Chapter 10

Sensitizing Plant Protein Kinases to Specific Inhibition by ATP-Competitive Molecules

Dor Salomon, Chao Zhang, Kevan M. Shokat, and Guido Sessa

Abstract

The highly conserved nature of the protein kinase catalytic domain and the low permeability of plant cell membranes pose a challenge to the development of specific inhibitors that target individual protein kinases in vivo. Here, we describe a chemical-genetic approach to specifically sensitize individual plant kinases to cell-permeable small molecules that do not inhibit wild-type kinases. In this approach, a single amino-acid substitution is introduced in the ATP-binding site of the enzyme enabling specific binding of ATP-competitive molecules. Cell-permeable molecules can then be used to specifically target the sensitized allele in transgenic *Arabidopsis thaliana* plants that do not express the wild-type form of the kinase. This strategy provides a useful tool for the functional characterization of protein kinases *in planta* and for the dissection of the signaling pathways in which they are involved.

Key words: Protein kinase, PP1, Gatekeeper, ATP-competitive molecules, Analog-sensitive, Arabidopsis, Kinase activity assay, Transgenic plants

1. Introduction

Members of the protein kinase superfamily regulate a wide variety of cellular processes and signal-transduction pathways in plants (e.g. 1–6). A major objective in the field of protein kinase research is to delineate the functional role of individual kinases. To accomplish this aim, strategies based on single knockout mutations and gene silencing are widely employed. However, such efforts may be compromised by lethality, in the case of essential genes, or by functional redundancy and cellular homeostasis resulting in no apparent phenotypic alterations.

To overcome these limitations, a chemical-genetic approach was developed to sensitize protein kinases to specific inhibition

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by cell-permeable ATP-competitive molecules (7, 8). A functionally silent substitution of a conserved, bulky hydrophobic residue, termed the *gatekeeper*, to a small residue (alanine or glycine) generates a novel pocket in the kinase ATP-binding site not present in any wild-type kinase. The sensitized mutant can be selectively and potently inhibited by ATP-competitive molecules containing substituents that occupy this additional pocket. Importantly, wild-type kinases are not inhibited by such inhibitors due to lack of the novel pocket. The PP1(1-*tert*-Butyl-3-*p*-tolyl-1*H*-pyrazolo[3,4-*d*] pyrimidine-4-ylamine)-derived inhibitors employed in this approach were shown to be cell permeable in various systems, including plants, yeast, and mammalian cell cultures (8–11, 17). Combined with the relative ease of generating transgenic Arabidopsis plants harboring the sensitized allele, this approach allows for modulation of the activity of individual kinases in the plant and for delineation of their biological function in vivo.

2. Materials

2.1. Site-Directed Mutagenesis

1. Quikchange II site-directed mutagenesis kit (Stratagene).

2.2. Purification of GST-Fusion Protein

1. Terrific broth (TB) medium: 1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4. Add filter-sterilized phosphate salts after autoclaving.
2. 1 M Isopropyl-β-D-thiogalactopyranoside (IPTG) in water, store in aliquots at –20°C.
3. Phosphate buffered saline (PBS) (10×): 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, and 18 mM KH_2PO_4, adjust to pH 7.4 with HCl, autoclave.
4. 100 mM Phenylmethylsulfonyl fluoride (PMSF, Roche) in isopropanol. Aliquot and store at –20°C. Thaw and vortex before use.
5. 5 mg/mL aprotinin (Sigma) and 5 mg/mL leupeptin (both Sigma) in water, stored in aliquots at –20°C.
6. Glutathione agarose beads (Sigma), incubate with water at 4°C overnight. After swelling, wash the beads with 10 volumes of water and resuspend as 50% (w/v) slurry in water. Store at 4°C.
7. Elution buffer: 50 mM Tris–HCl (pH 8.0), 5 mM reduced glutathione (Sigma).
8. Bradford protein assay solution (Bio-Rad).
9. Prestained molecular-weight markers.

2.3. In Vitro Kinase Activity Assay

1. Reaction buffer (10×): 500 mM Tris–HCl (pH 7.0), 10 mM dithiothreitol (DTT), and 100 mM $MgCl_2$ (see Note 1). Always prepare fresh.
2. ATP-mixture (10×): 200 μM ATP, 10 μCi [γ-^{32}P]-ATP (3,000 Ci/mmol; GE Healthcare).
3. 0.5 M EDTA in water. Adjust to pH 8.0 with NaOH, autoclave.

2.4. SDS-Polyacrylamide Gel Electrophoresis

1. Stacking buffer (4×): 500 mM Tris–HCl, 0.4% (w/v) sodium dodecyl sulfate (SDS), adjust to pH 6.8 with HCl.
2. Separating buffer (4×): 1.5 M Tris, 2% (w/v) SDS, adjust to pH 8.8 with HCl.
3. 40% acrylamide/bis solution (37.5:1 with 2.6% C). Acrylamide is a neurotoxin when unpolymerized and so care should be taken not to receive exposure.
4. *N,N,N′,N′*-Tetramethyl-ethylenediamine (TEMED, Bio-Rad). Use in a chemical fume hood to avoid vapor inhalation.
5. 10% (w/v) ammonium persulfate (APS) in water. Store in aliquots at −20°C.
6. Running buffer (10×): 250 mM Tris base, 2 M glycine, and 1% (w/v) SDS.
7. SDS-PAGE sample buffer (3×): 30% (v/v) glycerol, 15% (v/v) β-mercaptoethanol, 37.5% (v/v) stacking buffer (4×), and a few grains of bromophenol blue (Sigma).
8. SDS-PAGE equipment (such as Mini-PROTEAN mini-gel system (Bio-Rad)).

2.5. Coomassie Stain and Autoradiography

1. Coomassie solution: 0.2% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, and 7% (v/v) acetic acid.
2. Destaining solution: 10% (v/v) acetic acid, 20% (v/v) isopropanol.
3. Fuji medical X-ray film superRX (Fujifilm).

2.6. Identification of Specific and Potent Kinase Inhibitors

1. 10 mM Src family tyrosine kinase inhibitor PP1 (Fig. 1, derivatives are synthesized as described (12–14)), in dimethyl sulfoxide (DMSO), store in aliquots at −20°C.

2.7. Identification of Homozygous T-DNA Insertion Mutant for the Kinase of Interest

1. Arabidopsis T-DNA insertion line mutants, available from the Arabidopsis Biological Research Center (ABRC, http://arabidopsis.org) or the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.info).
2. Genomic DNA extraction buffer: 400 mM LiCl, 200 mM Tris–HCl (pH 9.0), 1% (w/v) SDS, and 25 mM EDTA.

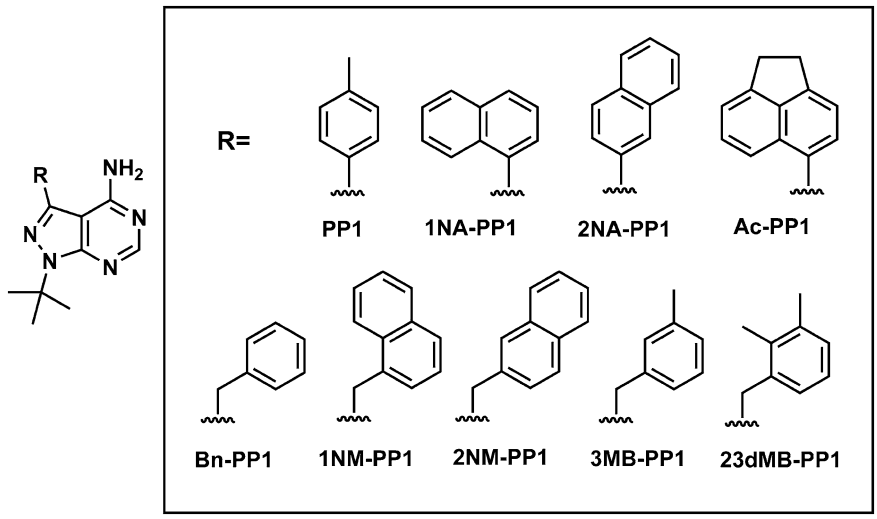

Fig. 1. Chemical structure of PP1 derivatives. PP1, 1-*tert*-Butyl-3-*p*-tolyl-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-ylamine; Bn-PP1, 3-Benzyl-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 1NA-PP1, 1-*tert*-Butyl-3-naphthalen-1-yl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 2NA-PP1, 1-*tert*-Butyl-3-naphthalen-2-y1-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 1NM-PP1, 1-*tert*-Butyl-3-naphthalen-1-ylmethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 2NM-PP1, 1-*tert*-Butyl-3-naphthalen-2-ylmethyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; Ac-PP1, 3-Acenaphthen-5-yl-1-*tert*-butyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 3MB-PP1; 1-*tert*-Butyl-3-(3-methyl-benzyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine; 23dMB-PP1; 1-*tert*-Butyl-3-(2,3-dimethyl-benzyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ylamine.

2.8. Generating Arabidopsis Lines Expressing the Sensitized Kinase

1. Silwet L-77 surfactant (Lehle Seeds).
2. Plant agar (gel strength >1,100 g/cm^2, Duchefa).
3. Murashige and Skoog (MS) medium including vitamins (Duchefa), used at a final concentration of 0.5% (w/v).
4. Luria broth (LB): 1% (w/v) tryptone, 1% (w/v) sodium chloride (NaCl), and 0.5% (w/v) yeast extract.
5. Domes to cover freshly transformed plants.

2.9. Effects of Kinase Inhibition in Planta

1. Inhibitor solution for plant treatment: dilute stock solution of the inhibitor (10 mM in DMSO) to 100 μM in water containing 0.01% (v/v) Silwet L-77. For mock treatment, use 1% (v/v) DMSO in water containing 0.01% (v/v) Silwet L-77.

3. Methods

The ATP-binding site of protein kinases is highly conserved and a targeted modification of its structure results in sensitization of the kinase to specific inhibition by ATP-competitive molecules. Site-directed mutagenesis is used to introduce in the ATP-binding site of the kinase of interest a functionally silent mutation that substitutes the bulky *gatekeeper* residue with a small residue such as

alanine or glycine. Identification of the *gatekeeper* residue in the ATP-binding site of the kinase of interest is readily achieved by using tools provided by the Kinase Sequence Database (http://sequoia.ucsf.edu/ksd/).

A kinase assay is used to verify in vitro that the bacterially expressed mutant kinase displays a similar catalytic activity as the wild-type enzyme, and to screen various PP1 analogs for their ability to inhibit activity of the sensitized kinase but not of the wild-type kinase. Measurement of half maximal inhibitory concentration (IC_{50}) values for the various inhibitors is important to compare their potency and to determine their working concentrations. The effect of inhibition of the kinase of interest by ATP-competitive molecules is tested in transgenic Arabidopsis plants expressing the sensitized kinase, while the nonspecific effect of the inhibitors can be assessed in the control experiment with plants expressing the corresponding wild-type kinase. This strategy allows for the study of physiological processes regulated by the individual kinase *in planta*.

3.1. Engineering a Sensitized Kinase Mutant

1. Identify the *gatekeeper* residue in the kinase of interest by using the online Kinase Sequence Database resource. This Web site includes detailed instructions for use of the database (see Note 2).
2. Design primers for mutation of the *gatekeeper* to either alanine or glycine in the kinase of interest according to the instructions of the site-directed mutagenesis kit (see Note 3).
3. Perform site-directed mutagenesis on a plasmid containing the coding sequence of the kinase of interest.
4. Transform the reaction products of the mutagenesis to transformation-competent *E. coli* cells and plate transformants on appropriate selective media.
5. For each mutant, extract plasmid DNA from 2 to 5 colonies and sequence the nucleotides surrounding the location of the desired mutation to verify its presence.

3.2. Purification of GST-Fusion Protein

1. Transform an IPTG-inducible expression vector containing a GST-fusion of the wild-type or the sensitized kinases into competent *E. coli* cells.
2. Grow a 5 mL overnight culture prepared from a fresh bacterial colony in TB containing appropriate antibiotics.
3. Inoculate 500 mL TB containing appropriate antibiotics with the overnight culture. Grow on a shaker at 37°C until OD_{600} of approximately 0.5–0.6 (see Note 4).
4. Induce expression of the fusion protein by adding IPTG to a final concentration of 0.5 mM and continue growing for 5 h at 28°C (see Note 4).

5. Pellet bacteria by centrifugation at 2,000 × *g* for 20 min at 4°C. Discard the supernatant.
6. Gently resuspend the pellet in 10 mL PBS containing 1 mM PMSF, 5 μg/mL leupeptin, and 5 μg/mL aprotinin (see Notes 5 and 6).
7. Sonicate by 30 cycles of a 2-s pulse and 1-s break. Repeat sonication twice (or more, if necessary) until change in the color and viscosity of the suspension is apparent. From this step on, all procedures should be carried out at 4°C.
8. Centrifuge the suspension at 12,000 × *g* for 20 min at 4°C.
9. In the meantime, transfer 300 μl of 50% (w/v) slurry of glutathione agarose beads to a new 1.5-mL tube by using a blunt tip. Add 300 μl of PBS to the bead slurry and centrifuge for 30 s at 500 × *g*. Discard supernatant and resuspend in 300 μl of PBS. Repeat wash step three times.
10. Add cleared supernatant from step 8 into a 50-mL conical tube containing the 300 μL of washed glutathione agarose beads from step 9. Incubate on an end-over-end rotator for 30 min at 4°C.
11. Load the suspension into a plastic column kept at 4°C. Be careful not to suspend the beads in the column. Discard the flow-through.
12. Wash the column with 10 mL of ice-cold PBS with protease inhibitors (as described in step 6).
13. Add 2 mL of ice-cold elution buffer to the column and collect ten 50-μL fractions sequentially (approximately two drops of flow-through per fraction).
14. Measure protein concentration by Bradford assay.
15. Analyze purity by SDS-PAGE and Coomassie stain as detailed below.

3.3. In Vitro Kinase Activity Assay

1. Prepare an 18 μL kinase reaction solution containing 2 μL of 10× reaction buffer and 1 μg of the purified GST-fusion of wild-type or sensitized kinases. Keep on ice.
2. Add 2 μL of the 10× ATP-mixture.
3. Incubate at room temperature for 15 min (see Note 7).
4. Stop reactions by the addition of EDTA to a final concentration of 10 mM.

3.4. SDS-PAGE

1. These instructions assume the use of a Mini-PROTEAN minigel system, but are adaptable to other gel-electrophoresis systems.
2. Prepare a 1.5-mm thick, 10% gel by mixing 2.5 mL of 4× separating buffer with 2.5 mL of acrylamide/bis solution, 5 mL of

water, 100 μL of 10% APS, and 10 μL of TEMED. Cast the gel and gently overlay it with 200 μL of water. The gel will polymerize in about 20 min.

3. Prepare the stacking gel by mixing 1.25 mL of 4× stacking buffer with 0.5 mL acrylamide/bis solution, 3.25 mL of water, 50 μL of 10% APS, and 5 μL of TEMED. Discard the water overlaying the separating gel, cast the stacking gel until it fills the space between the glasses, and insert the comb. Make sure that no air bubbles are caught between the comb and the gel. The stacking gel will polymerize within 20 min.
4. After gently removing the comb, assemble the gel into the gel unit. Add running buffer to the central chamber of the gel unit until full. Ensure that the buffer is not leaking and fill one third of the outer chamber with running buffer.
5. Add SDS-PAGE sample buffer (3×) to the kinase reactions and load up to 30 μL of each sample in a well. Include one well for molecular-weight markers.
6. Complete the assembly of the gel unit and connect to a power supply. Run the gel at 100 V through the stacking gel and at 120 V through the separating gel. The dye front will reach the bottom of the gel in approximately 1–2 h.

3.5. Coomassie Stain and Autoradiography

1. Disconnect the gel unit from the power supply and disassemble. Remove and discard the stacking gel.
2. Transfer the separating gel to a tray and incubate with Coomassie solution for 30 min with constant shaking.
3. Remove the Coomassie solution (see Note 8) and rinse with destaining solution.
4. Cover the gel with destaining solution and incubate for 1 h with constant shaking (see Note 9). If necessary, replace the used solution with fresh destaining solution, incubate the gel until blue protein bands are distinctly visible, and dry.
5. Expose the dry gel to an X-ray film and develop to visualize ^{32}P incorporation.
6. Quantify band intensities by scanning densitometry of the films, provided that care is taken to ensure that the signal has not saturated. Alternatively, the radioactivity signal can be quantified digitally by a phosphorimager.
7. Compare autophosphorylation activity of the wild-type and sensitized kinases. An example of results produced for the tomato (*Solanum lycopersicum*) MAP kinase LeMPK3 is shown in Fig. 2 (see Note 10).

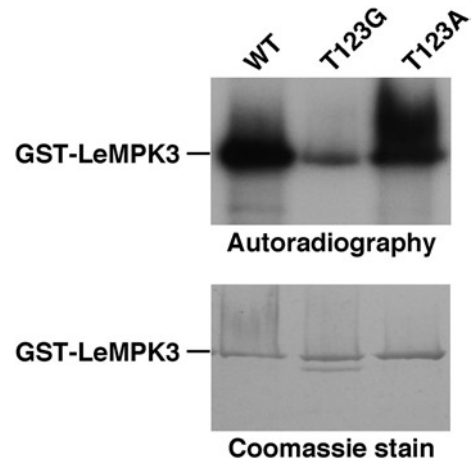

Fig. 2. In vitro kinase activity assay of wild-type and sensitized GST-LeMPK3. The tomato MAP kinase LeMPK3 (16) was expressed and purified from *E. coli* bacteria in wild-type and sensitized forms. The recombinant fusion proteins GST-LeMPK3, GST-LeMPK3(T123A), and GST-LeMPK3(T123G) were tested in vitro for autophosphorylation activity in a kinase assay in the presence of [γ-^{32}P]-ATP. The proteins were separated by SDS-PAGE, stained with Coomassie (*lower panel*) and the dry gel was autoradiographed (*upper panel*).

3.6. Identification of Specific and Potent Kinase Inhibitors

1. Perform in vitro kinase activity assays for wild-type and sensitized kinases. Before addition of the ATP-mix, add to the reaction mixture individual inhibitors to a final concentration of 1 μM. Include a control reaction with DMSO at a similar concentration as in the reactions containing the inhibitor.
2. Separate reactions by SDS-PAGE, stain the gel with Coomassie solution, and expose to X-ray film.
3. Quantify band intensities and compare reactions containing inhibitor with the DMSO control. Identify inhibitors that specifically inhibit the sensitized kinase, but not the wild-type. An example of inhibition of sensitized and wild-type LeMPK3 by various PP1 derivatives is shown in Fig. 3.
4. To determine potency of the specific inhibitor identified in step 3, perform in vitro kinase activity assays in the presence of increasing concentrations of the inhibitor. Typical concentrations range from 1 nM to 10 μM.
5. After developing the exposed X-ray film, quantify band intensities and compare reactions containing the inhibitor with a control reaction containing DMSO. Calculate percentage of kinase activity for reactions containing inhibitor relative to the control reaction set as 100% kinase activity.
6. Plot percentage of kinase activity (Y-axis) against a logarithm of inhibitor concentration (X-axis).
7. Identify the concentration of inhibitor causing 50% inhibition of kinase activity (this is the IC_{50} value of the inhibitor).
8. Select a mutant-specific inhibitor with the lowest IC_{50} value for subsequent analysis.

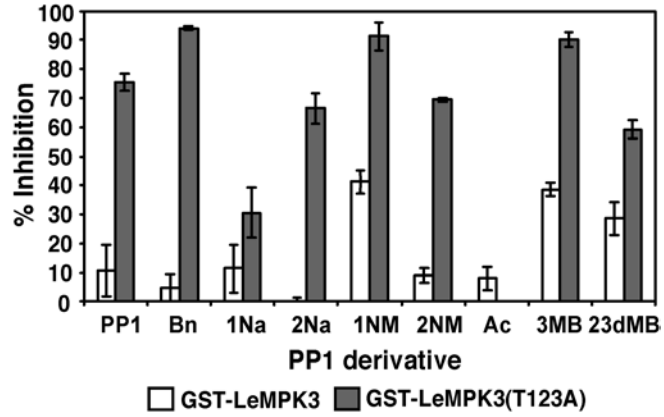

Fig. 3. Inhibition of wild-type and sensitized GST-LeMPK3 kinase activity by PP1 derivatives. In vitro kinase activity assays were performed for wild-type GST-LeMPK3 (*white bars*) and sensitized GST-LeMPK3(T123A) (*gray bars*) in the presence of [γ-^{32}P]-ATP and the indicated PP1 derivatives (1 μM). Percentage of inhibition (defined as $1 - V_1/V_0$) was calculated by determining the ratio of V_1 (activity in the presence of inhibitor)/V_0 (activity in the absence of inhibitor). Data are the means of three technical repeats ± SE. The assay was repeated at least two times for each inhibitor with similar results.

3.7. Identification of Homozygous T-DNA Insertion Mutant for the Kinase of Interest

1. Order Arabidopsis T-DNA insertion lines for the gene encoding the kinase of interest from the stock center. If the available mutant line is heterozygous for the insertion, isolate a homozygous line after selfing and analyzing the progeny as follows.
2. Grow 12–20 seeds (2 seeds in each 9 cm^2 pot) from the batch received from the stock center under long-day conditions (16 h light/8 h dark).
3. Harvest 3–4 young leaves and flowers from each 4-weeks-old plant and place in a 1.5-mL microcentrifuge tube. Immediately freeze in liquid nitrogen (samples can be stored at −80°C).
4. Grind the frozen tissue and add 500 μL of Genomic DNA extraction buffer.
5. Incubate on an end-over-end rotator (slow rotations) for 5 min at room temperature.
6. Centrifuge for 8 min at 16,000 × *g* at room temperature.
7. Transfer 350 μL of the cleared lysate to a clean 1.5-mL microcentrifuge tube and add 350 μL of isopropanol. Mix gently by inverting the tube ten times.
8. Centrifuge at 16,000 × *g* for 10 min at room temperature. Discard the supernatant and air-dry the pellet (it will take approximately 30 min).
9. Add 300 μL of water and incubate on an end-over-end rotator (at slow rotation) for 30 min at room temperature to allow DNA pellet to dissolve. Store at 4°C.
10. Use primers for PCR identification of a homozygous T-DNA insertion line designed by using the online T-DNA Primer

design tool of the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/tdnaprimers.2.html). Insert the name of your T-DNA insertion line (e.g. SALK_000001) and retrieve the output sequences for the left primer (LP) and right primer (RP) that are located on the gene sequences flanking the T-DNA insertion site.

11. Perform PCR amplification using 2 μL of genomic DNA preparations as template. For each genomic DNA to be tested, perform two reactions. The first reaction should contain LP and RP, and the second one should contain RP and the left-border primer of the T-DNA insertion (see Note 11). If the DNA sample tested is homozygous for the T-DNA insertion, a PCR amplicon is obtained only from the second reaction. However, if the DNA sample tested is heterozygous, a product is obtained from both reactions.
12. Harvest seeds from plants homozygous for the T-DNA insertion and allow them to dry overnight at room temperature. Store seeds at 4°C in a dry environment.

3.8. Generating Arabidopsis Lines Expressing the Sensitized Kinase

1. Grow 40–50 seeds (2 seeds in each 9 cm^2 pot) of the Arabidopsis homozygous mutant T-DNA insertion line for the gene of interest under long-day conditions (16 h light/8 h dark). Cover the soil with bridal veil or cheesecloth (the plants will flower after approximately 5 weeks).
2. Clip first bolts to encourage growth of secondary bolts and continue growing until plants have many immature flower clusters and not many fertilized siliques.
3. Grow 5 mL of overnight culture of *Agrobacterium tumefaciens* carrying a binary vector for the expression of the sensitized or the wild-type kinase (see Note 12). Cultures are prepared in LB medium containing appropriate antibiotics and inoculated with a fresh colony.
4. Inoculate 500 mL of LB containing appropriate antibiotics with the overnight culture. Grow at 28°C for 16 h.
5. Pellet cells by centrifugation at 3,700 × *g* for 15 min at 4°C. Discard the supernatant.
6. Resuspend cells in 200 mL of 5% (w/v) sucrose solution and transfer to a 250-mL beaker.
7. Add Silwet-L77 to a final concentration of 0.035% (v/v) and stir for 1 min.
8. Dip aboveground parts of plants in the bacterial suspension for 2–3 s.
9. Return dipped plants to tray and cover tray with a dome. Incubate in low light intensity condition for 16–24 h.
10. Remove the dome and continue growing plants under long-day conditions.

11. Repeat steps 3–10 for the same plants after 5–7 days and grow them until fertilized siliques are open.
12. To harvest the dry seeds, open siliques on top of a clean paper. Collect seeds from the paper to 1.5 mL tubes. Leave the tube open overnight at room temperature to dry seeds completely. Store seeds at 4°C in a dry environment.
13. Prior to germination, the collected seeds should be sterilized. For sterilization, dispense approximately 100–200 seeds into 1.5-mL tubes and place in a dessicator (leave tube caps open) within a chemical fume hood. In the middle of the dessicator place a beaker containing 100 mL of a 10% sodium hypochlorite solution. Add 5 mL of hydrochloric acid (HCl) and immediately close the dessicator. Incubate for 3 h.
14. Spread seeds (roughly 1,000) on 0.8% plant agar plate with 0.5% MS and appropriate antibiotics (of the T-DNA of the *Agrobacterium* transformation vector) to select for transformed seeds. Wrap plates with parafilm and incubate at 4°C overnight.
15. Transfer plate to long-day growth conditions for approximately 7 days. Transgenic seedlings will appear green and healthy, while nontransformed seedling will appear white and small.
16. Gently transplant each putative transgenic plant in soil and grow under long-day conditions.
17. Extract DNA from individual transformed plants as described above and test for the presence of the transgene by performing PCR on genomic DNA using primers that will amplify a segment of the T-DNA containing the desired transgenic kinase allele.
18. Harvest seeds from the transgenic plants (see Note 13).

3.9. Effects of Kinase Inhibition In Planta

1. Sow seeds of transgenic and wild-type Arabidopsis plants. Grow the plants to the desired developmental stage.
2. Apply inhibitor solutions either to the whole plant, by spraying, or locally, by using a brush with soft fine fibers.
3. Proceed with desirable assays to examine the effect of specific inhibition of the kinase of interest on selected phenotypes.

4. Notes

1. The choice and working concentration of the divalent cations (Mg^{2+} or Mn^{2+}) and the pH of the buffer in the kinase reaction should be optimized beforehand for the individual kinases.
2. It is advisable to use the GenBank GI number or the commonly used name of the protein kinase of interest to search for the appropriate alignment using the "Search by: protein name"

option. The resulting sequence alignment can be either downloaded as a Microsoft Excel file or directly viewed in HTML format with a Web browser. In these alignments, the *gatekeeper* residue is highlighted in red and other ATP-contacting amino acids are highlighted in green.

3. It is possible to design mutagenesis primers that either introduce a novel restriction site or eliminate an existing restriction site through auxiliary silent mutation adjacent to the primary mutation. The alteration of a restriction site allows for a quick screen for mutated plasmid via restriction enzyme digestion, prior to nucleic-acid sequencing.
4. Induction conditions and incubation time should be calibrated and optimized for each application.
5. The half-life of PMSF in aqueous solutions is approximately 30 min. Therefore, it is advisable to add PMSF to the PBS solution right before use.
6. At this point, the suspension can be frozen in liquid nitrogen and kept at −80°C for up to 1 month.
7. Optimal incubation time during which the change in initial velocity of the reaction is linear should be determined in preliminary experiments.
8. The Coomassie staining solution can be reused several times.
9. It is advisable to add a piece of tissue paper to the tray. This will shorten the destaining procedure, since the tissue paper will keep the washed dye out of the destaining solution.
10. Introduction of a mutation at the *gatekeeper* may affect activity of the kinase as shown in Fig. 2 for LeMPK3(T123G). It is, thus, advisable to substitute the *gatekeeper* residue with alanine and glycine and select the mutation with the lowest effect on kinase activity. If the kinase of interest does not tolerate any mutation at the *gatekeeper* residue, a second site suppressor mutation can be introduced to restore activity (15).
11. For lines obtained from SALK, it is advisable to use the following oligonucleotide as left-border primer: 5′-ATTTT GCCGATTTCGGAAC-3′.
12. Strains of *A. tumefaciens* commonly used are EHA105 or GV3101. The binary vector used for expression should contain a selectable marker different from that present in the T-DNA insertion to allow appropriate selection of transformed seeds. It is advisable to drive the expression of wild-type and sensitized alleles with the kinase endogenous promoter to maintain the original expression patterns.
13. Functionality of the wild-type or sensitized kinase should be evaluated in transgenic plants as compared to wild-type plants.

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