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Overexpression of Arabidopsis *MAP kinase kinase 7* leads to activation of plant basal and systemic acquired resistance

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Summary

There is a growing body of evidence indicating that mitogen-activated protein kinase (MAPK) cascades are involved in plant defense responses. Analysis of the completed *Arabidopsis thaliana* genome sequence has revealed the existence of 20 MAPKs, 10 MAPKKs and 60 MAPKKKs, implying a high level of complexity in MAPK signaling pathways, and making the assignment of gene functions difficult. The *MAP kinase kinase 7* (*MKK7*) gene of Arabidopsis has previously been shown to negatively regulate polar auxin transport. Here we provide evidence that MKK7 positively regulates plant basal and systemic acquired resistance (SAR). The activation-tagged *bud1* mutant, in which the expression of *MKK7* is increased, accumulates elevated levels of salicylic acid (SA), exhibits constitutive pathogenesis-related (*PR*) gene expression, and displays enhanced resistance to both *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 and *Hyaloperonospora parasitica* Noco2. Both *PR* gene expression and disease resistance of the *bud1* plants depend on SA, and partially depend on NPR1. We demonstrate that the constitutive defense response in *bud1* plants is a result of the increased expression of *MKK7*, and requires the kinase activity of the MKK7 protein. We found that expression of the *MKK7* gene in wild-type plants is induced by pathogen infection. Reducing mRNA levels of *MKK7* by antisense RNA expression not only compromises basal resistance, but also blocks the induction of SAR. Intriguingly, ectopic expression of *MKK7* in local tissues induces *PR* gene expression and resistance to *Psm* ES4326 in systemic tissues, indicating that activation of MKK7 is sufficient for generating the mobile signal of SAR.

Keywords: ***MAP kinase kinase 7***, ***bud1*** **mutant, systemic acquired resistance, salicylic acid, NPR1.**

Introduction

Plants, like other multicellular organisms, have innate defense mechanisms to combat microbial pathogens (Jones and Takemoto, 2004). These defense mechanisms function at different levels after the pathogen makes contact with the plant. At the infection site, defense responses are initiated by detecting two general classes of pathogen-derived molecules: pathogen-associated molecular patterns (PAMPs) and effector proteins that are delivered into the plant cell by the type-III secretion system of the pathogen (He, 1998; Nurnberger *et al.*, 2004). Recognition of PAMPs by plant PAMP receptors activates a defense mechanism that is referred to as 'basal' defense (Gomez-Gomez *et al.*, 1999; Ron and Avni, 2004). Pathogens can often overcome this initial defense mechanism by delivering effector molecules into the plant cell to interfere with normal cellular functions. However, the presence of some effectors can be detected by host resistance (R) proteins, thereby triggering a defense mechanism known as the hypersensitive response (HR) to limit pathogen growth (Dangl and Jones, 2001; Heath, 2000; Martin *et al.*, 2003). HR at the site of infection can also activate systemic acquired resistance (SAR), which provides protection against a broad spectrum of pathogens throughout the plant (Durrant and Dong, 2004; Ryals *et al.*, 1996).

Systemic acquired resistance requires the signal molecule salicylic acid (SA), which induces the accumulation of pathogenesis-related (PR) proteins (van Loon and van

Kammen, 1970; Malamy *et al.*, 1990; Van Loon and Van Strien, 1999). Plants expressing the SA-degrading salicylate hydroxylase (nahG) or mutants deficient in SA synthesis, such as *eds5* (also known as *sid1*) and *sid2* (also known as *eds16*), have impaired SAR (Gaffney *et al.*, 1993; Nawrath and Métraux, 1999; Nawrath *et al.*, 2002; Rogers and Ausubel, 1997; Wildermuth *et al.*, 2001). Genetic screens in Arabidopsis have identified several SAR-related mutants (Durrant and Dong, 2004). Among them, *npr1* (also known as *nim1* and *sai1*), which exhibits enhanced susceptibility to a wide range of pathogens such as *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 and *Hyaloperonospora parasitica* Noco2, was found to be insensitive to SA (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). Numerous studies have been conducted to understand the molecular function of NPR1, and to elucidate the signaling pathway downstream of SA (Dong, 2004). However, less is known about the signaling pathway upstream of SA.

There is a growing body of evidence indicating that MAPK cascades are involved in plant defense responses (Innes, 2001; Nakagami *et al.*, 2005; Pedley and Martin, 2005; Zhang and Klessig, 2001). SA and various pathogen-derived elicitors were shown to induce the tobacco mitogen-activated protein kinases (MAPKs), SA-induced protein kinase (SIPK) and wound-induced protein kinase (WIPK) (Zhang and Klessig, 1997). Expression of a constitutively active mutant of *NtMEK2*, which encodes an MAPK kinase (MAPKK) upstream of SIPK and WIPK, leads to multiple defense responses, including defense gene expression and HR-like cell death (Yang *et al.*, 2001). Both SIPK and WIPK can be activated by the Avr9/Cf-9 interaction (Romeis *et al.*, 1999). Silencing of *NPK1*, which encodes an MAPKK kinase (MAPKKK), interferes with the function of the disease-resistance (*R*) genes *N*, *Bs2* and *Rx* (Jin *et al.*, 2002). Silencing of *NTF6/NRK1* (an MAPK) or *MEK1/NQK1* (an MAPKK) attenuates *N*-mediated resistance to tobacco mosaic virus (Liu *et al.*, 2004). Recently, the NbMKK1–NbSIPK cascade was shown to control non-host resistance including HR cell death (Takahashi *et al.*, 2007b).

In tomato, systemin and several oligosaccharide elicitors were shown to activate LeMPK1 and LeMPK2 (Holley *et al.*, 2003). Silencing of genes encoding two MAPKKs (LeMKK2 and LeMKK3) and two MAPKs (LeMPK3 and one similar to Ntf6) compromises Pto-mediated resistance (Ekengren *et al.*, 2003). Both LeMKK2 and LeMKK4 can phosphorylate LeMPK1, LeMPK2 and LeMPK3 *in vitro* (Pedley and Martin, 2004). Silencing of *LeMAP3Kα* blocks both avrPto/Pto-mediated HR and disease-associated cell death (del Pozo *et al.*, 2004).

In Arabidopsis, a complete MAPK cascade (MEKK1, MKK4/MKK5 and MPK3/MPK6) and WRKY22/WRKY29 transcription factors were identified to function downstream of the PAMP receptor FLS2, a leucine-rich-repeat (LRR) receptor kinase (Asai *et al.*, 2002). Several laboratories recently reported that MEKK1 is required for flg22- and/or reactive oxygen species (ROS)-induced MPK4 activation (Ichimura *et al.*, 2006; Nakagami *et al.*, 2006; Suarez-Rodriguez *et al.*, 2007). MKK1 was also shown to be involved in flg22-induced activation of MPK4 (Mészáros *et al.*, 2006). MPK4 is a negative regulator of SAR. The *mpk4* mutant plants exhibit a constitutive SAR phenotype, including elevated levels of SA, constitutive expression of *PR* genes and increased resistance to pathogens (Petersen *et al.*, 2000). The MPK4 protein may regulate defense responses by phosphorylation of specific WRKY transcription factors (Andreasson *et al.*, 2005). The MKK3–MPK6 cascade was shown to play a role in jasmonate-dependent negative regulation of *ATMYC2/JASMONATE-INSENSITIVE1* (Takahashi *et al.*, 2007a). Additionally, the proteinaceous bacterial elicitor harpin can activate MPK4 and MPK6 (Desikan *et al.*, 2001). Silencing of *MPK6* by an intron-containing hairpin loop RNA (ihpRNA) compromises disease resistance (Menke *et al.*, 2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) by MPK6 induces ethylene biosynthesis (Liu and Zhang, 2004).

We identified a semidominant Arabidopsis activation-tagged mutant, *bud1*, in which the expression of the *MKK7* gene is increased (*t307* in Mou *et al.*, 2002; Dai *et al.*, 2006). Previous work has shown that the increased expression of *MKK7* in *bud1* or the repressed expression in *MKK7* antisense transgenic plants causes deficiency or enhancement in auxin transport, indicating that *MKK7* negatively regulates polar auxin transport (PAT) (Dai *et al.*, 2006). In this study, we show that the *bud1* mutant has an elevated level of SA, and exhibits constitutive *PR* gene expression and enhanced resistance to both *Psm* ES4326 and *H. parasitica* Noco2. Consistently, the expression of *MKK7* is induced by pathogen infection in wild-type plants. Silencing of *MKK7* by antisense RNA expression not only compromises basal resistance but also blocks the induction of SAR, demonstrating that MKK7 is a positive regulator required for both basal resistance and SAR. Moreover, ectopic expression of *MKK7* in local tissues induces *PR* gene expression and resistance to the bacterial pathogen *Psm* E4326 in systemic tissues, indicating that MKK7 activation may be involved in generating the mobile signal for SAR.

Results

The bud1 *mutant accumulates elevated levels of SA, and exhibits constitutive* PR *gene expression and enhanced resistance to both bacterial and oomycete pathogens*

The *bud1* mutant was previously generated using a sense/antisense RNA expression system (*t307* in Mou *et al.*, 2002). Increased expression of the *MKK7* gene in the activation-tagged *bud1* mutant causes deficiency in PAT, which in turn leads to the bushy and dwarf morphology of the *bud1*

mutant plants (Dai *et al.*, 2006). The morphology of *bud1* plants is reminiscent of constitutive defense response mutants such as *cpr1*, *ssi1*, and *mpk4*, which accumulate high levels of SA (Bowling *et al.*, 1994; Petersen *et al.*, 2000; Shah *et al.*, 1999). We therefore measured the concentration of free SA in *bud1* plants. As shown in Figure 1(a), *bud1* plants exhibited elevated levels of free SA, indicating that BUD1/MKK7 may act upstream of SA.

To test whether *bud1* displays constitutive defense responses, we crossed a defense response reporter gene containing the *BGL2* (β-1,3-glucanase 2; also known as *PR2*) promoter fused to the *GUS* coding region into the *bud1* mutant background (Bowling *et al.*, 1994). Figure 1(b) shows that the *BGL2:GUS* reporter gene was constitutively expressed in the *bud1* mutant. The molecular marker genes of plant defense responses, *PR1*, *PR2* and *PR5*, were also constitutively expressed in the *bud1* mutant (Figure 1c). We then tested the growth of bacterial pathogen *Psm* ES4326 in *bud1* and wild-type plants. As *bud1* homozygous plants are significantly smaller than wild type (Dai *et al.*, 2006), only *bud1* heterozygous plants were used for the test. Figure 1(d) shows that *bud1* heterozygous plants exhibited enhanced resistance to *Psm* ES3426. We also tested the growth of the oomycete pathogen *H. parasitica* Noco2 on *bud1* and wild-type seedlings. As *bud1* homozygous plants are sterile, a progeny population from *bud1* heterozygous plants was used for the test. At the seedling stage, the size of *bud1* heterozygous plants was similar to wild type, whereas *bud1* homozygous seedlings were smaller than wild type (data not shown). Therefore after *H. parasitica* Noco2 infection,

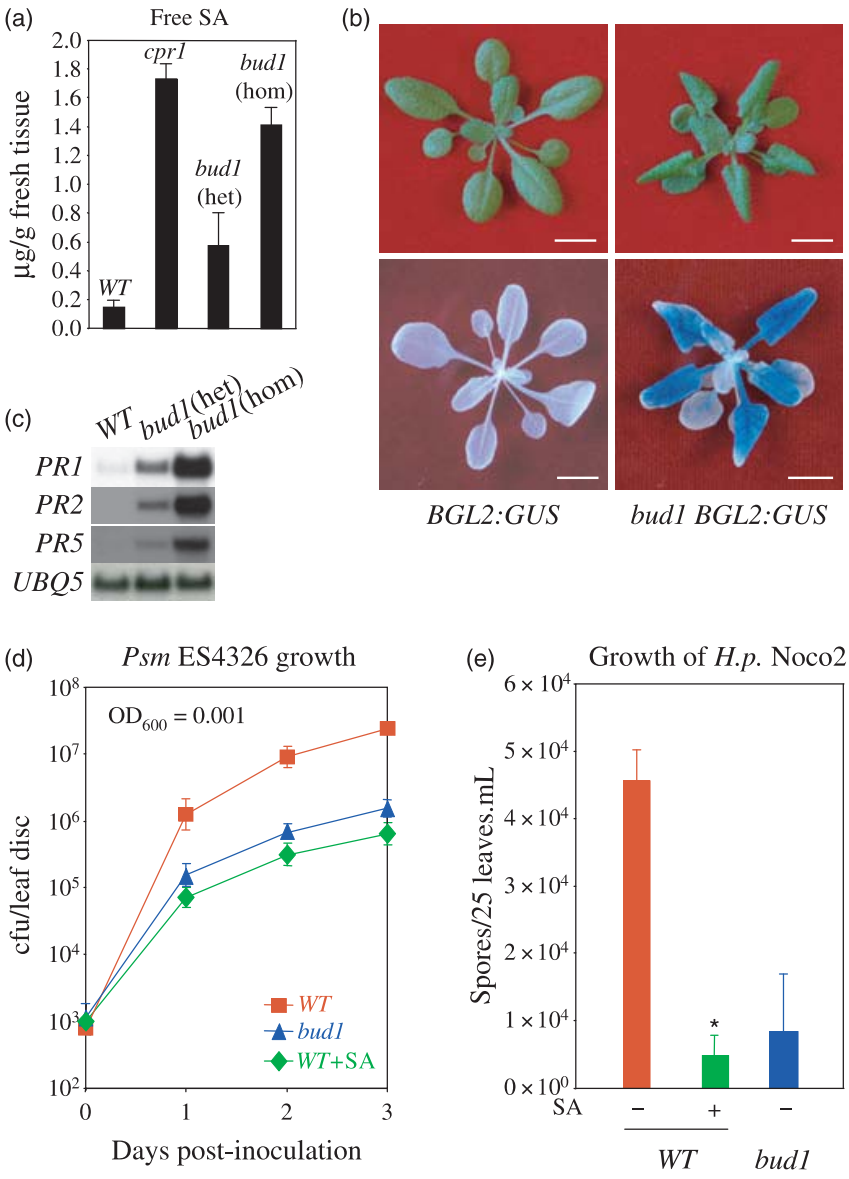

Figure 1. Constitutive defense responses in *bud1*.

(a) Free salicylic acid (SA) levels in wild-type, *cpr1*, *bud1* heterozygous and *bud1* homozygous plants. Four independent leaf samples were collected from each genotype. Each leaf sample was measured once. Data represent the means of the four samples with standard deviation.

(b) Morphology of *BGL2:GUS* (top left) and *bud1 BGL2:GUS* plants (top right), and expression of the *BGL2:GUS* reporter gene in wild-type (bottom left) and *bud1* heterozygous (bottom right) plants. Scale bars: 1 cm.

(c) RNA gel blot analysis of the expression of *PR1*, *PR2* and *PR5* in wild-type, *bud1* heterozygous and *bud1* homozygous plants.

(d) Growth of *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 in *bud1* heterozygote, wild type and wild type treated with 0.5 mM SA. Data represent the means of eight samples with standard deviation.

(e) Growth of *Hyaloperonospora parasitica* Noco2 on *bud1* heterozygote, wild type and wild type treated with 0.5 mM SA. *H. parasitica* Noco2 grew significantly less on the *bud1* plants than on the wild-type plants (*$P < 0.0003$). Three leaf samples (each containing 25 leaves from 10 plants) were collected from six pots (8 × 8 cm) of each genotype/treatment. Each leaf sample was counted six times using a hemacytometer. An average value was obtained from the six counts for each sample, and data represent the means of the resulting three values with standard deviation.

The experiment in (a) was repeated twice with similar results, and experiments in (d) and (e) were repeated three times with similar results.

bud1 heterozygous seedlings were collected to determine the spore numbers of the pathogen. As shown in Figure 1(e), *bud1* heterozygous plants also exhibited enhanced resistance to *H. parasitica* Noco2.

The constitutive defense responses in bud1 *plants depend on SA and partially depend on NPR1*

The *bud1* mutant plants accumulate elevated levels of SA and exhibit constitutive defense responses. To test whether SA signaling plays a role in the constitutive defense response of *bud1* plants, we crossed *bud1* with the SA-deficient mutant, *sid2* (*eds16*). The *bud1sid2* double mutant plants still exhibited *bud1* morphology (Figure 2a). However, the *bud1*-activated *PR1* gene expression was completely suppressed by *sid2* (Figure 2b). The *sid2* mutation also suppressed *bud1*-conferred resistance to *Psm* ES4326 (Figure 2c). Therefore, *bud1* activates SA-dependent defense response pathways in the mutant plants.

NPR1 is a key component of the SA-mediated signaling pathway, and the *npr1* mutation blocks SA-activated defense responses (Cao *et al.*, 1994). To examine whether *npr1* is epistatic to *bud1* we generated the *bud1npr1* double mutant. The double mutant retained the *bud1* morphology (Figure 2a). However, the expression of *PR1* and the resistance to *Psm* ES4326 were partially suppressed (Figure 2b,c). Thus, the *bud1* mutation activates both NPR1-dependent and NPR1-independent defense responses.

The constitutive defense responses in bud1 *plants is a result of increased expression of* MKK7

The morphological phenotype of *bud1* plants was reverted to wild type by reducing the mRNA levels of *MKK7* in the *bud1* plants with a *35S:MKK7* antisense transgene (Dai *et al.*, 2006). We found that the antisense transgene also suppressed the constitutive *PR1* expression in *bud1* plants (Figure 3a), indicating that *MKK7* overexpression is the cause for the mutant phenotype.

We attempted to recapitulate the constitutive defense response phenotype of *bud1* by overexpressing *MKK7* in *35S:MKK7* transgenic plants. Unfortunately, most of the seeds produced by the *35S:MKK7* transgenic plants were not viable (Dai *et al.*, 2006). To circumvent this problem, we generated a transgenic line expressing the *MKK7* gene under the control of the dexamethasone (DEX)-inducible promoter (DEX^{in}). DEX treatment of the DEX^{in}*:MKK7* transgenic plants not only induced the expression of *MKK7*, but also activated *PR1* gene expression and resistance to the bacterial pathogen *Psm* E4326 and the oomycete pathogen *H. parasitica* Noco2 (Figure 3b–d). These results indicate that the constitutive defense responses in the *bud1* plants are caused by increased expression of *MKK7*.

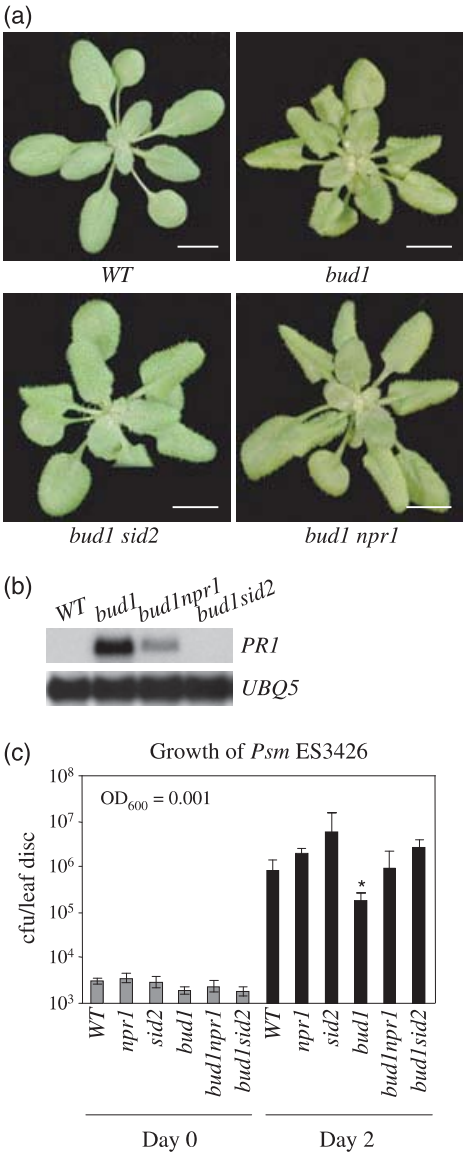

Figure 2. Epistasis analysis of *bud1*.
(a) Morphology of wild-type, *bud1* heterozygous, *bud1sid2* and *bud1npr1* plants. The *bud1sid2* and *bud1npr1* plants are in a *bud1* heterozygote background. Scale bars: 1 cm.
(b) RNA gel blot analysis of the expression of the *PR1* gene in the wild-type, *bud1* heterozygous, *bud1npr1* and *bud1sid2* plants.
(c) Growth of *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 in the *bud1* heterozygous, *bud1npr1* and *bud1sid2* plants compared with that in wild-type, *npr1* and *sid2* plants. *Psm* ES4326 grew significantly less in the *bud1* plants than in the *bud1npr1* and *bud1sid2* plants (*$P < 0.02$ and 0.001 respectively). Data represent the means of eight samples with standard deviation. The experiment was repeated three times with similar results.

The kinase activity of MKK7 is required for the protein to activate defense responses

We have previously shown that the *E. coli* expressed recombinant myelin basic protein (MBP)-MKK7 protein has *in vitro* autophosphorylation activity, whereas

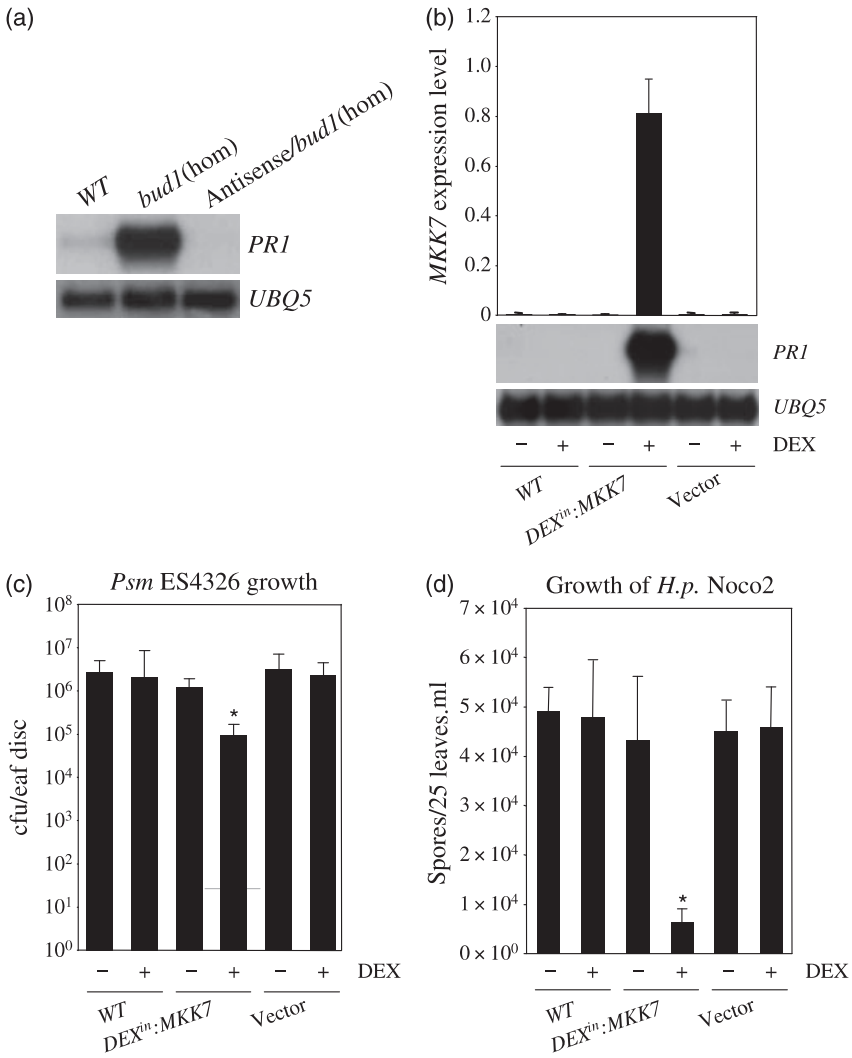

Figure 3. Overexpressing *MKK7* induces defense responses.
(a) RNA gel blot analysis of the expression of the *PR1* gene in wild-type, *bud1* homozygous and antisense plants. The antisense plants are in a *bud1* homozygote background.
(b) Quantitative PCR analysis of the expression of *MKK7* and RNA gel blot analysis of the expression of *PR1* in wild type, *DEX^{in}:MKK7* and pTA7001 vector transgenic plants treated either with or without dexamethasone (DEX). For *MKK7*, data represent the means of three samples with standard deviation.
(c) Growth of *Psm* ES4326 in wild-type, *DEX^{in}:MKK7*,and pTA7001 vector transgenic plants treated either with or without DEX. *Psm* ES4326 grew significantly less in the *DEX^{in}:MKK7* plants treated with DEX than in the mock (0.1% ethanol)-treated *DEX^{in}:MKK7* plants (*$P < 0.0001$). Data represent the means of eight samples with standard deviation.
(d) Growth of *Hyaloperonospora parasitica* Noco2 on wild-type, *DEX^{in}:MKK7* and pTA7001 vector transgenic plants treated either with or without DEX. *H. parasitica* Noco2 grew significantly less on the *DEX^{in}:MKK7* plants treated with DEX than on the mock-treated *DEX^{in}:MKK7* plants (*$P < 0.0002$). Data represent the means of three average values with standard deviation as described in Figure 1(e).
Experiments in (c) and (d) were repeated three times with similar results.

MBP-mkk7(K74R), in which a conserved Lys residue (K) at the position 74 of the ATP binding site in the kinase domain II was replaced with an Arg residue (R), does not (Dai *et al.*, 2006). The *35S:MKK7* transgenic plants overexpressing a wild-type *MKK7* transgene mimicked the *bud1* phenotype, whereas the *35S:mkk7*(K74R) transgenic plants showed the same morphology as wild type, even though the *mkk7*(K74R) transgene was highly expressed (Dai *et al.*, 2006). To test whether overexpression of *MKK7* in the *bud1* mutant increases MAPK activity *in vivo*, an in-gel kinase activity assay was performed using the MBP as an artificial substrate. As shown in Figure 4(a), overexpression of *MKK7* in *bud1* plants activated a kinase with a molecular weight (MW) of ~45 kDa, which is different from the calculated MW of MKK7 (~34 kDa), suggesting that a new kinase was activated in the *bud1* plants. The MKK7 kinase activity in the *bud1* plants was not detected in the in-gel kinase activity assay, probably because the kinase activity of wild-type MKK7 is much lower than that of the activated kinase.

To investigate whether the kinase activity of MKK7 is required for activation of defense responses, we first characterized the *35S:mkk7*(K74R) transgenic plants. No *PR1* gene expression or disease resistance were detected in the *35S:mkk7*(K74R) transgenic plants (data not shown). To further confirm that the kinase activity of MKK7 is required for the protein to activate defense responses, we generated transgenic plants containing a *DEX^{in}:mkk7*(K74R) transgene. In contrast to the *DEX^{in}:MKK7* transgenic plants, DEX treatment of the *DEX^{in}:mkk7*(K74R) transgenic plants did not induce *PR1* gene expression and resistance to *Psm* E4326 and *H. parasitica* Noco2, even though the *mkk7*(K74R) transgene was highly induced (Figure 4b–d). Note that the expression levels of the *GVG* gene in the *DEX^{in}:MKK7*, *DEX^{in}:mkk7*(K74R) and in the vector pTA7001 transgenic plants were similar (Figure S1), suggesting that the *PR1* gene expression in the *DEX^{in}:MKK7* transgenic plants was not caused by expression of the *GVG* gene (Kang *et al.*, 1999). These results demonstrate that the

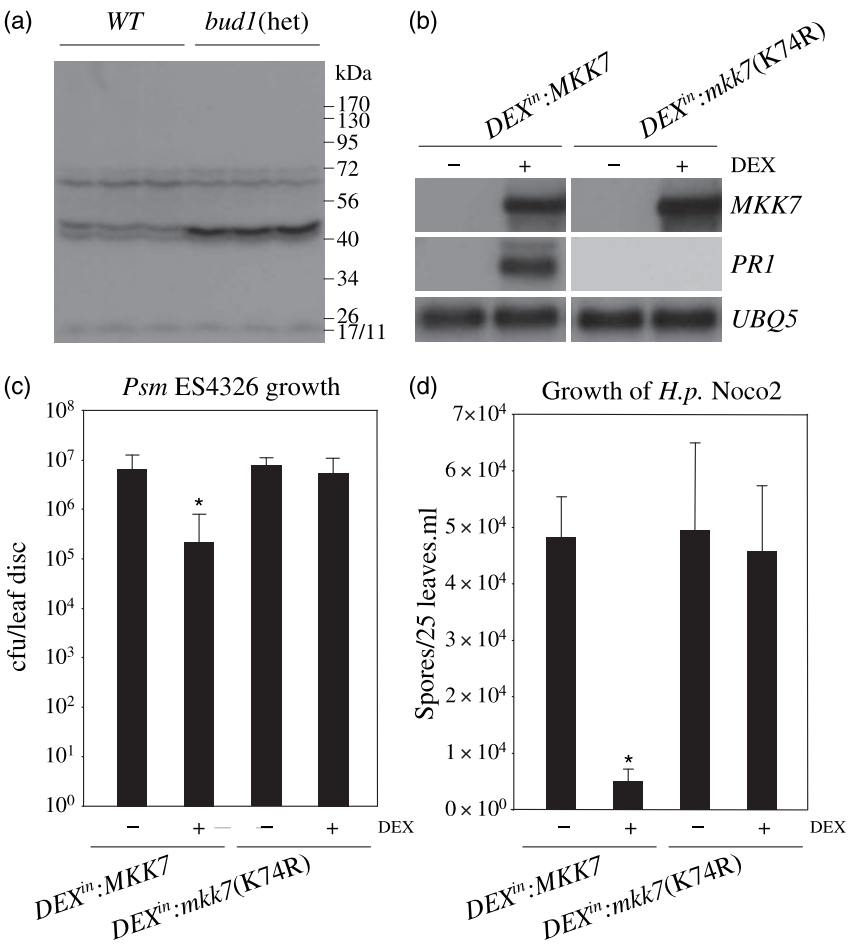

Figure 4. The kinase activity of MKK7 is essential for activation of defense responses.
(a) Kinase activity in wild-type and *bud1* plants (heterozygous) determined by an in-gel kinase activity assay using myelin basic protein (MBP) as a substrate.
(b) RNA gel blot analysis of the expression of *MKK7* or *mkk7*(K74R) and *PR1* in *DEX^{in}:MKK7* and *DEX^{in}:mkk7*(K74R) transgenic plants treated either with or without dexamethasone (DEX).
(c) Growth of *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 in *DEX^{in}:MKK7* and *DEX^{in}:mkk7*(K74R) transgenic plants treated either with or without DEX. *Psm* ES4326 grew significantly less in the *DEX^{in}:MKK7* plants treated with DEX than in the mock-treated *DEX^{in}:MKK7* plants (*$P < 0.0002$). Data represent the means of eight samples with standard deviation.
(d) Growth of *Hyaloperonospora parasitica* Noco2 on *DEX^{in}:MKK7* and *DEX^{in}:mkk7*(K74R) transgenic plants treated either with or without DEX. *H. parasitica* Noco2 grew significantly less on the *DEX^{in}:MKK7* plants treated with DEX than on the mock-treated *DEX^{in}:MKK7* plants (*$P < 0.0001$). Data represent the means of three average values with standard deviation as described in Figure 1(e).
Experiment in (a) and (b) was repeated twice with similar results, and experiments in (c) and (d) were repeated three times with similar results.

MKK7 kinase activity is essential for activation of defense responses.

The expression of the MKK7 *gene is induced by pathogen infection*

Although increased expression of *MKK7* confers *bud1* constitutive *PR* gene expression and enhanced disease resistance, this may not reflect the biological function of MKK7 in Arabidopsis. If MKK7 is involved in defense responses, either its expression or activity should be altered during pathogen infection. To test this hypothesis, a 1.694-kb DNA fragment of the *MKK7* promoter was amplified from wild-type genomic DNA by PCR, and was fused to the *GUS* gene to generate *MKK7:GUS* transgenic plants. As shown in Figure 5(a), expression of the *MKK7:GUS* reporter gene was induced by infection of an avirulent pathogen *P. syringae* pv. tomato (*Pst*) DC3000/*avrRpt2*. This observation was confirmed by examination of the mRNA levels of *MKK7* after *Pst* DC3000/*avrRpt2* infection using both quantitative PCR and RNA gel blot analysis (Figure 5b,c). Interestingly, *MKK7:GUS* expression appeared to be restricted to the vascular tissues, suggesting that MKK7 may be involved in systemic signaling.

To test whether other pathogens could induce *MKK7*, we infected the *MKK7:GUS* transgenic plants with *H. parasitica* Noco2 and *Psm* ES4326 as well as *Pst* DC3000/*avrRpt2*. As shown in Figure S2(a,b), although the avirulent pathogen *Pst* DC3000/*avrRpt2* induced *MKK7* expression 8 h after infection, both *H. parasitica* Noco2 and *Psm* ES4326 did not induce *MKK7*. The induction of *MKK7* by *Pst* DC3000/*avrRpt2* was further confirmed by quantitative PCR analysis of infected tissues collected at 0, 8, 16 and 24 h post-inoculation (Figure S2c). In addition, we also collected uninfected tissues (systemic tissues) of the plants inoculated with *Pst* DC3000/*avrRpt2* at 24 and 48 h post-inoculation, and subjected the tissues to quantitative PCR analysis. Consistent with the expression pattern revealed in Figure 5(a), *MKK7* was not induced in the systemic tissues (data not shown).

Silencing of MKK7 *not only compromises basal resistance, but also blocks the induction of SAR after inoculation with avirulent pathogens*

As increased expression of *MKK7* provides *bud1* with resistance to pathogens, loss-of-function mutants of *MKK7* may exhibit enhanced susceptibility. We therefore obtained five dSym (SM) transposon lines (Tissier *et al.*, 1999), in

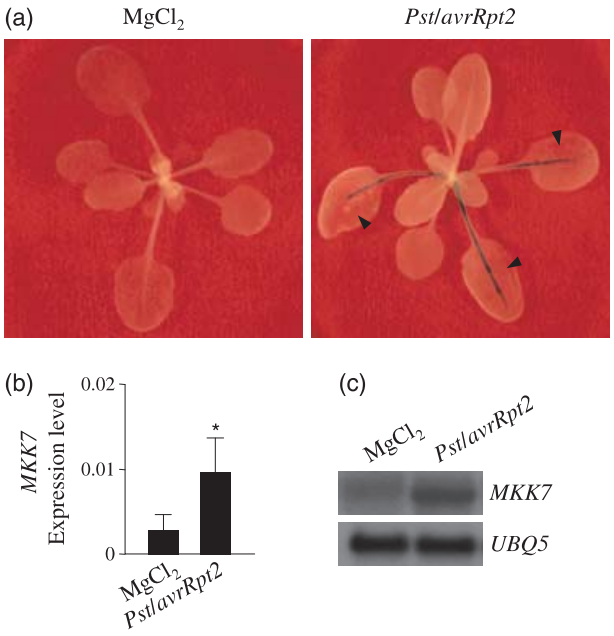

Figure 5. Induction of *MKK7* by *Pst* DC3000/*avrRpt2* infection.
Samples were taken 12 h after *Pseudomonas syringae* pv. tomato (*Pst*) DC3000/*avrRpt2* infection.
(a) Histochemical GUS assay of the *MKK7:GUS* transgenic plants infected with *Pst* DC3000/*avrRpt2*, showing the expression pattern of the *MKK7:GUS* reporter gene. Four independent *MKK7:GUS* transgenic lines were analyzed and a similar induction pattern was detected in all four lines. Arrowheads indicate the inoculated leaf halves.
(b) Quantitative PCR analysis of the expression of *MKK7* after *Pst* DC3000/*avrRpt2* infection. *Pst* DC3000/*avrRpt2* infection significantly induced *MKK7* compared with the mock inoculation (10 mM $MgCl_2$) (*$P < 0.03$). Data represent the means of three samples with standard deviation.
(c) RNA gel blot analysis of the expression of *MKK7* after *Pst* DC3000/*avrRpt2* infection. Note that the background expression level of *MKK7* in the wild type is higher than in Figure 4(a), because of the 3-day longer exposure time.

which the transposon insertions were shown in the coding region of *MKK7* (an intronless gene). We confirmed the transposon insertion sites (Figure S3a and Table S1) and identified homozygous transposon insertion plants using gene-specific primers (Figure S3b). We examined the mRNA levels of *MKK7* in the five SM transposon lines using both quantitative PCR and RNA gel blot analysis. The expression level of *MKK7* in the five SM transposon insertion lines was similar to that of wild type (Figure S3c). To confirm this result, we performed RT-PCR using three pairs of primers covering different regions of the *MKK7* cDNA (Figure S3a). RT-PCR products were detected from all the lines (Figure S3d), and the specificity of the RT-PCR reactions was confirmed by sequencing the RT-PCR products amplified with *MKK7F1* and *MKK7R1*, a pair of primers that cover the coding region of *MKK7* (data not shown). Consistent with the mRNA analysis results, no significant difference in defense responses between wild type and these transposon insertion lines was detected (data not shown).

Because we were unable to identify a knock-out mutant of *MKK7*, we focused on characterization of the previously generated *MKK7* antisense lines that are in the wild-type background (Dai *et al.*, 2006). As shown in Figure 6(a), although the expression of *MKK9*, a homolog of *MKK7*, was not affected in the antisense plants, the expression of *MKK7* was decreased to a lower level compared with that of wild type. As the expression of *MKK7* can be induced by *Pst* DC3000/*avrRpt2*, we tested the induction of *MKK7* in the antisense plants. As shown in Figure S4(a), the induction of *MKK7* by *Pst* DC3000/*avrRpt2* was completely blocked in the antisense plants. Additionally, an in-gel kinase activity assay revealed that the kinase activity at ~45 kDa, which was activated in the *bud1* plants (Figure 4a), was decreased to a lower level compared with wild type (Figure S4(b)), suggesting that this kinase activity may be related to MKK7 activity. Although we cannot exclude the possibility of silencing of additional genes, these results indicated that *MKK7* was silenced in the antisense plants.

To test whether silencing of *MKK7* compromises basal resistance, we monitored the growth of *Psm* ES4326 and *Xanthomonas campestris* pv. *campestris* (*Xcc*) ATCC33913 in the antisense plants. Compared with wild type, the antisense plants exhibited enhanced susceptibility to both *Psm* ES4326 and *Xcc* ATCC33913 infection (Figure 6b,c), indicating that *MKK7* is required for maintaining basal resistance to bacterial pathogens.

We also tested the ability of the antisense plants to develop SAR. After inoculation of lower leaves with the avirulent pathogen *Pst* DC3000/*avrRpt2*, the induction of *PR1* in the systemic tissues of the antisense plants was significantly decreased (Figure 6d). Furthermore, the avirulent pathogen-induced SAR resistance to the bacterial pathogen *Psm* ES4326 was dramatically decreased (Figure 6e). These results demonstrate that MKK7 is essential for the establishment of SAR.

MKK7 may be involved in generating the mobile signal in SAR

Grafting experiments performed in tobacco showed that SA accumulates in systemic tissue in response to a systemic signal that is produced at the site of primary infection and is transduced systemically (Vernooij *et al.*, 1994). The vascular expression of *MKK7* in response to pathogen infection suggests that MKK7 may be involved in systemic signaling. To test this possibility, we made use of the *DEX^{in}:MKK7* transgenic plants in which the expression of *MKK7* is controlled by DEX. Three lower leaves of the *DEX^{in}:MKK7* transgenic plants and the *DEX^{in}:mkk7*(K74R) transgenic plants were infiltrated with DEX to induce *MKK7* and *mkk7*(K74R) expression. As shown in Figure 7(a,c), the *MKK7* and *mkk7*(K74R) transgenes were induced in the DEX-treated local tissues but not in systemic tissues, suggesting

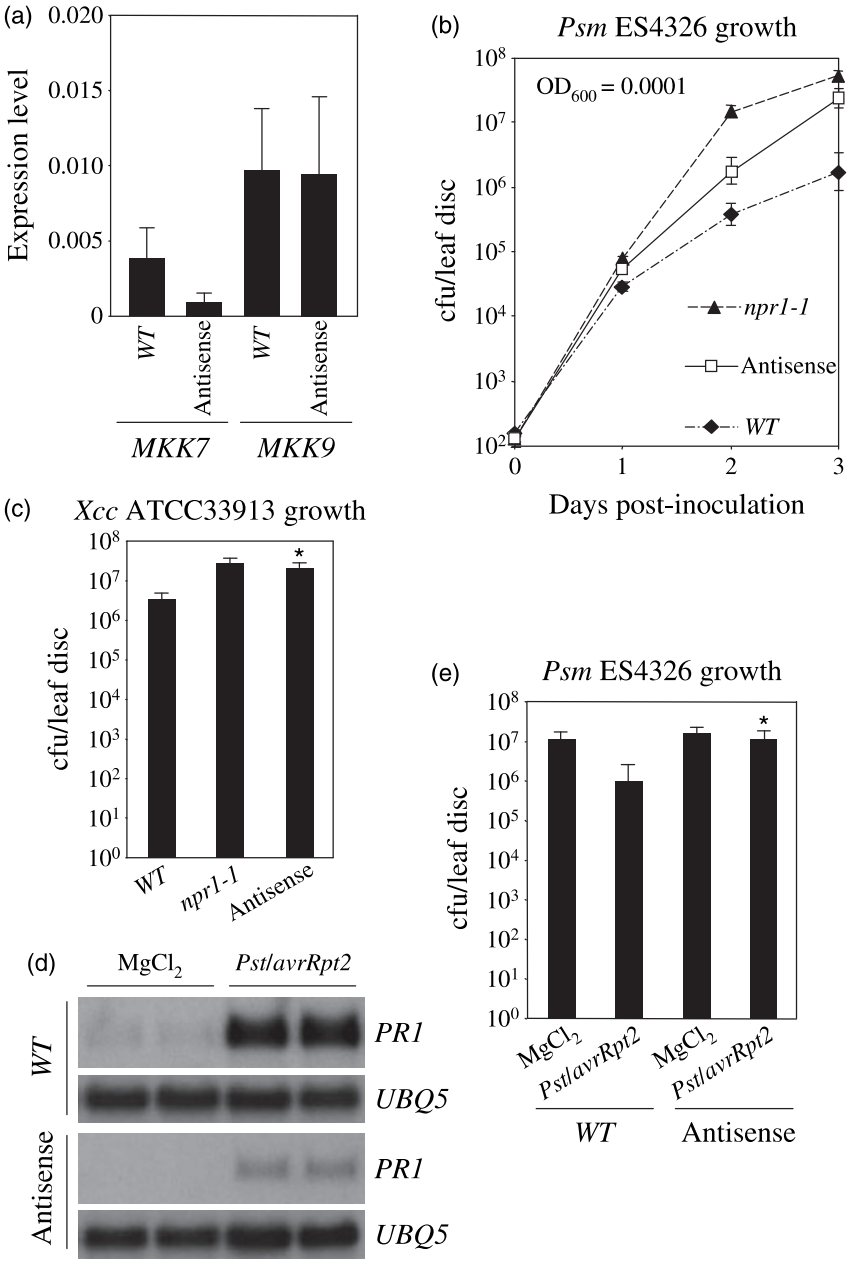

Figure 6. Characterization of *MKK7* antisense transgenic plants.

Four antisense lines were characterized with similar results, and results from a representative line were presented. Note that the antisense lines are in a wild-type background.

(a) Quantitative PCR analysis of the expression of *MKK7* and *MKK9* in the antisense plants. Data represent the means of three samples with standard deviation.

(b) Growth of *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 in wild-type, *npr1-1* and antisense plants.

(c) Growth of *Xanthomonas campestris* pv. *campestris* (*Xcc*) ATCC33913 in wild-type, *npr1-1* and antisense plants. *Xcc* ATCC33913 grew significantly more in the antisense plants than in the wild-type plants (*$P < 0.0001$).

(d) RNA gel blot analysis of the expression of *PR1* in systemic tissues of wild-type and antisense plants inoculated with *P. syringae* pv. tomato (*Pst*) DC3000/*avrRpt2* in local tissues.

(e) Growth of *Psm* ES4326 in systemic tissues of wild-type and antisense plants inoculated with *Pst* DC3000/*avrRpt2* in local tissues. *Psm* ES4326 grew significantly more in the systemic tissues of the antisense plants than in that of the wild-type plants (*$P < 0.006$).

Data in (b), (c) and (e) represent the means of eight samples with standard deviation. Experiments in (b), (c), (d) and (e) were repeated three times with similar results.

that DEX did not spread systemically, which is consistent with previous studies (Aoyama and Chua, 1997). Interestingly, resistance to the bacterial pathogen *Psm* ES4326 was induced in the systemic tissues of the *DEX^{in}:MKK7* transgenic plants, but not in those of the *DEX^{in}:mkk7*(K74R) transgenic plants (Figure 7b). Additionally, *PR1* gene expression was induced not only in the DEX-treated local tissues, but also in the systemic tissues of the *DEX^{in}:MKK7* transgenic plants (Figure 7c). These results showed that ectopic expression of *MKK7* in local tissues is sufficient to induce SAR in systemic tissues, demonstrating a critical role for MKK7 in generating the systemic signal of SAR.

Discussion

Plant–pathogen interaction often triggers defense responses to protect plants from further pathogen damage (Dangl and Jones, 2001; Hammond-Kosack and Jones, 1996). The signals that plants receive from pathogen infections are amplified and transduced to the nucleus to switch gene expression profiles (Nimchuk *et al.*, 2003). The MAPK cascades have been implicated in this signal amplification and transduction process (Pedley and Martin, 2005). Characterization of the Arabidopsis mutant *bud1* in this study demonstrates that the MAPK cascade, of which MKK7 is a

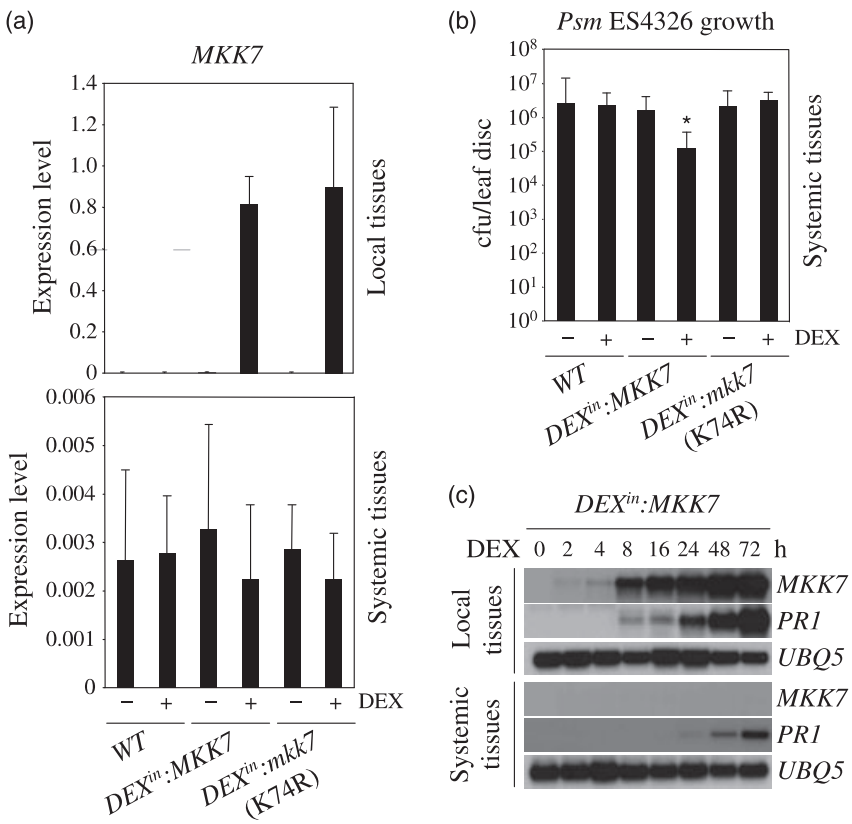

Figure 7. Ectopic expression of *MKK7* in local tissues induces systemic acquired resistance (SAR) in systemic tissues.
(a) Quantitative PCR analysis of the expression of *MKK7* or *mkk7*(K74R) in local tissues (top) and systemic tissues (bottom) of wild-type, *DEX*^{in}*:MKK7* and *DEX*^{in}*:MKK7*(K74R) transgenic plants with or without application of dexamethasone (DEX) in local tissues. Data represent the means of three samples with standard deviation.
(b) Growth of *Pseudomonas syringae* pv. maculicola (*Psm*) ES4326 in systemic tissues of wild-type, *DEX*^{in}*:MKK7* and *DEX*^{in}*:MKK7*(K74R) transgenic plants either with or without application of DEX in local tissues. *Psm* ES4326 grew significantly less in the systemic tissues of the *DEX*^{in}*:MKK7* plants treated with DEX than in that of the mock-treated *DEX*^{in}*:MKK7* plants (*$P < 0.0003$). Data represent the means of eight samples with standard deviation. The experiment was repeated three times with similar results.
(c) RNA gel blot analysis of the expression of *MKK7* and *PR1* in local tissues and systemic tissues of the *DEX*^{in}*:MKK7* transgenic plants at the indicated time points after application of DEX in local tissues. The *UBQ5* gene was used as a loading control. The experiment was repeated twice with similar results.

part, plays a critical role in regulating plant basal resistance and SAR.

The activation-tagged *bud1* mutant is a dwarf, exhibiting constitutive defense responses. This phenotype could be a pleiotropic effect of disturbances of normal cell function caused by increased expression of *MKK7*. However, four lines of evidence argue against this possibility. First, *bud1* does not exhibit necrotic lesions (data not shown), suggesting that the overall cell function in *bud1* is not disrupted. Second, the expression of *MKK7* in wild type is induced by pathogen infection. Increased expression of *MKK7* in *bud1* may mimic the upregulation of *MKK7* after pathogen infection. Third, the kinase activity of MKK7 is required for all the *bud1* phenotypes (Dai *et al.*, 2006; this study), indicating that an MAPK cascade(s) is activated in *bud1*. Finally, silencing of *MKK7* by antisense not only compromises basal resistance to *Psm* ES4326 and *Xcc* ATCC33913, but also blocks induction of SAR, demonstrating that MKK7 is required for both basal resistance and SAR.

The Arabidopsis MKK7 was placed into group D of plant MAPKK, based on sequence alignment (MAPK Group, 2002). Members in group D from other plant species have been reported to play a role in plant defense responses. For example, LeMKK4, encoded by an ortholog of *MKK7* in tomato, phosphorylates LeMPK1, LeMPK2 and LeMPK3 *in vitro* (Pedley and Martin, 2004). When overexpressed in leaves, LeMKK4 elicits cell death and activates LeMPK2 and LeMPK3. The three MAPKs, LeMPK1, LeMPK2 and LeMPK3, have been implicated in different aspects of plant defense responses (Ekengren *et al.*, 2003; Holley *et al.*, 2003). In tobacco, using virus-induced silencing, it has been shown that the *MKK7* ortholog, *NbMKK1*, controls non-host resistance including HR cell death (Takahashi *et al.*, 2007b). These results support the conclusion that MKK7 plays a function in plant defense responses.

MKK7 may affect basal resistance and SAR through SA synthesis. Consistent with this hypothesis, the defense phenotype in *bud1* was completely suppressed by the SA-deficient mutation *sid2*. Two different mechanisms may explain how MKK7 regulates SA synthesis. One possibility is that, like EDS1 and PAD4, MKK7 may function as a component in a signal amplification loop affecting SA synthesis (Feys *et al.*, 2001). MKK7 may also function in generating the mobile systemic signal of SAR; perception of the systemic signal leads to SA synthesis. Although these two mechanisms are not exclusive, evidence here favors the latter. First, pathogen infection of *MKK7:GUS* transgenic plants induces *GUS* gene expression in the midribs (vascular tissues) of the local tissues, but not in systemic tissues (Figure 3a). Second, ectopic expression of *MKK7* in local tissues not only activates defense responses in the local tissues, but also induces SAR in systemic tissues, suggesting that activation of MKK7 in local tissues is sufficient to induce SAR. Together, these results indicate that pathogen

infection activates MKK7 in the local tissues, which leads to the production of a signal that is transduced systemically to induce SAR in systemic tissues.

In Arabidopsis, there are fewer MAPKKs (10) than MAPKs (20) and MAPKKKs (60) (MAPK Group, 2002). This suggests that various signal transduction pathways may converge at the MAPKK levels in the MAPK cascades. MKK7, an MAPKK, not only functions as a negative regulator of plant PAT (Dai *et al.*, 2006), but also functions as a positive regulator of plant basal resistance and SAR, suggesting that MKK7 may serve as a crosstalk point between auxin signaling and defense responses.

Crosstalk between auxin and plant defense responses has been known for many years. Most microbial pathogens possess the capacity to synthesize indole-3-acetic acid (IAA) (Fett *et al.*, 1987; Wichner and Libbert, 1968). However, this capacity has been shown to be important for the pathogenicity of only a few pathogens such as *P. syringae* pv. *savastanoi*, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* (Liu *et al.*, 1982; Offringa *et al.*, 1986; Surico *et al.*, 1985). Using *Xcc* ATCC33913, a strain that does not synthesize IAA itself, O'Donnell *et al.* (2003)showed that the pathogen was able to induce the host plant to produce IAA by upregulating host genes involved in IAA synthesis. These results indicate that pathogens may perturb auxin homeostasis of the host plant to promote disease. Characterization of Arabidopsis *dth9* and *sgt1b*/*eta3* mutants suggests that auxin homeostasis is one of the components participating in the regulation of plant defense responses (Gray *et al.*, 2003; Mayda *et al.*, 2000). Both *dth9* and *sgt1b*/*eta3* mutants are more susceptible to pathogen invasion and insensitive to exogenous auxin application (Gray *et al.*, 2003; Mayda *et al.*, 2000; Tör *et al.*, 2002). Recently, a flg22-induced microRNA (miRNA) was shown to restrict *P. syringae* growth by repressing auxin signaling in Arabidopsis (Navarro *et al.*, 2006). In this study, we show that increased expression of *MKK7* in the *bud1* mutant not only causes deficiency in plant PAT, but also leads to constitutive defense responses, suggesting that the MKK7 MAPK cascade(s) is likely to be involved in regulating both auxin homeostasis and defense responses.

Because the kinase activity of MKK7 is required for all the *bud1* phenotypes, one or more MAPK cascades may be activated in *bud1*. Identification of other components in the MKK7 MAPK cascade(s), and its downstream effectors, will bring us more insight into the signal amplification and transduction pathways in plant defense responses.

Experimental procedures

Plant materials and growth conditions

The wild type used was the Columbia ecotype (Col-0). The mutant alleles used were *sid2-2* (*eds16-1*) and *npr1-1*. The *BGL2:GUS* transgenic line has been described by Bowling *et al.* (1994). The heterozygous *bud1* plants were used to produce plants for the experiments.

The *bud1 BGL2:GUS* lines were generated using pollen from the *BGL2:GUS* transgenic plants to fertilize the heterozygous *bud1* plants. The *bud1* heterozygotes were identified in the F_2 generation by the *bud1* morphological phenotype. The *BGL2:GUS* homozygotes were identified in the F_3 generation using a histochemical GUS assay. The *bud1sid2* and *bud1npr1* double mutants were generated using pollen from *sid2-2* or *npr1-1* plants to fertilize the heterozygous *bud1* plants. The *bud1* heterozygotes were identified in the F_2 generation as described above. The cleaved amplified polymorphic sequence markers for *sid2-2* and *npr1-1* were used to confirm homozygosity at the *sid2* and *npr1* loci.

Arabidopsis seeds were sown on autoclaved soil (Metro-Mix 200; Grace-Sierra, Malpitas, CA, USA) and vernalized at 4°C for 3 days. Plants were germinated and grown at 22°C under a 16-h light/8-h dark regime.

Pathogen infection

Infection of plants with *Psm* ES4326 or *H. parasitica* Noco2 was performed as described previously (Clarke *et al.*, 1998). For *Psm* ES4326 infection, between four and eight infected leaves were collected for each genotype, treatment or time point to determine *in planta* growth of the pathogen. For *H. parasitica* Noco2 infection, 25 leaves from 10 plants were harvested to determine the degree of infection. After vigorous vortex-mixing in 1 ml of H_2O, two 10-μl aliquots from each sample were examined with a hemacytometer to determine the number of spores. Three samples for each genotype, treatment or time point were assayed to obtain a standard deviation.

For SAR induction, three lower leaves on each plant were inoculated with an avirulent bacterial pathogen *Pst* DC3000/*avrRpt2* (OD_{600} = 0.02). The upper uninfected systemic leaves were collected 2 days later for *PR1* gene expression analysis. After 3 days, the uninfected systemic leaves were challenge-inoculated with *Psm* ES4326 (OD_{600} = 0.001). Eight leaves were collected on day 3 to examine the growth of the pathogen.

To determine the expression pattern of *MKK7*, three half leaves on each *MKK7:GUS* plant were inoculated with *Pst* DC3000/*avrRpt2* (OD_{600} = 0.02). The inoculated plants were collected for histochemical GUS assay after 24 h.

Infection of the bacterial pathogen *Xcc* ATCC33913 was performed following the protocol used for *Psm* ES4326 (Clarke *et al.*, 1998). Briefly, the bacteria cell suspension (in 10 mM $MgCl_2$, OD_{600} = 0.005) was infiltrated into leaves with a 1-ml syringe. After 4 days, eight leaves were collected to determine the *in planta* growth of the pathogen.

Histochemical GUS assay

Soil-grown plants (3–4-weeks old) with or without pathogen treatment were stained for GUS activity as described by Fan and Dong (2002). Briefly, plants were submerged in a solution containing 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl glucuronide in 0.1 M Na_2HPO_4, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide/ferrocyanide and 0.06% Triton X-100, and were vacuum infiltrated for 5 min. After incubation at 37°C for 16 h, the staining solution was removed and the samples were cleared of chlorophyll by sequential changes of 75% and 95% ethanol.

In-gel kinase activity assay

The in-gel kinase activity assay was performed as described by Ren *et al.* (2002). In brief, protein was extracted from 2- to 3-week-old plants by homogenizing in extraction buffer [50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM Na_3VO_4, 10 mM NaF, 50 mM glycerophosphate, 10 mM DTT, 5% glycerol and protease inhibitors: 50 μg ml^{-1} L-1-tosylamido-2-phenylethylchloromethyl ketone (TPCK), 50 μg ml^{-1} N-alpha-p-tosyl-L-lysine chloromethyl ketone (TLCK) and 0.6 mM phenylmethanesulphonyl fluoride (PMSF)]. About 20 μg of total protein was separated by electrophoresis on 10% SDS-polyacrylamide gels embedded with 0.1 mg ml^{-1} myelin basic protein (MBP) in separating gel as a substrate for the kinase. After electrophoresis, the SDS was removed from the gel by washing with washing buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} bovine serum albumin and 0.1% Triton X-100) three times for 30 min each at room temperature (22–23°C). The proteins were then renatured in 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4 and 5 mM NaF at 4°C overnight with three changes of the buffer. The gel was incubated at room temperature in 100 ml of reaction buffer (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4) for 30 min. Phosphorylation was performed for 1.5 h at room temperature in 30 ml of the same buffer with 200 nM ATP plus 50 μCi of [γ-^{32}P]ATP (6000 Ci $mmol^{-1}$). The reaction was stopped using a solution with 5% trichloroacetic acid (w/v) and 1% sodium pyrophosphate (w/v). The gel was then washed using the same solution for 6 h at room temperature with five changes of solution to remove unincorporated radioactivity. The gel was then dried with a gel dryer (Model 583; Bio-Rad, http://www.bio-rad.com) and subjected to autoradiography. Prestained size markers (Fisher Scientific, http://www.fisher.co.uk) were used to calculate the size of kinases.

SA measurement

Soil-grown plants (3–4-weeks old) were used to measure the concentration of free SA using a previously described protocol (Schmelz *et al.*, 2003).

RNA analysis, RT-PCR and quantitative PCR

RNA extraction and RNA gel blot analysis were carried out as described by Cao *et al.* (1997). For reverse transcription (RT), total RNA was treated with Dnase I (Gibco, http://www.invitrogen.com) at 37°C for 30 min. After inactivation of the DNase, RT was performed using SUPERSCRIPT First-strand Synthesis System (Gibco) and 2 μg of the DNase-treated RNA in a 20-μl reaction. Aliquots of the resulting RT reaction product were used for RT-PCR and quantitative PCR. For RT-PCR, amplification of cDNA was performed with 2 μl of RT product in a 50-μl reaction. The three pairs of primers used for amplification reactions of *MKK7* were *MKK7F1* (5′-ATGGCTCTTGTTCGTAAACG-3′) and *MKK7R1* (5′-AAGACTTTCACGGAGAAAAGG-3′), *MKK7F2* (5′-GCACTTGCGCTTACAT-3′) and *MKK7R2* (5′-GAAAAGGGTGACCGAGA-3′), and *MKK7F3* (5′-GTAAAGAATCGAGTGAGAGG-3′) and *MKK7R3* (5′-AATTGCGATTTGGGTCACCC-3′). Primers used for the *GVG* gene were *GVGF* (5′-GACAATCAAGCGGAAACCTG-3′) and *GVGR* (5′-TCATGCATGGAGTCCAGAAG-3′). All PCR reactions were performed under the following conditions: 94°C for 3 min, 35 cycles (94°C for 1 min, 56°C for 1 min, 72°C for 1 min), and a final extension at 72°C for 10 min.

Quantitative PCR was performed using SYBR Green protocol (Applied Biosystems, http://www.appliedbiosystems.com) with 1-μM primers and a 0.2-μl aliquot of RT product in a total of 10 μl per reaction. Reactions were run and analyzed on a Lightcycler (Roche, http://www.roche.com) according to the manufacturer's instructions. A standard curve was made by determining the threshold cycle (C_t) values for a dilution series of the RT reaction product for each primer pair. For each reaction, the C_t was determined by setting the threshold within the logarithmic amplification phase. The relative quantity of a gene is expressed in relation to ubiquitin 5 (*UBQ5*) using the formula $2^{[C_t(UBQ5) - C_t(GENE)]}$, where 2 represents perfect PCR efficiency. Quantitative PCR reactions were performed in triplicate to obtain a standard deviation. The primers used were *MKK7F3* and *MKK7R3*, as described above, *MKK9F* (5′-AGTTTAGGAGCTTCGTTGAG-3′) and *MKK9R* (5′-AGTTTAGGAGCTTCGTTGAG-3′), and *UBQ5F* (5′-GACGCTTCATCTCGTCC-3′) and *UBQ5R* (5′-GTAAACGTAGGTGAGTCCA-3′).

Plasmid construction and plant transformation

To fuse the *MKK7* promoter with the *GUS* reporter gene, a 1.694-kb DNA fragment of the *MKK7* promoter was amplified from wild-type genomic DNA by PCR using primers *Xba*I-*MKK7PF* (5′-GCTCTAGAAGTGATTTGGTAGGAGCC-3′) and *Sma*I-*MKK7PR* (5′-TCCCCCGGGAGAGTGATGATGGTGATCG-3′). The PCR products were digested with *Xba*I and *Sma*I and cloned into *Xba*I/*Sma*I-digested pBI101 vector.

To generate transgenic plants expressing *MKK7* under the control of *DEX*^{in}, *MKK7* cDNA was amplified from wild-type genomic DNA by PCR using the primers *Sal*I-*MKK7F* (5′-GCGTCGACCTCTCTTCTATTTCCATGGC-3′) and *Spe*I-*MKK7R* (5′-GGACTAGTACAAGCAGTCGGATCTAAAG-3′). The PCR products were digested with *Sal*I and *Spe*I, and were cloned into *Xho*I/*Spe*I-digested pTA7001 vector (Aoyama and Chua, 1997). The *mkk7*(K74R) mutant was generated by site-directed mutagenesis in the pTA7001-MKK7 construct using a PCR-based Quick-Change site-directed mutagenesis kit (Stratagene, http://www.stratagene.com). The presence of the expected mutation in the pTA7001-*mkk7*(K74R) was verified by DNA sequencing.

The T-DNA plasmids were introduced into Agrobacterium strain GV3101(pMP90) by electroporation, and were transformed into Arabidopsis plants (ecotype Columbia) using the floral-dip method.

DEX treatment

Leaves of the *DEX*^{in}*:MKK7* and *DEX*^{in}*:mkk7*(K74R) transgenic plants were infiltrated with 0.01 mM DEX in 0.1% ethanol solution or 0.1% ethanol using a 1-ml syringe. After 24 h, the infiltrated leaves were either collected for *PR1* gene expression analysis or inoculated with *Psm* ES4326 (OD_{600} = 0.001) for the resistance test. For *H. parasitica* Noco2 infection, 7-day-old *DEX*^{in}*:MKK7* and *DEX*^{in}*:mkk7*(K74R) transgenic plants were sprayed with 0.01 mM DEX in 0.1% ethanol solution plus 0.01% Tween-20, or 0.1% ethanol plus 0.01% Tween-20. After 24 h the seedlings were infected with *H. parasitica* Noco2 as described above.

To test whether local application of DEX can induce SAR in these transgenic plants, three lower leaves on each plant were infiltrated with 0.01 mM DEX. Three days after DEX treatment, the uninfiltrated systemic leaves were challenge-inoculated with *Psm* ES4326 (OD_{600} = 0.001). Eight leaves were collected 3 days post-inoculation to examine the growth of the pathogen. For *PR1* expression, after DEX treatment, both local tissues and systemic tissues were collected at different time points for RNA gel blot analysis.

Statistical methods

All statistical analyses were performed with the data analysis tools (t-TEST: two samples assuming unequal variances) in the Microsoft

EXCEL program of Microsoft Office 2004 for Macintosh (Microsoft, http://www.microsoft.com).

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. The expression of *GVG* in the *DEX^{in}:MKK7*, *DEX^{in}:mkk7*(K74R) and in the vector pTA7001 transgenic plants.

Figure S2. Induction of *MKK7* by different pathogen infections.

Figure S3. The expression of *MKK7* in the five SM transposon lines.

Figure S4. Further characterization of the *MKK7* antisense plants.

Table S1. The T-DNA or transposon insertion site in the SALK and SM lines.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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