanol, 10% glacial acetic acid, and 0.12–0.25% Coomassie blue R-250 in water; destaining solution is 10–50% methanol and 10% glacial acetic acid in water. After staining, the gel can be fixed with fixing solution (25–50% methanol and 7–10% acetic acid) for 45 min to 1 h and then soaked in anti-cracking solution (7% methanol, 7% glacial acetic acid, and 1% glycerol) for 5 min. Place the gel on a sheet of Whatman® 3MM filter paper and cover with cellophane paper or plastic wrap and dry at 80°C for 30–120 min under a vacuum using a conventional gel dryer with safety Woulff bottle and (oil) vacuum pump; dry completely. The solutions must be disposed into the radioactive waste, if necessary. Photo- and autoradiograph. In general, a Coomassie stain could be used to check for equal amounts of substrate in each samples. In our special case, however, where not only a doublet of histone H1 runs at about 30–34 kDa but also multiple variants of CDKA;1 at 34 kDa, that might be difficult. The same is true for Ponceau S staining of blotted Western membranes.

36. Equilibration of different membranes: nitrocellulose is briefly immersed in water and then in transfer buffer. Immobilon (Millipore) is briefly wetted in methanol, then kept for 2 min in water, and moved for 5–10 min into transfer buffer; PVDF should be wetted briefly in 60% methanol and then in transfer buffer, and assembled with the glossy side toward the gel.
37. The membrane can be dried between Whatman® filter paper and optionally stained with Ponceau S red dye (either 0.1–0.22% Ponceau S in 1–3% acetic acid or 2% Ponceau S in 30% trichloroacetic acid (TCA) and 30% sulfosalicyclic acid, can be re-used multiple times). Immerse membrane in stain, shake for 10 min, and destain by rinsing with water. In general, a Ponceau S stain could be used to check for equal amounts of substrate in each samples. The same is true for Coomassie staining, see also Fig. 2 and Note 31.
38. The primary antibody can be saved for subsequent experiments in the presence of 0.02% sodium azide (NaN_3, stock 10%, store at room temperature) and stored at 4°C. NaN_3 is very toxic and must be strictly kept away from heavy metal salts under all circumstances because explosive azides can form that are highly sensitive to touch and warmth. Primary antibodies conserved like this can be used for many blots over several months; however, the length of exposure to film upon ECL treatment might have to be adjusted.
39. On a 12% SDS-PAGE, the labeled histone H1 will appear as a doublet of two distinct bands running at ~32 and 34 kDa, compare to Fig. 2.

40. Given the limited linear response of X-ray film, densitometry is an inaccurate way to measure the incorporation of label. It will provide only a rough guide to the relative amount of kinase activity in each sample. A much better way to quantify the kinase activities would be to use liquid scintillation counting for either ^{33}P or ^{32}P.

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References

1. Morgan, D. O. (2007) The cell cycle: principles of control, New Science Press Ltd in association with Oxford University Press, London.
2. Iwakawa, H., Shinmyo, A., and Sekine, M. (2006) *Arabidopsis* CDKA;1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. Plant J 45, 819–31.
3. Nowack, M. K., Grini, P. E., Jakoby, M. J., Lafos, M., Koncz, C., and Schnittger, A. (2006) A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. Nat Genet 38, 63–7.
4. Inze, D. (2007) Cell Cycle Control and Plant Development, Vol. 32, Wiley-Blackwell, Oxford.
5. Dissmeyer, N., Nowack, M. K., Pusch, S., Stals, H., Inze, D., Grini, P. E., and Schnittger, A. (2007) T-loop phosphorylation of *Arabidopsis* CDKA;1 is required for its function and can be partially substituted by an aspartate residue. Plant Cell 19, 972–85.
6. Harashima, H., Shinmyo, A., and Sekine, M. (2007) Phosphorylation of threonine 161 in plant cyclin-dependent kinase A is required for cell division by activation of its associated kinase. Plant J 52, 435–48.
7. Kaldis, P. (2006) Cell cycle regulation, Springer, Berlin; London.
8. Shimotohno, A., and Umeda, M. (2007) CDK phosphorylation, in The cell cycle control and plant development; Annual plant reviews, 32 (Inze, D., Ed.), Blackwell Publ., Oxford [u.a.].
9. Morgan, D. O. (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13, 261–91.
10. Dissmeyer, N., Weimer, A. K., Pusch, S., De Schutter, K., Kamei, C. L., Nowack, M. K., Novak, B., Duan, G. L., Zhu, Y. G., De Veylder, L., and Schnittger, A. (2009) Control of cell proliferation, organ growth, and DNA damage response operate independently of dephosphorylation of the *Arabidopsis* Cdk1 homolog CDKA;1. Plant Cell 21, 3641–54.
11. Dissmeyer, N., Weimer, A. K., De Veylder, L., Novak, B., and Schnittger, A. (2010) The regulatory network of cell-cycle progression is fundamentally different in plants versus yeast or metazoans. Plant Signal Behav 5, 1613–8.

12. Bleeker, P. M., Hakvoort, H. W., Bliek, M., Souer, E., and Schat, H. (2006) Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant Holcus lanatus. Plant J 45, 917–29.
13. Ellis, D. R., Gumaelius, L., Indriolo, E., Pickering, I. J., Banks, J. A., and Salt, D. E. (2006) A novel arsenate reductase from the arsenic hyperaccumulating fern Pteris vittata. Plant Physiol 141, 1544–54.
14. Gould, K. L., and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature 342, 39–45.
15. Krek, W., and Nigg, E. A. (1991) Mutations of p34cdc2 phosphorylation sites induce premature mitotic events in HeLa cells: evidence for a double block to p34cdc2 kinase activation in vertebrates. Embo J 10, 3331–41.
16. Krek, W., Marks, J., Schmitz, N., Nigg, E. A., and Simanis, V. (1992) Vertebrate p34cdc2 phosphorylation site mutants: effects upon cell cycle progression in the fission yeast Schizosaccharomyces pombe. J Cell Sci 102 (Pt 1), 43–53.
17. Ducommun, B., Brambilla, P., Felix, M. A., Franza, B. R., Jr., Karsenti, E., and Draetta, G. (1991) cdc2 phosphorylation is required for its interaction with cyclin. Embo J 10, 3311–9.
18. Atherton-Fessler, S., Parker, L. L., Geahlen, R. L., and Piwnica-Worms, H. (1993) Mechanisms of p34cdc2 regulation. Mol Cell Biol 13, 1675–85.
19. Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci USA 103, 1988–93.
20. Michard, E., Dreyer, I., Lacombe, B., Sentenac, H., and Thibaud, J. B. (2005) Inward rectification of the AKT2 channel abolished by voltage-dependent phosphorylation. Plant J 44, 783–97.
21. Kanamaru, K., Wang, R., Su, W., and Crawford, N. M. (1999) Ser-534 in the hinge 1 region of *Arabidopsis* nitrate reductase is conditionally required for binding of 14-3-3 proteins and *in vitro* inhibition. J Biol Chem 274, 4160–5.
22. Hass, C., Lohrmann, J., Albrecht, V., Sweere, U., Hummel, F., Yoo, S. D., Hwang, I., Zhu, T., Schafer, E., Kudla, J., and Harter, K. (2004) The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*. Embo J 23, 3290–302.
23. Leibfried, A., To, J. P., Busch, W., Stehling, S., Kehle, A., Demar, M., Kieber, J. J., and Lohmann, J. U. (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. Nature 438, 1172–5.
24. Kirchler, T., Briesemeister, S., Singer, M., Schutze, K., Keinath, M., Kohlbacher, O., Vicente-Carbajosa, J., Teige, M., Harter, K., and Chaban, C. (2010) The role of phosphorylatable serine residues in the DNA-binding domain of *Arabidopsis* bZIP transcription factors. Eur J Cell Biol 89, 175–83.
25. Wang, X., Goshe, M. B., Soderblom, E. J., Phinney, B. S., Kuchar, J. A., Li, J., Asami, T., Yoshida, S., Huber, S. C., and Clouse, S. D. (2005) Identification and functional analysis of *in vivo* phosphorylation sites of the *Arabidopsis* BRASSINOSTEROID-INSENSITIVE1 receptor kinase. Plant Cell 17, 1685–703.
26. Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M. B., Huber, S. C., and Clouse, S. D. (2008) Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. Dev Cell 15, 220–35.
27. Kim, T. W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J. X., Sun, Y., Burlingame, A. L., and Wang, Z. Y. (2009) Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat Cell Biol 11, 1254–60.
28. Hardtke, C. S., Gohda, K., Osterlund, M. T., Oyama, T., Okada, K., and Deng, X. W. (2000) HY5 stability and activity in *Arabidopsis* is regulated by phosphorylation in its COP1 binding domain. Embo J 19, 4997–5006.
29. Otterhag, L., Gustavsson, N., Alsterfjord, M., Pical, C., Lehrach, H., Gobom, J., and Sommarin, M. (2006) *Arabidopsis* PDK1: identification of sites important for activity and downstream phosphorylation of S6 kinase. Biochimie 88, 11–21.
30. Zegzouti, H., Anthony, R. G., Jahchan, N., Bogre, L., and Christensen, S. K. (2006) Phosphorylation and activation of PINOID by the phospholipid signaling kinase 3-phosphoinositide-dependent protein kinase 1 (PDK1) in *Arabidopsis*. Proc Natl Acad Sci USA 103, 6404–9.
31. Sirichandra, C., Gu, D., Hu, H. C., Davanture, M., Lee, S., Djaoui, M., Valot, B., Zivy, M., Leung, J., Merlot, S., and Kwak, J. M. (2009) Phosphorylation of the *Arabidopsis* AtrbohF NADPH oxidase by OST1 protein kinase. FEBS Lett 583, 2982–6.
32. Takeda, S., Gapper, C., Kaya, H., Bell, E., Kuchitsu, K., and Dolan, L. (2008) Local positive

feedback regulation determines cell shape in root hair cells. Science 319, 1241–4.

33. Dinesh-Kumar, S. P., Tham, W. H., and Baker, B. J. (2000) Structure-function analysis of the tobacco mosaic virus resistance gene N. Proc Natl Acad Sci USA 97, 14789–94.
34. Hildebrandt, J. D., Day, R., Farnsworth, C. L., and Feig, L. A. (1991) A mutation in the putative Mg(2+)-binding site of Gs alpha prevents its activation by receptors. Mol Cell Biol 11, 4830–8.
35. Chen, S. Y., Huff, S. Y., Lai, C. C., Der, C. J., and Powers, S. (1994) Ras-15A protein shares highly similar dominant-negative biological properties with Ras-17N and forms a stable, guanine-nucleotide resistant complex with CDC25 exchange factor. Oncogene 9, 2691–8.
36. Wu, T. H., and Marinus, M. G. (1994) Dominant negative mutator mutations in the mutS gene of Escherichia coli. J Bacteriol 176, 5393–400.
37. Jelic, M., Soll, J., and Schleiff, E. (2003) Two Toc34 homologues with different properties. Biochemistry 42, 5906–16.
38. Aronsson, H., Combe, J., Patel, R., and Jarvis, P. (2006) *In vivo* assessment of the significance of phosphorylation of the *Arabidopsis* chloroplast protein import receptor, atToc33. FEBS Lett 580, 649–55.
39. Oreb, M., Zoryan, M., Vojta, A., Maier, U. G., Eichacker, L. A., and Schleiff, E. (2007) Phospho-mimicry mutant of atToc33 affects early development of *Arabidopsis thaliana*. FEBS Lett 581, 5945–51.
40. Anderson, J. C., Pascuzzi, P. E., Xiao, F., Sessa, G., and Martin, G. B. (2006) Host-mediated phosphorylation of type III effector AvrPto promotes Pseudomonas virulence and avirulence in tomato. Plant Cell 18, 502–14.
41. Xiao, F., Giavalisco, P., and Martin, G. B. (2007) Pseudomonas syringae type III effector AvrPtoB is phosphorylated in plant cells on serine 258, promoting its virulence activity. J Biol Chem 282, 30737–44.
42. Champagne, J., Laliberte-Gagne, M. E., and Leclerc, D. (2007) Phosphorylation of the termini of Cauliflower mosaic virus precapsid protein is important for productive infection. Mol Plant Microbe Interact 20, 648–58.
43. Citovsky, V., McLean, B. G., Zupan, J. R., and Zambryski, P. (1993) Phosphorylation of tobacco mosaic virus cell-to-cell movement protein by a developmentally regulated plant cell wall-associated protein kinase. Genes Dev 7, 904–10.
44. Van Leene, J., Stals, H., Eeckhout, D., Persiau, G., Van De Slijke, E., Van Isterdael, G., De Clercq, A., Bonnet, E., Laukens, K., Remmerie, N., Henderickx, K., De Vijlder, T., Abdelkrim, A., Pharazyn, A., Van Onckelen, H., Inze, D., Witters, E., and De Jaeger, G. (2007) A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Mol Cell Proteomics 6, 1226–38.
45. Nieuwland, J., Menges, M., and Murray, J. A. (2007) The plant cyclins, in Cell Cycle Control and Plant Development (Inze, D., Ed.), John Wiley & Sons Inc.
46. Boruc, J., Van den Daele, H., Hollunder, J., Rombauts, S., Mylle, E., Hilson, P., Inze, D., De Veylder, L., and Russinova, E. (2010) Functional modules in the *Arabidopsis* core cell cycle binary protein-protein interaction network. Plant Cell 22, 1264–80.
47. Van Leene, J., Hollunder, J., Eeckhout, D., Persiau, G., Van De Slijke, E., Stals, H., Van Isterdael, G., Verkest, A., Neirynck, S., Buffel, Y., De Bodt, S., Maere, S., Laukens, K., Pharazyn, A., Ferreira, P. C., Eloy, N., Renne, C., Meyer, C., Faure, J. D., Steinbrenner, J., Beynon, J., Larkin, J. C., Van de Peer, Y., Hilson, P., Kuiper, M., De Veylder, L., Van Onckelen, H., Inze, D., Witters, E., and De Jaeger, G. (2010) Targeted interactomics reveals a complex core cell cycle machinery in *Arabidopsis thaliana*. Mol Syst Biol 6, 397.
48. Lorca, T., Labbe, J. C., Devault, A., Fesquet, D., Capony, J. P., Cavadore, J. C., Le Bouffant, F., and Doree, M. (1992) Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. Embo J 11, 2381–90.
49. Bourne, Y., Watson, M. H., Hickey, M. J., Holmes, W., Rocque, W., Reed, S. I., and Tainer, J. A. (1996) Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. Cell 84, 863–74.
50. Brown, N. R., Noble, M. E., Endicott, J. A., and Johnson, L. N. (1999) The structural basis for specificity of substrate and recruitment peptides for cyclin-dependent kinases. Nat Cell Biol 1, 438–43.
51. DeLano, W. L. (2005), DeLano Scientific LLC, San Carlos, CA, USA.
52. Brizuela, L., Draetta, G., and Beach, D. (1987) p13suc1 acts in the fission yeast cell division cycle as a component of the p34cdc2 protein kinase. Embo J 6, 3507–14.
53. Arion, D., Meijer, L., Brizuela, L., and Beach, D. (1988) cdc2 is a component of the M phase-specific histone H1 kinase: evidence for identity with MPF. Cell 55, 371–8.
54. Azzi, L., Meijer, L., Reed, S. I., Pidikiti, R., and Tung, H. Y. (1992) Interaction between the cell-cycle-control proteins p34cdc2 and

p9CKShs2. Evidence for two cooperative binding domains in p9CKShs2. Eur J Biochem 203, 353–60.

55. De Veylder, L., Segers, G., Glab, N., Casteels, P., Van Montagu, M., and Inze, D. (1997) The *Arabidopsis* Cks1At protein binds the cyclin-dependent kinases Cdc2aAt and Cdc2bAt. FEBS Lett 412, 446–52.
56. Stals, H., Casteels, P., Van Montagu, M., and Inze, D. (2000) Regulation of cyclin-dependent kinases in *Arabidopsis thaliana*. Plant Mol Biol 43, 583–93.
57. Landrieu, I., Casteels, P., Odaert, B., De Veylder, L., Portetelle, D., Lippens, G., Van Montagu, M., and Inze, D. (1999) Recombinant production of the p10CKS1At protein from *Arabidopsis thaliana* and 13C and 15N double-isotopic enrichment for NMR studies. Protein Expr Purif 16, 144–51.
58. Weinl, C., Marquardt, S., Kuijt, S. J., Nowack, M. K., Jakoby, M. J., Hülskamp, M., and Schnittger, A. (2005) Novel Functions of Plant Cyclin-Dependent Kinase Inhibitors, ICK1/KRP1, Can Act Non-Cell-Autonomously and Inhibit Entry into Mitosis. Plant Cell.
59. Koncz, C., and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. Mol Gen Genet 204, 383–396.
60. Brooks, G. (2005) Cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors: detection methods and activity measurements. Methods Mol Biol 296, 291–8.
61. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–5.
62. Harlow, E., and Lane, D. (1988) Antibodies: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
63. Bögre, L., Zwerger, K., Meskiene, I., Binarova, P., Csizmadia, V., Planck, C., Wagner, E., Hirt, H., and Heberle-Bors, E. (1997) The cdc2Ms Kinase Is Differently Regulated in the Cytoplasm and in the Nucleus. Plant Physiol 113, 841–852.
64. Fantes, P., and Brooks, R. (1993) The Cell cycle: a practical approach, IRL Press at Oxford University Press, Oxford; New York.
65. Pagano, M. (1996) Cell cycle: materials and methods, Berlin: Springer, c1996.
66. Dunphy, W. G. (1997) Cell cycle control, Vol. 283, Academic Press, San Diego.
67. Pines, J., Jackman, M., and Simpson, K. (2001) Assays for CDK activity and DNA replication in the cell cycle. Curr Protoc Cell Biol Chapter 8, Unit 8 2.
68. Carter, A. N. (2001) Assays of protein kinases using exogenous substrates. Curr Protoc Mol Biol Chapter 18, Unit 18 7.
69. Slater, R. J. (2002) Radioisotopes in biology: a practical approach, Oxford University Press, Oxford; New York.
70. Ballance, P. E., Day, L. R., Morgan, J., and Association of University Radiation Protection, O. (1992) Phosphorus-32: practical radiation protection, H and H Scientific Consultants, Leeds.
71. Meisenhelder, J., and Bursik, S. (2007) Safe use of radioisotopes. Curr Protoc Mol Biol Appendix 1, Appendix 1F.
72. Karimi, M., Inze, D., and Depicker, A. (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci 7, 193–5.
73. Sambrook, J., and Russell, D. W. (2001) Molecular cloning: a laboratory manual, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
74. Clough, S. J., and Bent, A. F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16, 735–43.
75. Berendzen, K., Searle, I., Ravenscroft, D., Koncz, C., Batschauer, A., Coupland, G., Somssich, I. E., and Ulker, B. (2005) A rapid and versatile combined DNA/RNA extraction protocol and its application to the analysis of a novel DNA marker set polymorphic between *Arabidopsis thaliana* ecotypes Col-0 and Landsberg erecta. Plant Methods 1, 4.
76. Connell-Crowley, L., and Harper, J. W. (1995) Bacterial expression and *in vitro* assay of cell cycle kinases, in Cell Cycle – Materials and Methods (Pagano, M., Ed.) pp 157–168, Springer, Berlin; New York.
77. Gordon, J. A. (1991) Use of vanadate as protein-phosphotyrosine phosphatase inhibitor. Methods Enzymol 201, 477–82.
78. Matejovicova, M., Mubagwa, K., and Flameng, W. (1997) Effect of vanadate on protein determination by the coomassie brilliant blue microassay procedure. Anal Biochem 245, 252–4.
79. Shareef, M. M., and Shetty, K. T. (1998) Effect of vanadate on different forms of Coomassie brilliant blue and protein assay. Anal Biochem 258, 143–6.
80. Prince, A. M., and Andrus, L. (1992) PCR: how to kill unwanted DNA. Biotechniques 12, 358–60.
81. Schönthal, A. H. (2004) Measuring cyclin-dependent kinase activity. Methods Mol Biol 281, 105–24.