Methods

Immunoprecipitation, protein kinase assay and western blotting. Bmk1 and Mek5 were immunoprecipitated from solubilized[7] HeLa cells as described[19]. Bmk1 kinase activity was assayed by an immune complex protein kinase assay and quantified as described[7]. Phosphorylation of Bmk1 results in a shift in its electrophoretic mobility detectable by western blotting, and we have consistently seen a correlation between the shifted fraction of phosphorylated Bmk1 and the activity of Bmk1 as measured by protein kinase assay[6,7] (Fig. 1a–c). On western blots, the relative percentage activation of Bmk1 was calculated by dividing the density of the upper phosphorylated Bmk1 band by the total density of the upper and lower Bmk1 bands, and multiplying by 100. Activation of endogenous ERK was measured in cell lysates by western blotting using an anti-ERK antibody (Santa Cruz Biotechnology) or a phospho-specific anti-ERK antibody (New England Biolabs), respectively. Relative activation was determined by measuring the density of each phosphorylated ERK band relative to that of untreated cells whose activity was taken as unity.

Expression vectors and recombinant adenoviruses. Raf-1(BXB), Ras(V12G) and Ras(T17N) pSRα vectors were gifts from M. Karin[10,20,21]. The cDNAs for the various forms of Erk2, Mek1, Mek5 and Bmk1 (ref. 7), were subcloned into the *Hin*dIII/*Xba*I site of the vector pAd/RSV[22]. Recombinant adenoviruses expressing the various forms of Erk2, Mek1, Mek5 and Bmk1 were generated as described[23–25]. The control adenovirus was generated using the empty vector. Viral DNA was isolated and insertion of the appropriate cDNA was confirmed by PCR[22]. High-titre recombinant adenoviral stocks of $\sim 10^{11}$ PFU ml^{-1} were produced in 293 cells and purified by density gradient ultracentrifugation[23].

Proliferation and cell cycle analysis. MCF10A cells were grown in DMEM/F12 medium supplemented with 10% FCS, 10 ng ml^{-1} EGF, 5 μg ml^{-1} insulin and 0.5 μg ml^{-1} hydrocortisone. To ensure optimal expression of recombinant proteins, MCF10A cells were infected with recombinant adenovirus at an MOI of 10^3 PFU per cell. Two days after infection, cells were plated into 96-well dishes at 2×10^3 cells per well using serum-free medium (DMEM/F12) containing 1 mg fetuin, 10 μg transferrin, 5 μg insulin and 0.5 μg hydrocortisone per ml) with or without 100 ng ml^{-1} of EGF. Growth was monitored 3 days later using an MTT-based cell-proliferation assay (Boehringer) as specified by the supplier. Preliminary experiments established a linear correlation between the absorbance measured in the MTT assay and actual cell number over a range of 200–50,000 MCF10A cells per well. All assays were done in triplicate within this linear range.

HeLa cells were incubated with or without recombinant adenovirus at an MOI of 10^3 PFU per cell. 48 h after infection, cells were synchronized at the G1–S phase boundary by a double thymidine black as described[26]. Cells were released from this double thymidine block for various times and analysed by flow cytometry after staining nuclear DNA with propidium iodide[27]. In a separate experiment, MCF10A cells were starved for 48 h in unsupplemented DMEM/F12 medium. Some cells were exposed to growth factors by addition of DMEM/F12 medium containing 10% fetal calf serum, 10 ng ml^{-1} EGF, 5 μg ml^{-1} insulin and 0.5 μg ml^{-1} hydrocortisone. After 15 h, the percentage of cells in S phase was measured in triplicate by adding 10 μM bromodeoxyuridine (Boehringer) directly to the culture medium for 45 min. The cells were then collected, stained with propidium iodide and anti-BrdU fluorescein isothiocyanate (FITC)-conjugated antibody (Boehringer). The fraction of cells in S phase was determined by flow cytometry[28,29].

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1. Yarden, Y. & Ullrich, A. Growth factor receptor tyrosine kinases. *Annu. Rev. Biochem.* **57,** 443–478 (1988).
2. Ullrich, A. & Schlesinger, J. Signal transduction by receptors with tyrosine kinase activity. *Cell* **61,** 203–212 (1990).
3. Su, B. & Karin, M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr. Opin. Immunol.* **8,** 402–411 (1996).
4. Johnson, G. L. & Vaillancourt, R. R. Sequential protein kinase reactions controlling cell growth and differentiation. *Curr. Opin. Cell Biol.* **6,** 230–238 (1994).
5. Katz, M. E. & McCormick, F. Signal transduction from multiple Ras effectors. *Curr. Opin. Genet. Dev.* **7,** 75–79 (1997).
6. Abe, J.-I., Kushuhara, M., Ulevitch, R. J., Berk, B. C. & Lee, J.-D. Big mitogen-activated protein kinase 1 (BMK1) is a redox-sensitive kinase. *J. Biol. Chem.* **271,** 16586–16590 (1996).
7. Kato, Y. *et al.* BMK1/ERK5 regulates serum-induced early gene expression through transcription factor MEF2C. *EMBO J.* **16,** 7054–7066 (1997).
8. Li, J. J. & Herskowitz, I. Isolation of ORC6 a component of the yeast origin recognition complex by a one-hybrid system. *System* **262,** 1870–1874 (1993).
9. Lange-Carter, C. A. & Johnson, G. L. Ras-dependent growth factor regulation of MEK kinase in PC12 cells. *Science* **265,** 1458–1461 (1994).
10. Minden, A. *et al.* Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* **266,** 1719–1723 (1994).
11. Zhou, G., Bao, Z. Q. & Dixon, J. E. Components of a new human protein kinase signal transduction pathway. *J. Biol. Chem.* **270,** 12665–12669 (1995).
12. Soule, H. D. *et al.* Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* **50,** 6075–6086 (1990).
13. Pages, G. *et al.* Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc. Natl Acad. Sci. USA* **90,** 8319–8323 (1993).
14. Kovary, K. & Bravo, R. The jun and fos protein families are both required for cell cycle progression in fibroblasts. *Mol. Cell Biol.* **11,** 4466–4472 (1991).
15. Barr, M. M., Tu, H., Van Aeist, L. & Wigler, M. Identification of Ste4 as a potential regulator of Byr2 in the sexual response pathway of *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **16,** 5597–5603 (1996).
16. Libermann, T. A. *et al.* Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin. *Nature* **313,** 144–147 (1985).
17. Velu, T. J. *et al.* Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene. *Science* **238,** 1408–1410 (1987).
18. Merlino, G. T. *et al.* Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science* **224,** 417–419 (1984).
19. Han, J. *et al.* Characterization of the structure and function of a novel MAP kinase kinase (MKK6). *J. Biol. Chem.* **271,** 2886–2891 (1996).
20. White, M. A. *et al.* Multiple Ras functions can contribute to mammalian cell transformation. *Cell* **80,** 533–541 (1995).
21. Minden, A., Lin, A., Claret, F.-X., Abo, A. & Karin, K. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81,** 1147–1157 (1995).
22. Huang, S. *et al.* Apoptosis signaling pathway in T cells is composed of ICE/Ced-3 family proteases and MAP kinase kinase 6b. *Immunity* **6,** 739–749 (1997).
23. Chang, M. W., Barr, E., Lu, M. M., Barton, K. & Leiden, J. M. Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. *J. Clin. Invest.* **96,** 2260–2268 (1995).
24. Bett, A. J., Haddara, W., Prevec, L. & Graham, F. L. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc. Natl Acad. Sci. USA* **91,** 8802–8806 (1994).
25. Jomary, C. *et al.* Adenovirus-mediated gene transfer to murine retinal cells *in vitro* and *in vivo*. *FEBS Lett.* **347,** 117–122 (1994).
26. Stein, G. S. *et al.* in *A Laboratory Handbook* (ed. Celis, J.) 282–287 (Danish Centre for Human Genome Research, Academic, San Diego, 1994).
27. Draetta, G. & Beach, D. Activation of cdc2 protein kinase during mitosis in human cells: cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54,** 17–26 (1988).
28. Hoy, C. A., Seamer, L. C. & Shimke, R. T. Thermal denaturation of DNA for immunochemical staining of incorporated bromodeoxyuridine (BrdUrd): critical factors that affect the amount of fluorescence and the shape of BrdUrd/DNA histogram. *Cytometry* **10,** 718–725 (1989).
29. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. & Craig, R. W. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51,** 6304–6311 (1991).

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Suppression of auxin signal transduction by a MAPK cascade in higher plants

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The plant hormone auxin activates many early response genes that are thought to be responsible for diverse aspects of plant growth and development[1]. It has been proposed that auxin signal transduction is mediated by a conserved signalling cascade consisting of three protein kinases: the mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK)[2]. Here we show that a specific plant MAPKKK, NPK1 (ref. 3), activates a MAPK cascade that leads to the suppression of early auxin response gene transcription. A mutation in the kinase domain abolishes NPK1 activity, and the presence of the carboxy-terminal domain diminishes the kinase activity. Moreover, the effects of NPK1 on the activation of a MAPK and the repression of early auxin response gene transcription are specifically eliminated by a MAPK phosphatase[4]. Transgenic tobacco plants overexpressing the NPK1 kinase domain produced seeds defective in embryo and endosperm development.

These results suggest that auxin sensitivity may be balanced by antagonistic signalling pathways that use a distinct MAPK cascade in higher plants.

A transient expression system using maize mesophyll protoplasts has been developed to elucidate the molecular mechanisms of gene expression and intracellular signal transduction stimulated by sugars, light and the plant hormone abscisic acid[5–9]. To determine whether the system is suitable for the study of auxin signalling, we tested the auxin inducibility of a well-characterized early response gene promoter, GH3 (ref. 10), in maize protoplasts. The protoplasts transfected with a construct carrying the coding region of a green fluorescent protein (sGFP)[11] driven by the soybean GH3 promoter (GH3–sGFP) showed bright fluorescence on induction with different active auxin forms, NAA (Fig. 1a) or IAA (data not shown). In contrast, auxin did not affect the expression of sGFP controlled by the maize chlorophyll *a/b* binding protein gene promoter (CAB5)[5] (Fig. 1a). The auxin inducibility of the GH3 promoter in maize protoplasts was verified by using another reporter gene encoding *Escherichia coli* β-glucuronidase (GUS) (Fig. 1b).

To confirm that the early auxin responses are conserved among higher plants, we tested an auxin responsive element, ER7 (ref. 12), which was found in most early auxin response gene promoters[1,12]. The ER7 promoter element was inserted upstream of the GUS gene driven by a 35S minimal promoter. The ER7 synthetic promoter conferred auxin inducibility in the system, whereas the mutated ER7 element or the minimal promoter alone could not be induced by auxin (Fig. 1b). The data demonstrate that maize mesophyll

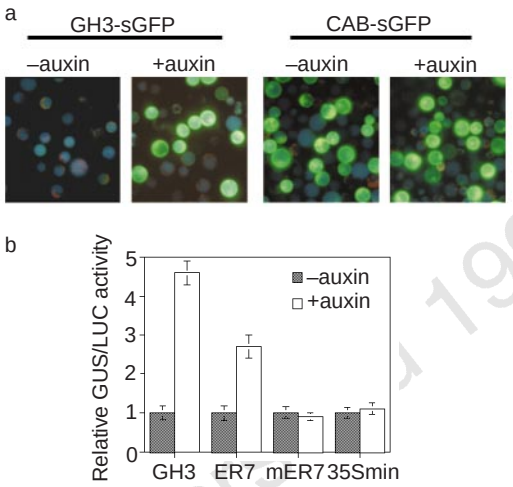

Figure 1 Auxin responses in maize protoplasts. **a**, Visualization of auxin responses. Protoplasts were transfected with plasmid DNA carrying GH3–sGFP or CAB5–sGFP and incubated without or with auxin. **b**, The GH3 promoter and the ER7 auxin responsive element are regulated in maize protoplasts. The protoplasts were transfected with plasmid DNA carrying GH3–GUS (GH3), ER7–GUS (ER7), mutated ER7–GUS (mER7), or GUS controlled by the CaMV 35S minimal (–72) promoter (35Smin). The protoplasts were incubated without or with auxin. GUS/LUC activity of the protoplasts transfected with each construct and incubated without auxin was set to 1.

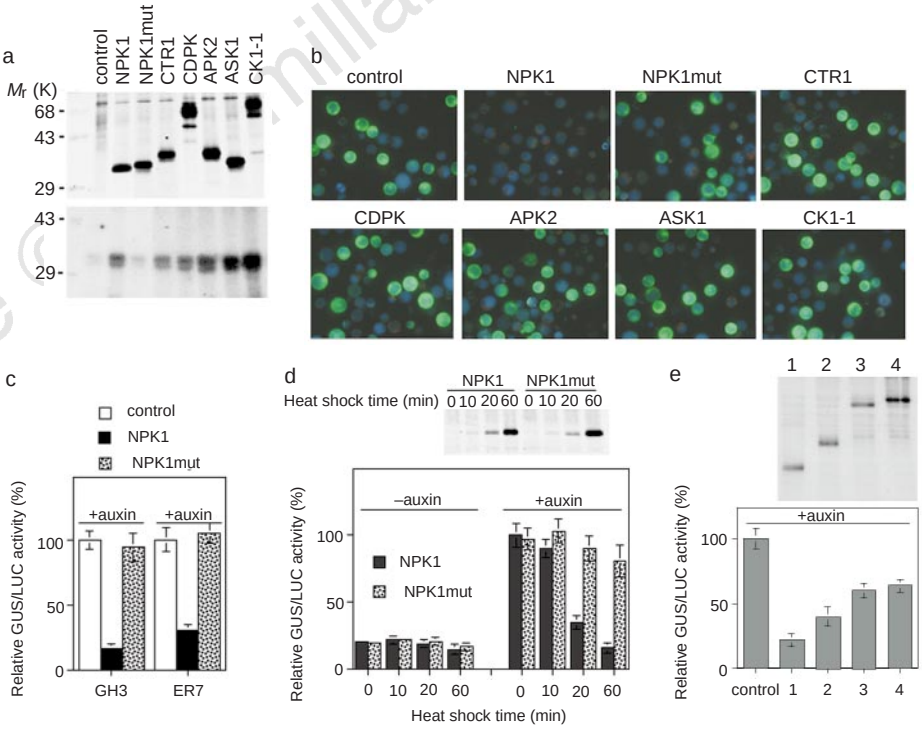

Figure 2 NPK1 represses the auxin-inducible promoters. **a**, Analyses of protein kinases in maize protoplasts. Protoplasts were transfected with vector DNA (control) or an effector construct carrying various protein kinases (NPK1, CTR1, CDPK, APK2, ASK1, CK1-1) or NPK1 K109M mutant (NPK1mut). Top, the effector protein expression level; bottom, effector kinase activity. **b**, NPK1 represses the GH3 promoter. Protoplasts were co-transfected with the GH3–sGFP reporter and an effector construct carrying the kinases indicated or vector DNA (control), and incubated with auxin to induce the GH3 promoter. **c**, NPK1 represses auxin-responsive promoters. Protoplasts were co-transfected with GH3–GUS (GH3), or ER7–GUS (ER7) reporter and an effector construct carrying NPK1 or NPK1mut, or vector DNA (control). **d**, NPK1 protein levels correlate with the inhibitory effect. The expression of wild-type (NPK1) or mutated (NPK1mut) kinase domain is controlled by a heat-shock-inducible promoter (HSP)[14]. Protoplasts were co-transfected with the GH3–GUS reporter and HSP–NPK1 or HSP–NPK1mut effector. Expression of the NPK1 or NPK1mut protein was induced at 42 °C. The protoplasts from each treatment were divided to determine the effector protein expression level (top) and to measure GUS/LUC activity (bottom). **e**, Analysis of the putative regulatory domains of NPK1. Protoplasts were co-transfected with the GH3–GUS reporter and vector DNA (control), or one of the effector constructs carrying the coding region of NPK1 kinase domain only (lane 1), N terminus and kinase domain (lane 2), kinase domain and C terminus (lane 3) or full-length NPK1 protein (lane 4). The protoplasts were divided to determine the effector protein expression level (top) and to measure GUS/LUC activity (bottom).

protoplasts respond to physiological levels of auxin, and that the early auxin responses are probably conserved in both monocotyledonous and dicotyledonous plants.

It has been reported that auxin activates a MAPK in tobacco[2]. The presence of NPK1, a tobacco MAPKKK, in fast-growing cells[3] suggested that NPK1 could mediate auxin signalling. To determine whether NPK1 is involved in auxin signal transduction, we tested the effect of NPK1 on the GH3 promoter and the ER7 promoter element. The NPK1 kinase domain was tagged with a double haemagglutinin epitope (DHA)[8] and cloned into a plant expression vector with a derivative of the CaMV35S promoter (not affected by auxin)[6,8,9]. The NPK1 construct was co-transfected with the GH3–sGFP or GH3–GUS construct into maize protoplasts. Expression and kinase activity of the NPK1 protein were confirmed in transfected protoplasts (Fig. 2a). Surprisingly, NPK1 blocked auxin activation of the GH3 promoter and the ER7 promoter element (Fig. 2b, c). However, the activities of many auxin-insensitive promoters, including the promoters of CAB, actin, ubiquitin, and CaMV35S genes, were not affected by NPK1 (data not shown). To show that the kinase activity of NPK1 is necessary for the repression, a kinase-inactive mutant (K109M) was created by site-directed mutagenesis. The mutation did not affect the expression of the NPK1 protein but abolished the protein kinase activity (Fig. 2a) and the negative effect of NPK1 on the GH3 promoter and the ER7 promoter element in the presence of auxin (Fig. 2b, c).

To demonstrate that the inhibitory effect was specific to NPK1, we tested the effect of other serine/threonine protein kinases that belong to five different classes, including another plant MAPKKK, *Arabidopsis* CTR1 (ref. 13). These five kinases were expressed and displayed protein kinase activity in maize protoplasts (Fig. 2a), but did not block auxin signalling (Fig. 2b).

To test the effect of different NPK1 protein levels on the GH3 promoter activity, we used a heat-shock promoter[14] to control the amount of the NPK1 protein in the transfected protoplasts. The active and mutated NPK1 protein levels correlated well with the duration of heat shock (Fig. 2d). In the absence of auxin, NPK1 could not activate the GH3 promoter. In the presence of auxin, the inverse correlation between the NPK1 protein level and GH3 promoter activity supports the idea that NPK1 acts as a negative regulator in auxin signal transduction (Fig. 2d).

NPK1 consists of a conserved kinase domain, a short amino terminus and a long carboxy-terminal sequence[3]. To investigate the function of the regions outside the kinase domain, we created several NPK1 deletions and tested their effect on GH3 promoter activity. All constructs showed similar levels of protein expression in transfected maize protoplasts (Fig. 2e, top). The kinase region alone or the kinase domain with the N terminus inhibited the GH3 promoter more effectively than the N-terminal deletion or the full-length NPK1 (Fig. 2e, bottom). The data are consistent with the previous observation that the NPK1 protein lacking the C terminus was more effective in the yeast complementation assay[3]. It is likely that the regions outside the NPK1 kinase domain regulate kinase activity, and signals antagonizing auxin responses are required to completely activate the full-length NPK1. Because several signals, such as cell-wall-derived oligosaccharides, cytokinin, ABA and ethylene[15], can interfere with auxin responses in plants, future work will be directed towards identifying the physiologically relevant signals that control NPK1 activity and counteract the auxin responses.

Because NPK1 is a MAPKKK, it is expected to induce a protein phosphorylation cascade resulting in the activation of a MAPK. Although several plant MAPKs have been shown to be induced by stress, hormone and elicitor signals[2,16,17], their activation by a phosphorylation cascade has not been demonstrated in plant cells. We performed an in-gel MAPK activity assay[2,17] with extracts prepared from transfected protoplasts (Fig. 3a, top). Protoplasts transfected with the NPK1 kinase domain had significantly higher endogenous MAPK activity (relative molecular mass, M_r, of 44K) than protoplasts transfected with the mutated NPK1 or an empty vector. The expression of the full-length NPK1 increased the putative MAPK activity only marginally, consistent with the observation that the full-length protein repressed the GH3 promoter mildly (Fig. 2e). CTR1 also activated an endogenous kinase (Fig. 3a), implying the existence of another unrelated MAPK cascade in maize protoplasts.

To verify that NPK1 expression resulted in the activation of a MAPK, we performed kinase activity assays with the proteins immunoprecipitated with an antibody raised against two conserved domains of a mammalian MAPK. The MAPK activity of the protoplasts transfected with the active NPK1 was significantly higher than that of the cells transfected with the mutated NPK1 (Fig. 3a, bottom). These data are consistent with the results of the in-gel MAPK activity assay (Fig. 3a, top), and indicate that tobacco NPK1 can induce a kinase cascade and activate an endogenous MAPK in maize protoplasts.

To determine whether the 44K MAPK is involved in the repression of early auxin response genes, we tested the effect of a MAPK-specific phosphatase (MKP). Protein phosphatases that can specifically dephosphorylate and inactivate MAPKs have been reported in a variety of eukaryotes and are evolutionarily conserved[4]. A mouse MPK1 (ref. 4), highly specific to MAPKs, was cloned into the plant expression vector and expressed in maize protoplasts. The expression of MKP1, but not other plant protein phosphatases (PP) that belong to the three serine/threonine classes (PP1, PP2A[6], and PP2C[9]; Fig. 3b, top), resulted in the complete elimination of the NPK1 effects, including the NPK1-dependent activation of a MAPK (Fig. 3b, bottom) and the repression of the GH3 promoter (Fig. 3c).

To assess the function of NPK1 at a whole-plant level, we generated transgenic tobacco plants overexpressing the NPK1

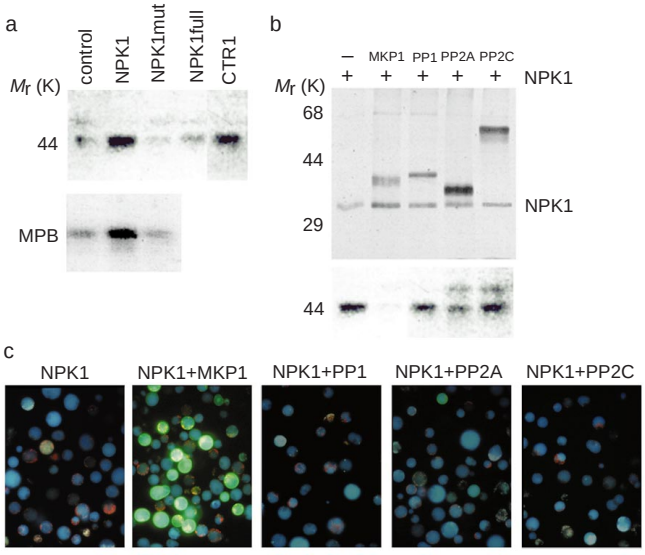

Figure 3 NPK1 activates a MAPK. **a**, MAPK activity assays. Maize protoplasts were transfected with vector DNA (control) or constructs carrying the coding region of NPK1 kinase domain (NPK1), mutated NPK1 kinase domain (NPK1mut), full-length NPK1 (NPK1full) or CTR1 kinase domain (CTR1). In-gel (top) and immunocomplex (bottom) kinase activity assays were performed with cell lysates from the transfected protoplasts. **b**, MAPK phosphatase inactivates NPK1-induced MAPK. Protoplasts were co-transfected with NPK1 and MAPK phosphatase (MKP1) or serine/threonine protein phosphatase (PP1, PP2A, PP2C) effector constructs. The transfected protoplasts were divided to determine the effector protein expression level (top) and to perform the in-gel kinase activity assay (bottom). **c**, MKP1 abolishes NPK1 repression of auxin-inducible transcription. Protoplasts were co-transfected with the GH3–sGFP reporter and NPK1, or NPK1 plus the phosphatases indicated, and incubated in a medium with auxin.

kinase domain. It was anticipated that overexpression of NPK1, as an auxin antagonist, could be lethal. Indeed, in the strongest transgenic line about 75% of the seeds never germinated, whereas seeds from the wild-type control (Fig. 4a) and many other tobacco lines carrying other transgenes (data not shown) germinated normally. Closer examination showed that some transgenic seeds had visible defects in embryo and endosperm (Fig. 4b); for example, many transgenic seeds were hollow with little endosperm and retarded embryos, and in the most severe cases transgenic seeds completely lacked both endosperm and embryo (Fig. 4b). The number of the defective seeds in each line (Fig. 4a) correlated with the level of the transgene expression (Fig. 4c), suggesting that the seed phenotype was due to transgene expression and enhanced NPK1-dependent MAPK activity. Similarly, ectopic activation of a MAPK cascade can disrupt embryo development by inducing mitotic arrest at the M phase in *Xenopus*[18].

Surprisingly, the surviving NPK1 transgenic seeds produced plants of wild-type appearance. These plants had a high level of ectopic NPK1 mRNA (Fig. 4c) but we could not detect the ectopic DHA-tagged NPK1 protein after several protein blot analyses, whereas the control transgenic line expressing the DHA-tagged MEK1 showed a strong signal (Fig. 4d). We hypothesize that the truncated NPK1 protein is unstable and cannot accumulate to a level sufficient to cause grossly abnormal phenotypes. This is in agreement with a recent report that MEKK1, a mammalian MAPKKK, is degraded rapidly after processing and activation[19]. In tobacco cells the NPK1 protein is subjected to a fast turnover after activation at the end of M phase in the cell cycle[20], and is only detectable at low levels in dividing cells[21]. Thus, accumulation of NPK1 protein might be tightly regulated. This could explain why we observed the most dramatic effect of NPK1 during embryogenesis and seed development, when rapid cell divisions occur and more NPK1 proteins could accumulate to block cell-cycle progression. Recent analyses of auxin-resistant mutants in *Arabidopsis* suggested a crucial role for protein degradation in auxin signalling and cell-cycle control. For example, several auxin-resistant mutants (*axr1*, *tir1*) show defects in protein degradation processes[22,23]. Many auxin-inducible proteins, such as SAUR and Aux/IAA, are highly unstable, and some of them function as negative regulators of auxin-mediated transcription[1,24,25]. Our data provide another indication that cell cycle, protein turnover and auxin signalling are connected.

It has been shown that conserved MAPK cascades mediate several vital functions in mammals and yeast, such as cell proliferation, cell death and stress responses[26]. Although many plant MAPK, MAPKK and MAPKKK homologues have been identified on the basis of sequence conservation and functional complementation in yeast, their precise physiological functions in plants are mostly unknown[16]. Here we demonstrate that a plant MAPKKK can activate a MAPK cascade controlling gene expression, act as a negative regulator in the auxin signal transduction pathway, and potentially disturb embryogenesis in transgenic tobacco. The finding of three NPK1-like protein kinases in *Arabidopsis*[27] suggests that this distinct MAPKKK is conserved in higher plants and that a selection for the desired NPK1 mutants could be problematic owing to potential functional redundancy or lethality. Our data suggest that the tightly regulated protein turnover might complicate the use of transgenic plants in the functional analysis of the NPK1-like MAPKKKs. The protoplast transient expression system offers an alternative strategy for investigating the functions of known genes, especially those encoding tightly regulated proteins, with great specificity and flexibility. □

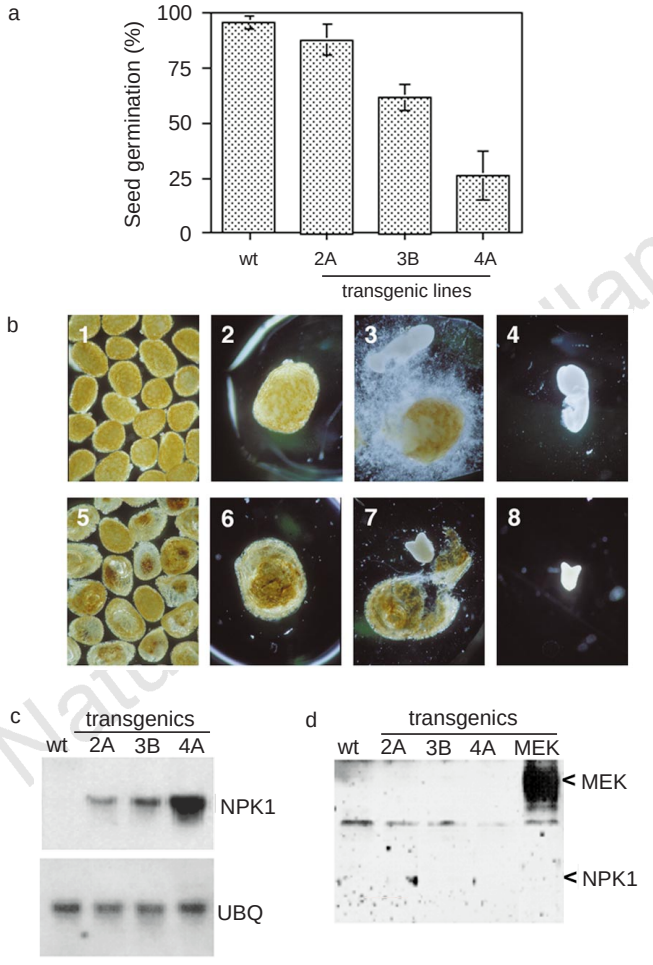

Figure 4 Analyses of NPK1 transgenic tobacco plants. **a**, Seed germination. Wild-type (wt) and three independent transgenic lines (2A, 3B and 4A) were examined. The results are the means of triplicate samples, 100 seeds each, ±s.d. **b** NPK1 transgenic seeds display defects in embryo and endosperm. The wt (1–4) and 4A (5–8) seeds were soaked for 24 hours in water. The seeds are shown as a population (1 and 5), a typical single seed (2 and 6) and dissected (3 and 7). A representative embryo from wt (4) or 4A (8) is shown. At least 10 seeds from each population were analysed. **c**, RNA blot analysis of the transgene expression. The NPK1 probe hybridized with the transgene RNA only. Ubiquitin (UBQ)[30] expression was used as a control. **d**, Protein blot analysis of the transgene expression. The same amount of protein (50 μg per lane) was fractionated by SDS-PAGE (12%). An anti-haemagglutinin antibody was used to detect DHA-tagged transgene proteins. A tobacco transgenic line overexpressing a DHA-tagged MEK protein (MEK) was used as a positive control.

Methods

Reporter constructs. The 749-base pair (bp) soybean GH3 promoter[10] was fused to a synthetic green fluorescent protein gene[11] to create the GH3–sGFP reporter construct. A synthetic ER7 element, TTGTCTCCCAAAGGGAGA-CAA, or mutated ER7, TTGTCTCCCAAAGGGAGAtAA[12], was inserted in front of the CaMV 35S minimal promoter (−72)[6]. The synthetic promoters were fused to a GUS gene to create ER7–GUS and mER7–GUS reporter constructs. Three clones of each construct gave identical results when tested for auxin induction.

Effector constructs. NPK1, NPK1 deletions and CTR1 were obtained by the polymerase chain reaction (PCR) from tobacco cDNA and an *Arabidopsis* cDNA library, respectively. The kinase-inactive NPK1 mutant (K109M) was generated by PCR using the following primers: TACTCGCTATAAtG-GAGGTTTCGAT and CGCAATCGAAACCTCCaTTATAGCGAGTA. The mutation was confirmed by DNA sequencing. All PCR products, and the coding regions of protein kinases from *Arabidopsis* (CDPK (ref. 8), APK2 (ref. 8), ASK1 (ref. 8) and CK1-1 (ref. 28)) and protein phosphatases (mouse MPK1 (ref. 4), maize PP1 (ref. 6), maize PP2A (ref. 6) and *Arabidopsis* PP2C (ref. 9)) were tagged with two copies of the haemagglutinin epitope (DHA) and inserted into a plant expression vector containing the 35SC4PPDK promoter and the *nos* terminator[8,9]. Three to four independent effector clones were tested and gave identical results.

Protoplast transient expression. The preparation and electroporation of etiolated maize mesophyll protoplasts have been described[5,6]. In each electroporation, 2×10^5 protoplasts were transfected with 30 μg of plasmid DNA carrying a reporter construct alone or with 30 μg of plasmid DNA carrying an effector construct or a vector DNA control. The transfected protoplasts were incubated in medium (5×10^4 ml^{-1}) with (+ auxin) or without (− auxin) 1 μM NAA for 14 hours in the dark. GFP fluorescence was observed under ultraviolet irradiation by fluorescent microscopy[14]. A construct carrying the maize CAB5 promoter[5] fused to the luciferase gene (CAB–LUC) was used as an internal control in each transfection where the GH3–GUS or ER7–GUS reporter construct was tested. In each sample the GUS activity[5] of the cell lysate was divided by the LUC activity[8], thereby normalizing the data for variation in experimental conditions (number of cells, transformation efficiency and cell viability). Our results are shown as the means of triplicate samples ± the standard deviation. All transient expression experiments were repeated three times with similar results.

Effector protein expression level. Transfected maize protoplasts (10^5) were incubated for 5 hours with [^{35}S]-methionine (200 μCi ml^{-1}). The DHA-tagged effectors were immunoprecipitated with an anti-haemagglutinin antibody[8], separated by SDS-PAGE (10%) and visualized by fluorography.

Protein kinase activity assays. Cell lysates from 10^5 transfected protoplasts were used for immunoprecipitation with an anti-haemagglutinin antibody[8]. The immunoprecipitated proteins were assayed with [γ-^{32}P]ATP and casein as a substrate. The [^{32}P]-casein was separated by SDS-PAGE (10%) and visualized by autoradiography.

MAPK activity assays. The transfected protoplasts (10^5) were incubated for 5 h before collection. For the in-gel kinase activity assay, protoplasts extracts containing 7 μg of protein were fractionated in 10% SDS-PAGE gel embedded with 0.25 mg ml^{-1} of myelin basis protein (MBP), a MAPK substrate. The protein denaturing, renaturing and kinase activity in the gel were performed as described[17]. For immunocomplex kinase activity assay, maize endogenous MAPKs were immunoprecipiated from protoplast lysates with an anti-ERK (pan-ERK) antibody (Transduction Laboratory)[8] and assayed for MAPK activity with MBP as a substrate[17]. The [^{32}P]-MBP was separated by SDS-PAGE (15%) and visualized by autoradiography.

Transgenic plants. A construct including the 35SC4PPDK promoter[6,8,9], the kinase domain of NPK1, a DHA tag and a *nos* terminator was inserted into the pART27 binary vector[29]. The resulting plasmid was introduced into *Agrobacterium tumefaciens* EHA105 and the transformation was performed with *Nicotiana tabacum* SR1 leaf discs[11]. Several kanamycin-resistant plants were selected from three independent transformation experiments. The seeds were examined by light microscopy. Seedlings two weeks old were used for RNA blot and protein blot analyses[30].

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1. Abel, S. & Theologis, A. Early genes and auxin action. *Plant Physiol.* **111,** 9–17 (1996).
2. Mizoguchi, T. *et al.* Characterization of two cDNAs that encode MAP kinase homologues in *Arabidopsis thaliana* and analysis of the possible role of auxin in activating such kinase activities in cultured cells. *Plant J.* **5,** 111–122 (1994).
3. Banno, H. *et al. NPK1*, a tobacco gene that encoded a protein with a domain homologous to yeast BCK1, STE11, and Byr2 protein kinases. *Mol. Cell. Biol.* **13,** 4745–4752 (1993).
4. Sun, H. *et al. MKP-1* (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase *in vivo*. *Cell* **75,** 487–493 (1993).
5. Sheen, J. Metabolic repression of transcription in higher plants. *Plant Cell* **2,** 1027–1038 (1990).
6. Sheen, J. Protein phosphatase activity is required for light-inducible gene expression in maize. *EMBO J.* **12,** 3497–3505 (1993).
7. Jang, J.-C. & Sheen, J. Sugar sensing in higher plants. *Plant Cell* **6,** 1665–1679 (1994).
8. Sheen, J. Ca^{2+}-dependent protein kinases and stress signal transduction in plants. *Science* **274,** 1900–1902 (1996).
9. Sheen, J. Mutational analysis of protein phosphatase 2C involved in abcisic acid signal transduction in higher plants. *Proc. Natl Acad. Sci. USA* **95,** 975–980 (1998).
10. Hagen, G. *et al.* Auxin-induced expression of the soybean GH3 promoter in transgenic tobacco plants. *Plant Mol. Biol.* **17,** 567–579 (1991).
11. Chiu, W. *et al.* Engineered GFP as a vital reporter in plants. *Curr. Biol.* **6,** 325–330 (1996).
12. Ulmasov, T. *et al.* ARF1, a transcription factor that binds to auxin response elements. *Science* **276,** 1865–1868 (1997).
13. Kieber, J. J. *et al.* CTR1, a negative regulator of the ethylene response pathway in arabidopsis, encoded a member of the RAF family of protein kinases. *Cell* **72,** 427–441 (1993).
14. Sheen, J. *et al.* Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* **8,** 777–784 (1995).
15. John, M. *et al.* Cell signalling by oligosaccharides. *Trends Plant Sci.* **2,** 111–115 (1997).
16. Hirt, M. Multiple roles of MAP kinases in plant signal transduction. *Trends Biol. Sci.* **2,** 11–15 (1997).
17. Zhang, S. & Klessig, D. Salicylic acid activates a 48-kD MAP kinase in tobacco. *Plant Cell* **9,** 809–824 (1997).
18. Takenaka, K. *et al.* Activation of the protein kinase p38 in the spindle assembly checkpoint and mitotic arrest. *Science* **280,** 599–602 (1998).
19. Widmann, C. *et al.* MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. *Mol. Cell. Biol.* **18,** 2416–2429 (1998).
20. Machida, Y. *et al.* Plant MAP kinase cascade that is mediated by MAPKKK-related kinase NPK1: Possible involvement in the regulation of the M phase (40th NIBB Conf. Stress response, 1998).
21. Nakashima, M. *et al.* The expression pattern of the gene for NPK1 protein kinase related to mitogen-activated protein kinase kinase kinase (MAPKKK) in tobacco plant: correlation with cell proliferation. *Plant Cell Physiol.* **39,** 690–700 (1998).
22. Leyser, O. Auxin signalling: Protein stability as a versatile control target. *Curr. Biol.* **8,** R305–R307 (1998).
23. del Pozo, J. C. *et al.* The ubiquitin-related protein RUB1 and auxin response in arabidopsis. *Science* **280,** 1760–1763 (1998).
24. Guilfoyle, T. J. Aux/IAA proteins and auxin signal transduction. *Trends Plant Sci.* **3,** 205–207 (1998).
25. Ulmasov, T. *et al.* Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9,** 1963–1971 (1997).
26. Kyriakis, J. M. & Avruch, J. Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271,** 24313–24316 (1996).
27. Nishihama, R. *et al.* Possible involvement of different splicing in regulation of the activity of *Arabidopsis* ANP1 that is related to mitogen-activated protein kinase kinase kinases. *Plant J.* **12,** 39–48 (1997).
28. Klimczal, L. J. *et al.* Multiple isoforms of arabidopsis casein kinase I combine conserved catalytic domains with variable carboxyl-terminal extensions. *Plant Physiol.* **109,** 687–696 (1995).
29. Gleave, A. P. A versatile binary vector system with a T-DNA organizational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20,** 1203–1207 (1992).
30. Jang, J.-C. *et al.* Hexokinase as a sugar sensor in higher plants. *Plant Cell* **9,** 5–19 (1997).

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Ribonuclease E is a 5′-end-dependent endonuclease

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The selective degradation of messenger RNAs enables cells to regulate the levels of particular mRNAs in response to changes in the environment. Ribonuclease (RNase) E (ref. 1), a single-strand-specific endonuclease[2–4] that is found in a multi-enzyme complex known as the 'degradosome'[5–7], initiates the degradation of many mRNAs in *Escherichia coli*[3,8,9]. Its relative lack of sequence specificity and the presence of many potential cleavage sites in mRNA substrates[2,3] cannot explain why mRNA decay frequently proceeds in a net 5′-to-3′ direction[9–11]. I have prepared covalently closed circular derivatives of natural substrates, the *rpsT* mRNA encoding ribosomal protein S20 (ref. 2) and the 9S precursor to 5S ribosomal RNA[1,12], and find that these derivatives are considerably more resistant to cleavage *in vitro* by RNase E than are linear molecules. Moreover, antisense oligo-deoxynucleotides complementary to the 5′ end of linear substrates significantly reduce the latter's susceptibility to attack by RNase E. Finally, natural substrates with terminal 5′-triphosphate groups are poorly cleaved by RNase E *in vitro*, whereas 5′ monophosphorylated substrates are strongly preferred (compare with ref. 13). These results show that RNase E has inherent vectorial properties, with its activity depending on the 5′ end of its substrates; this can account for the direction of mRNA decay in *E. coli*, the phenomenon of 'all or none' mRNA decay, and the stabilization provided by 5′ stem–loop structures[14–17].

If initiation of mRNA decay by RNase E begins only at or near the 5′ end of an mRNA[14–17], then blocking or sequestering the 5′ end of a substrate might spare it from attack. Circular RNAs lack a free 5′ terminus; I prepared circular derivatives of *rpsT* mRNA by self-ligation, catalysed by DNA ligase[18] in the presence of ATP and a 'bridging' oligodeoxynucleotide complementary to 12 residues at the 5′ and 3′ ends of the substrate. A 3′ extension permitted efficient annealing (compare lanes 1 and 7 in Fig. 1a). Up to 20% of the input