Appendix S1. Experimental procedures

In-gel kinase assay
At the indicated time points, plant material was frozen in liquid nitrogen, ground in a mortar and proteins were extracted in a buffer containing 50 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 1 mM Na₃VO₄, 10 mM NaF, 50 mM β-glycerophosphate, 5 µg.ml⁻¹ leupeptin, 1 mM PMSF. After centrifugation at 13,000 rpm, supernatant aliquots were stored at -20°C. Thirty µg of protein extract in SDS-PAGE sample buffer were separated by electrophoresis on 10% SDS-polyacrylamide gel containing 0.25 mg. ml⁻¹ myelin basic protein. After electrophoresis, gels were washed at room temperature for 1 h in buffer A (50 mM Tris-HCl pH8, 20 % propan-2-ol), 1 h in buffer B (50 mM Tris-HCl pH8, 5 mM β-mercaptoethanol), 1 h in buffer C (50 mM Tris-HCl pH8, 6M guanidine-HCl, 5 mM β-mercaptoethanol) and overnight in buffer D at 4°C (50 mM Tris-HCl pH8, 5 mM β-mercaptoethanol, 0.04% Tween 20). Then the gels were equilibrated for 30 min at room temperature in reaction buffer (40 mM HEPES pH7.5, 0.1 mM EGTA, 20 mM MgCl₂, 2mM DTT) followed by 1.5 h in the reaction buffer supplemented with 15 µM ATP and 10 µCi γ⁻³²P-ATP. The reaction was stopped by washing the gels with 5% (w/v) trichloroacetic acid and 1% (w/v) tetrasodiumpyrophosphate. Unincorporated γ⁻³²P-ATP was removed by extensive gel washing in the same solution for 2.5 h. The gels were dried and exposed in a phosphorimager cassette (Amersham Biosciences, Piscataway, NJ, USA). Prestained SDS-PAGE standards were used to calculate the apparent sizes of the kinases (Biorad, Hercules, CA, USA).

Immunoprecipitation and immunoblot analysis
The following primary antibodies were used: anti-phospho-p44/42 MAP kinase antibody (Cell signaling, Danvers, MA, USA), anti-MPK6 and anti-MPK3 (kindly provided by Shuqun Zhang). To identify kinases as MAPK, they were first immunoprecipitated using an anti-phospho-p44/42 MAP kinase antibody. To perform this experiment, the time at which the strongest kinase activity was observed was used. Immunoblots were performed using an anti-phospho-p44/42 MAP kinase antibody and plant specific antibodies raised against MPK3 and MPK6 to identify the two immunoprecipitated kinases as MPK3 and MPK6.

Immunoprecipitation was performed as previously described (Droillard et al. 2002). Proteins from crude extracts (100 µg) were incubated with 12 µL of anti-phospho-p44/42 MAP kinase antibody in a final volume of 500 µL in immunoprecipitation buffer (20 mM Hepes pH 7.5, 5 mM EDTA, 2 mM EGTA, 5 mM DTT, 60 mM β-glycerophosphate, 100 µM Na₃VO₄, 1 mM PMSF, 5 µg.ml⁻¹ leupeptin, 0.5% Triton X-100 (v/v), 0.5% Nonidet P-40 (v/v) and 150 mM NaCl) at 4°C overnight on a carrousel. Then, 30 µL of Protein-A was added and incubation continued for one additional hour. After centrifugation at 2,500 rpm during 2 min, the immunoprecipitate was washed three times with immunoprecipitation buffer and two times with protein extraction buffer, and then resuspended in SDS-PAGE sample buffer. Samples were heated at 95°C for 5 min and subjected to the in-gel kinase assay as described above.
For immunoblot analysis, protein extracts (20 µg protein) were subjected to 10% SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membrane Optitran BA-S85 (Schleicher & Schuell, Dassel, Germany) by wet transfer using transfer buffer (25mM Tris-HCl, 192 mM glycine, 20% MeOH (v/v)). Blots were blocked in Tris-buffered saline with Tween 20 (TBST; 10 mM Tris-HCl ph7.5, 150 mM NaCl, 0.1% Tween20 (v/v)) and 1% BSA for 1h. Antibodies were diluted in TBST with 1% BSA to 1:1,000, 1:5,000, 1:10,000 for anti-phospho-p44/42 MAP kinase, anti-MPK3 and anti-MPK6 antibodies, respectively. Protein gel blots were incubated with diluted antibodies overnight at 4°C. Blots were washed (3 x 10 min) in TBST and subsequently incubated with polyclonal goat anti-rabbit immunoglobulins conjugated with horseradish peroxidase (Dakocyтомation, Glostrup, Denmark) diluted 1:5,000 in TBST with 1% BSA (w/v). Blots were washed (3 x 10 min) with TBST. Immunocomplexes were visualized using Super signal west pico chemiluminescence substrate according to manufacturer’s instructions (Pierce, Rockford, IL, USA).

Reference:
