An Arabidopsis Mitogen-Activated Protein Kinase Cascade, MKK9-MPK6, Plays a Role in Leaf Senescence1[C][W][OA]

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Leaf senescence is a developmentally programmed cell death process that constitutes the final step of leaf development, and it can be regulated by multiple environmental cues and endogenous signals. The mitogen-activated protein kinase (MAPK) cascades play diverse roles in intracellular and extracellular signaling in plants. Roles of the MAPK signaling module in leaf senescence are unknown. Here, a MAPK cascade involving MKK9-MPK6 is shown to play an important role in regulating leaf senescence in Arabidopsis (Arabidopsis thaliana). Both MKK9 and MPK6 possess kinase activities, with MPK6 an immediate target of MKK9, as revealed by in vitro, in vivo, and in planta assays. The constitutive and inducible overexpression of MKK9 causes premature senescence in leaves and in whole Arabidopsis plants. The premature senescence phenotype is suppressed when MKK9 is overexpressed in the mpk6 null background. When either MKK9 or MPK6 is knocked out, leaf senescence is delayed.

Leaf senescence is the final phase of leaf development, in which leaf cells undergo active degenerative processes, including the degradation of chlorophylls, proteins, and other macromolecules. The released nutrients are transferred to actively growing young leaves and developing fruits and seeds (Gan, 2007). Differential gene expression is believed to play an important role in leaf senescence. Analysis of a leaf senescence EST database (dbEST) indicated that approximately 10% (approximately 2,500) of the Arabidopsis (Arabidopsis thaliana) genes are expressed in senescent leaves (Guo et al., 2004). Leaf senescence can be induced by endogenous signals, including age, developmental cues, and plant growth regulators (Gan, 1995; Gan and Amasino, 1995; Grbic and Bleecker, 1995; Riefler et al., 2006). Senescence can also be initiated prematurely by a number of exogenous environmental stresses, including light and temperature stress, dehydration, nutrient stress, and pathogen infection (Beers and McDowell, 2001; Pic et al., 2002; Xiong et al., 2005; Hopkins et al., 2007). When the endogenous signals or environmental stresses are perceived by the plant, the signals are subsequently transmitted, resulting in changes in gene expression and/or physiological activities. One of the universal signaling pathways involved in the response to external stimuli is the mitogen-activated protein kinase (MAPK) cascade.

The MAPK cascade has been shown to be involved in various biotic and abiotic stress responses, hormone responses, cell proliferation, differentiation, and developmental processes in plants (Nakagami et al., 2005). There are 20 MAPKs, 10 MAPKKs, and more than 60 MAPKKKs in the Arabidopsis genome (MAPK Group, 2002). The 20 MAPKs can be divided into four groups according to their sequences and structures (MAPK Group, 2002). Three MAPKs, namely MPK3 and MPK6 of group A and MPK4 of group B, have been extensively studied. They are involved mainly in stress responses and can be activated by a diverse set of stresses, including pathogens, osmotic stress, cold stress, and oxidative stress (Ichimura et al., 2000; Yuasa et al., 2001; Asai et al., 2002; Teige et al., 2004; Mishra et al., 2006). Recently, MPK1, MPK2, MPK7, and MPK14 of group D were identified as downstream substrates of MKK3, as revealed by yeast two-hybrid analysis (Doczi et al., 2007). There is no information on the functions of group C MPKs to date.

Like MAPKs, the 10 MAPKKs can also be divided into four groups (MAPK Group, 2002). Five MAPKKs, MKK1 and MKK2 of group A, MKK3 of group B, and

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MKK4 and MKK5 of group C, appear to be mainly involved in stress responses (Asai et al., 2002; Matsuoka et al., 2002; Teige et al., 2004; Doczi et al., 2007). Very little is known regarding the function of the group D MAPKKs, which include MKK7, MKK8, MKK9, and MKK10. MKK7 was recently shown to be a negative regulator of polar auxin transport (Dai et al., 2006) and a positive regulator of the plant basal resistance and systemic acquired resistance (Zhang et al., 2007). It has been shown that a mutation in MKK9 results in enhanced seedling stress tolerance, suggesting that MKK9 may act as a negative regulator of abiotic stress responses (Alzwiya and Morris, 2007). Most recently, Yoo et al. (2008) reported that the MKK9-MPK3/MPK6 cascades promote ethylene-insensitive3 (EIN3)-mediated transcription in ethylene signaling.

It is known that genes encoding components of signal transduction pathways such as the MAPK cascades and the Ca^{2+} signaling pathway are expressed in senescing leaves. For example, a senescence-associated receptor kinase gene has been cloned and shown to be expressed during senescence in bean (Phaseolus vulgaris) leaves (Hajouj et al., 2000). A transcriptome analysis revealed that there are 15 genes related to the MAPK phosphorylation relay system in senescing leaves in Arabidopsis (Guo et al., 2004). Whether and how these genes and any other genes in signaling play any roles in controlling leaf senescence have not been reported thus far.

Here, we report a new signaling pathway that involves MKK9 and MPK6 and plays an important role in leaf senescence in Arabidopsis. We demonstrate not only that both MKK9 and MPK6 possess kinase activities but, more importantly, that MPK6 is an immediate downstream target of MKK9 via various in vitro, in vivo, and in planta biochemical and molecular genetic analyses. Both loss-of-function and gain-of-function analyses reveal that the MKK9-MPK6 cascade positively regulates leaf senescence in Arabidopsis.

RESULTS

**MKK9 Encodes an Active Kinase**

An Arabidopsis leaf senescence EST database revealed 15 genes that are apparent components of MAPK signal cascades in which MKK9 is included (Guo et al., 2004). We performed RNA gel blot analysis to investigate the steady state levels of MKK9 expression in Arabidopsis. As shown in Figure 1, MKK9 is up-regulated with the progression of leaf senescence. To investigate whether the product of the annotated MKK9 possesses protein kinase activity, in vitro kinase assays were performed. MKK9 could autophosphorylate and phosphorylate myelin basic protein (Fig. 2). Maltose-binding protein and an inactive form of MKK9, MKK9KR, in which a conserved Lys residue (K) at position 76 of the ATP-binding site of MKK9 was replaced with an Arg residue (R), showed neither autophosphorylation nor protein kinase activity toward myelin basic protein (Fig. 2). In-gel kinase assays were also performed, and the results were consistent with the in vitro assay (Supplemental Fig. S1). These data indicate that MKK9 is a functional kinase and that its ATP-binding site is critical for its kinase activity.

**Senescence Is Delayed in Detached Leaves of the mkk9 Knockout Line**

To investigate the role of MKK9 in leaf senescence, we performed loss-of-function analyses. A T-DNA insertional null mutant, mkk9-1 (SALK_017378), was obtained from the Salk T-DNA Collection (http://signal.salk.edu; Supplemental Fig. S2). The mkk9-1 mutant plants grew and developed normally, with a subtle delay in leaf senescence, in our growth conditions (data not shown). However, 4 d after detachment, leaves of mkk9-1 plants showed significantly delayed senescence compared with the age-matched leaves of wild-type plants (Fig. 3A). Consistent with the delayed senescence phenotype, both the ratio of variable fluorescence to maximal fluorescence (F_{v}/F_{m}) and chlorophyll levels in detached leaves of mkk9-1 plants were higher than in counterpart leaves of wild-type plants (Fig. 3, B and C).

**MKK9 Restores the Knockout Mutant Plants to Wild Type**

To confirm that the T-DNA insertion in MKK9 is responsible for the delayed senescence phenotype observed in the detached leaves, we performed a complementation test. The wild-type copy of MKK9, including the 1,678-bp promoter region, was intro-
duced into the mkk9-1 mutant plants. The leaves detached from the MKK9 complementation lines senesced in the same manner as the wild-type leaves, both phenotypically (Fig. 4A) and in terms of changes in the $F_v/F_m$ (Fig. 4C). Reverse transcription (RT)-PCR analysis showed that the introduced MKK9 was expressed in leaves of the complementation lines 6 d after detachment, which was the same pattern observed in detached wild-type control leaves (Fig. 4B). These data confirmed that the loss of MKK9 expression in the mkk9-1 mutant was the cause of the delayed senescence phenotype.

Figure 2. MKK9 encodes an active kinase. In vitro kinase activity assay of MKK9. Affinity-purified MBP, MBP-MKK9, or MBP-MKK9KR was incubated in the presence of [$\gamma$-32P]ATP with or without myelin basic protein. After SDS-PAGE, the reaction products were analyzed as indicated. Top, Coomassie Brilliant Blue staining; bottom, autoradiography.

Constitutive and Inducible Overexpression of MKK9 Causes Precocious Leaf Senescence

To further investigate the role of MKK9 in leaf senescence, two different types of gain-of-function experiments were performed. First, the constitutive 35S promoter was used to direct MKK9 in transgenic Arabidopsis. Of the 184 T1 transformants, 71 transgenic plants died 30 d after germination. The remaining plants were classified into the following four categories: type a plants were severely dwarfed, with curly leaves that displayed an early leaf senescence phenotype, and did not produce any seeds; type b plants had a stature bigger than type a plants and also exhibited an early senescence phenotype; type c plants were similar in size to wild-type plants and also exhibited an early senescence phenotype; type d plants appeared as normal as wild-type plants (Fig. 5A; Table I). RNA gel blot analysis revealed that type d plants showed no expression of MKK9, presumably due to “position effects.” Types a, b, and c plants had elevated expression levels of MKK9, with type a plants having the higher levels and type c having the lower levels (Fig. 5B). In summary, the MKK9 transcript levels appeared to be correlated well with the earliness in leaf senescence and the severity of the reduced stature; the higher the expression, the earlier the leaves senesce. It is reasonable to assume that the MKK9 expression levels in those dead transgenic plants were elevated to such high levels that cells were prompted to senesce very precociously.

The yellowing processes observed in types a, b, and c plants are senescence processes, because senescence-associated genes (SAGs) such as SAG12 (Noh and Amasino, 1999; Pontier et al., 1999), SAG13 (Lohman et al., 1994), SEN4 (Park et al., 1998), WRKY6 (Robatzek and Somssich, 2001), and AtANP (Guo and Gan, 2006) were expressed in these plants (Fig. 5B). It should be

Figure 3. Delayed senescence in detached leaves of mkk9 and mpk6 mutant plants. A. Phenotypes of detached leaves of wild-type (Col-0), mkk9-1, and mpk6 mutant plants. The seventh and eighth leaves were kept in light (100–120 μmol m$^{-2}$ s$^{-1}$) for 6 d. B and C, $F_v/F_m$ (B) and chlorophyll content (C) of leaves shown in A. Mean values of six samples ± s are shown. Asterisks indicate significant differences between Col-0 and the mutants (Student’s t test, $P < 0.01$). FW, Fresh weight. [See online article for color version of this figure.]
noted that SAG12 is a highly senescence-specific gene (Gan, 1995); it expresses in senescing tissues only but not in hypersensitive response-related cell death (Noh and Amasino, 1999; Pontier et al., 1999) and thus has been widely used as a senescence-specific marker that distinguishes senescence-related cell death from hypersensitive response-related cell death (Robatzek and Somssich, 2001; He and Gan, 2002; Miao et al., 2004; Guo and Gan, 2006; Lim et al., 2007; Nakagawa et al., 2007; Wu et al., 2008).

To avoid potential complications in interpreting the pleiotropic phenotype caused by constitutive overexpression of MKK9, we further used the glucocorticoid-regulated transcriptional induction system (Aoyama and Chua, 1997) to overexpress MKK9 for assessing the role of MKK9 in leaf senescence. Four days after dexamethasone (DEX; a synthetic glucocorticoid) treatment, the transgenic lines harboring both DEX:\MKK9 (MKK9 fused with the DEX-inducible promoter) and pTA7001 showed precocious leaf yellowing in fully expanded leaves (Fig. 6A). pTA7001 encodes a recombinant transcription factor that binds to the promoter to induce the expression of a gene of interest (e.g. MKK9 here) upon DEX application. RNA gel blot analysis revealed that MKK9 in these lines was induced by DEX (Fig. 6B). In contrast, the parental ecotype Columbia (Col-0) line and the line harboring pTA7001 alone did not show MKK9 expression (Fig. 6B) or any precocious senescence 4 d after DEX treatment (Fig. 6A). The precocious leaf yellowing in lines containing DEX:\MKK9 and pTA7001 was a senescence process, because the SAGs described above were expressed in the yellowing leaves (Fig. 6G). Consistent with an early visible yellowing phenotype (Fig. 6A), chlorophyll level and \( F_v/F_m \) in individual leaves (sixth leaf) of the line containing both DEX:\MKK9 and pTA7001 at 4 d after DEX induction were lower than in counterpart leaves of the age-matched other lines (Fig. 6, C and D). In our detachment analyses, leaves of

Figure 4. Complementation of mkk9-1 with MKK9. A, Phenotypes of detached leaves of the wild type (Col-0), the mkk9-1 mutant, and mkk9-1 transformed with MKK9. The seventh and eighth leaves were detached and kept in light for 6 d. B, RT-PCR analysis of the expression of MKK9 of plants described in A. ACT3 served as an internal standard. C, \( F_v/F_m \) of leaves shown in A. Mean values of five samples ± se are shown. \( F_v/F_m \) was significantly different between the wild type and the mkk9-1 mutant (Student’s t test, \( P < 0.01 \)). The difference between the wild type and the mkk9-1 + MKK9 complementation plants was not significant. [See online article for color version of this figure.]

Figure 5. Constitutive overexpression of MKK9 in the Col-0 and mpk6-2 backgrounds. A, Phenotypes of 35S:MKK9, 35S:MKK9KR transgenic, and wild-type (Col-0) Arabidopsis plants. Bar = 1 cm. a, b, c, and d are different types of 35S:MKK9 transgenic plants based on their stature and senescence phenotypes as described in the text; e is the wild-type (Col-0) plant; f is a 35S:MKK9KR transgenic plant. B, RNA gel blot analysis of the expression of MKK9 and some senescence marker genes in plants shown in A. C, RNA gel blot analysis of the expression of MKK9 and some senescence marker genes in the mpk6-2 mutant plants that were transformed with 35S:MKK9. c28 is a randomly selected type c plant. Similarly, d19 and d20 are two randomly selected type d plants.
this line also showed remarkable precocious senescence (Supplemental Fig. S3). These and the above data suggest that MKK9 is sufficient to cause leaf senescence.

The kinase activity is critical for MKK9 to exert its biological functions. When MKK9KR, an MKK9 mutant that does not possess kinase activity (Fig. 2), was constitutively overexpressed, all of the 63 T1 transgenic Arabidopsis lines displayed a wild-type-like phenotype with no observable early senescence (type f plants in Fig. 5). Similarly, no alterations in senescence and other developmental processes were observed in transgenic plants that overexpressed MKK9KR after DEX induction (Fig. 6; Supplemental Fig. S3).

MPK6 Is a Target of MKK9

In a typical MAPK cascade, MAPKKs function as activators of specific MAPKs. To identify potential downstream MAPKs of MKK9, we first performed in vitro phosphorylation assays. The MKK9WT, MKK9EE (a constitutively active form of MKK9 created by changing the two putative phosphorylation site Ser residues to Glu residues), and five MAPKs (MPK1, MPK3, MPK4, MPK6, and MPK7) exhibited autophosphorylation (Supplemental Fig. S4A). These MAPKs (except MPK4) were chosen because they were up-regulated during leaf senescence based on our RNA gel blot analysis (data not shown) and previous study (van der Graaff et al., 2006). To avoid the complexity of autophosphorylation, we used the inactive forms (KR) of the MAPKs as the substrates of MKK9 in the in vitro phosphorylation assays (Supplemental Fig. S4B). As shown in Figure 7A (and Supplemental Fig. S4A), among the MAPKs we examined, MPK6 was the only one that was phosphorylated in our experiments. MKK9EE displayed higher kinase activity on MPK6 than MKK9WT did (Fig. 7A).

The above in vitro data suggest that MPK6 is a downstream target of MKK9 in Arabidopsis. To further test this, we performed in vivo phosphorylation experiments and gel mobility shift assays. mkk9-1 knockout protoplasts were cotransformed with both 3SS promoter-driven GFP-tagged MKK9 and MPK6. When the protoplasts showed GFP production, proteins were extracted and then treated with or without λ-protein phosphatase (λ-PPase) and/or phosphatase inhibitor. As shown in Figure 7B, the mobility shift of MPK6 was observed only when MKK9 and MPK6 were cotransformed (lanes 4 and 6). After λ-PPase treatment, phosphorylated MPK6 was dephosphorylated and migrated as a single band with the same mobility as the faster migrating band (lane 5), suggesting that the phosphorylation of MPK6 in the protoplasts was executed by MKK9.

Table 1. Phenotypic categorization of 35S:MKK9 transgenic plants in the Col-0 or mpk6 mutant background

<table>
<thead>
<tr>
<th>Type</th>
<th>35S:MKK9/Col-0</th>
<th>35S:MKK9/mpk6-2</th>
</tr>
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<tbody>
<tr>
<td>Died</td>
<td>71 (38.6%)</td>
<td>16 (15.5%)</td>
</tr>
<tr>
<td>a</td>
<td>35 (19.0%)</td>
<td>15 (14.6%)</td>
</tr>
<tr>
<td>b</td>
<td>33 (17.9%)</td>
<td>21 (20.4%)</td>
</tr>
<tr>
<td>c</td>
<td>36 (19.6%)</td>
<td>23 (22.3%)</td>
</tr>
<tr>
<td>d</td>
<td>9 (4.9%)</td>
<td>28 (27.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>184 (100%)</td>
<td>103 (100%)</td>
</tr>
</tbody>
</table>

aSignificant differences (χ² test, P < 0.001).
we performed further analysis on the activation of MPK6 by MKK9 in planta. We generated MKK9WT and MKK9EE transgenic Arabidopsis plants in which MKK9 could be induced by DEX treatment (Fig. 7C, top). Without DEX induction (0 h), the MPK6 kinase activity was low (Fig. 7C, bottom). Three hours after DEX treatment (and thus induction of MKK9), MPK6 kinase activity was increased, and the increase in activity was enhanced with the progression of DEX induction. MPK6 showed higher kinase activity in MKK9EE plants than in MKK9WT plants after DEX induction. Because the levels of MPK6 protein were not increased (protein gel blot in Fig. 7C), the increase in MPK6 activity was most likely due to the activation by MKK9.

**mpk6 Null Mutants Phenocopy mkk9 with Delayed Senescence in Detached Leaves**

If MPK6 functions downstream of MKK9, loss-of-function mutant plants should phenocopy mkk9. To test this, we obtained two T-DNA insertion lines of MPK6 that were named mpk6-2 (SALK_073907) and mpk6-3 (SALK_127507). RT-PCR analyses revealed that these two lines are knockout mutants (Supplemental Fig. S5). Under our growth conditions, there were no obvious differences between the mpk6 knockout mutants and wild-type plants in terms of growth and development, except for a reduced fertility observed in the mpk6 plants, which is consistent with a recent report (Bush and Krysan, 2007). We then performed the experiments involving the detached leaves, similar to those involving the mkk9 plants as discussed above. Senescence of leaves of the mpk6-2 and mpk6-3 plants was delayed in terms of visible sign of yellowing and the F_v/F_m (Fig. 3).

**Precocious Senescence Caused by MKK9 Overexpression Is Suppressed in mpk6 Knockout Plants**

The constitutive overexpression of MKK9 directed by the 35S promoter caused precocious senescence in leaves and in whole plants (Col-0 background; Fig. 5, A and B; Table I). We performed similar experiments by transforming 35S:MKK9 into the mpk6 knockout plants. The rationale was that if MKK9 functions via MPK6 (i.e. the MKK9-MPK6 cascade) in promoting senescence, overexpressing MKK9 in the mpk6 knockout genetic background would not cause precocious senescence, as seen in the Col-0 background. Although we still obtained five groups of plants as described above (compare with Fig. 5A), the percentage of plants that died was only 15.5%, a substantial reduction compared with 38.6% in the Col-0 background (Table I). In contrast, the percentage of the type d plants that died was only 15.5%, a substantial reduction compared with 38.6% in the Col-0 background (Table I). In contrast, the percentage of the type d plants (phenotypically normal) was 27.2% in the mpk6 background, a significant increase from 4.9% in the Col-0 background (Table I). RNA gel blot analysis showed that MKK9 was highly expressed in some of the type d plants (e.g. type d plant 20, or d20 in Fig. 5C). Similarly, some of the type c plants showed very high levels of MKK9 (e.g. c28 in Fig. 5C); such high levels of MKK9 would have caused earlier senescence, more severe dwarfness, and even lethality at the early stage of development in the wild-type (Col-0) background, as shown in Figure 5, A and B. These data indicated that mpk6 could partially suppress the phenotype caused...
by constitutive overexpression of MKK9. However, the incomplete suppression suggested that there could be an additional target(s) of MKK9.

DISCUSSION

Leaf senescence is a complex developmental phase that involves both degenerative and nutrient recycling processes. It can be induced by an array of environmental cues, such as drought, darkness, extreme temperatures, and pathogen attack, and endogenous factors, including age, ethylene, jasmonic acid, salicylic acid, abscisic acid, and reproductive development. How these signals are perceived, transmitted, and ultimately executed at the biochemical and molecular levels is poorly understood. The MAPK cascade has been shown to be involved in various biotic and abiotic stress responses, hormone actions, cell proliferation, differentiation, and developmental processes in plants (Nakagami et al., 2005). Whether MAPK cascades play any roles in controlling leaf senescence has not been investigated. In this report, we have demonstrated the interaction of MKK9 and MPK6 in Arabidopsis at the biochemical, physiological, cell biological, and molecular genetic levels. Both genes possess kinase activities, and the MKK9-MPK6 cascade plays an important role in controlling leaf senescence.

We provide several lines of evidence that MKK9 has an important role in regulating leaf senescence. The first line of evidence comes from our gain-of-function analyses. When MKK9 was constitutively overexpressed under the direction of the 35S promoter, up to 39% of the resulting transgenic plants died at 30 d after germination (Table I), with nearly 57% displaying a premature leaf senescence phenotype (Fig. 5; a + b + c in Table I). Fewer than 5% developed like wild-type plants. We were unable to determine the expression levels of MKK9 in the small seedlings that died (38.6%) due to inadequate amounts of tissue; however, the transcript levels of MKK9 in the surviving plants correlated well with an early senescence phenotype. The higher the MKK9 expression, the earlier the leaves senesced (Fig. 5B). There were no detectable MKK9 transcripts in the normally developed plants (5%; Fig. 5B). The interpretation of the data obtained in the 35S promoter-directed constitutive expression lines is strongly supported by the experiments involving inducible overexpression of MKK9. As shown in Figure 6B, the MKK9 transcripts were induced to accumulate following DEX treatment, and consequently, the rosette leaves senesced precociously (Fig. 6A) compared with other control plants. In these inducible expression experiments, there was no pleiotropy that was observed in the 35S:MKK9 plants. In addition, either constitutive or inducible overexpression of MKK9KR did not cause early senescence (Figs. 5 and 6; Supplemental Fig. S3). The second line of evidence comes from the loss-of-function experiments. Senescence in detached leaves of mkk9 null mutant lines was delayed compared with that of wild-type leaves (Fig. 3). Third, the delayed leaf senescence phenotype of the mkk9 null plants was restored to wild type after an intact MKK9 gene was introduced into the mkk9-1 null plants in a complementation test (Fig. 4). Fourth, RNA gel blot analysis revealed that the MKK9 transcripts are barely detectable in leaves without visible signs (yellowing) of senescence but are readily detected in leaves with yellowing tips. The transcript level increases with the progression of senescence (Fig. 1). The senescence-specific expression pattern is consistent with a role of MKK9 in leaf senescence.

MPK6 has been shown to be involved in responses to salt and cold stresses, to jasmonic acid, and to ethylene (Liu and Zhang, 2004; Teige et al., 2004; Takahashi et al., 2007). Our loss-of-function analysis revealed that MPK6 plays an important part in regulating leaf senescence. Leaves of both mpk6-2 and mpk6-3, two T-DNA insertion null lines, had a prolonged lifespan similar to that of the mkk9-1 null mutant (Fig. 3).

How do MKK9 and MPK6 exert their functions in regulating leaf senescence? Typically, a MAPKK acts by phosphorylating, and thus activating, its downstream MAPKs. To identify potential downstream MAPKs, we generated active, inactive, and constitutively active forms of MKK9 and five MPKs (MPK1, MPK3, MPK4, MPK6, and MPK7) in Escherichia coli and performed a series of in vitro kinase assays (Fig. 7A; Supplemental Fig. S4). Among the five MAPKs tested, MKK9 only phosphorylated MPK6. We further demonstrated that MKK9 was able to phosphorylate MPK6 when both genes were coexpressed in Arabidopsis mkk9 null mutant protoplasts (Fig. 7B). The reason for using the mkk9 null mutant was to avoid any basal MKK9 activity. In addition to the evidence from the in vitro and in vivo experiments, our in planta analysis also revealed that MKK9 increased MPK6 activity. Specifically, when MKK9EE was induced in Arabidopsis leaves, the MPK6 activity in the leaves was increased, and the longer the induction of MKK9EE, the higher the MPK6 activity (Fig. 7C). The fourth line of evidence supporting that both MKK9 and MPK6 function as the MKK9-MPK6 signaling cascade in controlling leaf senescence comes from the gain-of-function experiments. As shown in Figure 5 and Table I, the 35S promoter-directed constitutive overexpression caused a 39% lethality rate at very early seedling stages (presumably due to high levels of MKK9 expression) and a 57% rate of precocious senescence, and only less than 5% (that did not have detectable MKK9 transcripts presumably due to position effects) developed normally. In contrast, when the same construct was transferred into the mpk6 null background, the percentage of dead plants was reduced from 39% to less than 16% and the percentage of plants that developed normally increased from less than 5% to more than 27% (Table I). The lack of downstream MPK6 blocked, at least partially, the MKK9 function in promoting senescence.
The partial suppression of leaf senescence promoted by MKK9 overexpression suggests that there are other downstream targets of MKK9. In fact, Yoo et al. (2008) most recently reported that, in addition to MPK6, MPK3 is also a target of MKK9 that plays an important role in ethylene signaling. Our in vitro assays revealed that the MKK9 expressed in E. coli was unable to phosphorylate MPK3 (Supplemental Fig. S4). This difference may have resulted from the different approaches used. There are fewer MAPKs than MAPKs and MAPKKKs in the Arabidopsis genome. This suggests that the MAPKKks serve as entry routes to many upstream signals (from MAPKKKs) and as bifurcation points for the activation of multiple downstream MAPKs (Cardinale et al., 2002). For instance, MKK2 activates MPK4 and MPK6 (Teige et al., 2004), MKK3 activates MPK6 and MPK7 (Doczi et al., 2007; Takahashi et al., 2007), and both MKK4 and MKK5 can activate MPK3 and MPK6 (Asai et al., 2002), although there are also reports that one MAPKK phosphorylates one MAPK only: MKK1 activates MPK4 (Matsuoka et al., 2002) and MKK6 activates MPK13 (MellIan et al., 2004).

In contrast to the one MAPKK-multiple MAPKs discussed above, one MAPK can be a direct target of multiple MAPKks. Previous studies and our data show that MPK6 could be activated by six MAPKKs: MKK2 (group A), MKK3 (group B), MKK4 and MKK5 (group C), and MKK7 and MKK9 (group D; Asai et al., 2002; Teige et al., 2004; Takahashi et al., 2007; Yoo et al., 2008). It is interesting that MPK6, depending on its different activators (MAPKKs), may have different biological functions. The MKK2-MPK6, MKK3-MPK6, and MKK4/MKK5-MPK6 cascades are involved in salt/cold stress signaling, in JA signaling, and in the ethylene signaling pathway, respectively (Liu and Zhang, 2004; Teige et al., 2004; Takahashi et al., 2007; Yoo et al., 2008). It is interesting that MPK6, depending on its different activators (MAPKKs), may have different biological functions. The MKK2-MPK6, MKK3-MPK6, and MKK4/MKK5-MPK6 cascades are involved in salt/cold stress signaling, in JA signaling, and in the ethylene signaling pathway, respectively (Liu and Zhang, 2004; Teige et al., 2004; Takahashi et al., 2007; Yoo et al., 2008). The MKK9-MPK3/MKK6 cascade promotes EIN3-mediated transcription in ethylene signaling (Yoo et al., 2008). The function of the MKK7-MPK6 cascade is yet to be determined. This report clearly shows that the MKK9-MPK6 cascade plays an important role in the regulation of leaf senescence. The MAPKks-MPK6 cascade may thus serve as a signaling node of convergence among these different signaling pathways and senescence.

In summary, we have found that MKK9 directly phosphorylates and activates MPK6 and that the MKK9-MPK6 cascade plays an important role in the regulation of leaf senescence. In addition to MPK6, there may be other MAPKs that are targets of MKK9, as suggested by the fact that knockout of MPK6 can only partially suppress the precocious senescence phenotype caused by MKK9 overexpression. Not only does MPK6 have multiple functions in addition to regulating leaf senescence, as discussed above, but MKK9 may also play multiple roles in other processes. In fact, we have observed that MKK9 can be induced by wounding, paraquat, and hydrogen peroxide (data not shown). mkk9 mutant seedlings show enhanced abscisic acid and salt tolerance compared with wild-type seedlings (Alzwiya and Morris, 2007). MPK6 is also induced by wounding, paraquat, and hydrogen peroxide and is involved in salt stress signaling (Yuasa et al., 2001; Menke et al., 2004; Teige et al., 2004). Because MKK9-MPK3/MPK6 cascades have been clearly demonstrated in ethylene signaling (Yoo et al., 2008), it is possible that the MKK9-MPK6 pathway mediates ethylene-regulated leaf senescence. Further studies addressing this possibility and such questions as how the MKK9-MPK6 cascade regulates leaf senescence and other physiological processes, which MAPKkks activates MKK9, and what the immediate target of MPK6 is will help us fully understand the biological functions and the underlying mechanisms of the MKK9-MPK6 cascade.

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Treatments

The Arabidopsis (Arabidopsis thaliana Col-0) genetic background was used in this study. Seeds were sown on petri dishes containing 0.5% Murashige and Skoog salts and 0.8% (w/v) phytagar (Sigma; http://www.sigmaaldrich.com) with the appropriate antibiotics. Two-week-old seedlings were transplanted to Cornell University mix soils (3:2:1 peat moss:vermiculite:perlite). Plants were grown at 22°C with 60% relative humidity under constant light (100–120 μmol m⁻² s⁻¹ light from a mixture of fluorescent and incandescent bulbs). The T-DNA insertion lines and transgenic lines were grown side by side with the wild type and other appropriate control lines unless indicated otherwise.

Glucocorticoid treatments were performed as described previously (Aoyama and Chua, 1997). Three-week-old plants grown in pots were sprayed with 30 μM DEX. Four days after treatment, the leaves of these plants were used for molecular and physiological analysis.

For detached leaf senescence analysis, leaves from a 3-week-old Arabidopsis plant were excised and either placed on moist filter papers or floated on 30 μM DEX in petri dishes with the adaxial leaf surface facing up. The plates were kept in light (100–120 μmol m⁻² s⁻¹) at 22°C.

Analyses of the MKK9 and MPK6 T-DNA Insertion Lines

One T-DNA insertion line for MKK9 (SALK_017378) and two T-DNA insertion lines for MPK6 (SALK_073907 and SALK_127507) were obtained from the Salk T-DNA Collection. SALK_073907 and SALK_127507 have previously been described as mpk6-2 and mpk6-3, respectively (Liu and Zhang, 2004; Bush and Krysan, 2007). Gene-specific primers G2165, G2420 (for mkk9-1), G2709, and G2710 (for mpk6-2 and mpk6-3) and T-DNA left border primer G2325 were used for genotyping the putative null lines. All primer sequences can be found in Supplemental Table S1.

Molecular Cloning and Plasmid Construction

Open reading frames of MKK9 and various MPKs were PCR amplified from Arabidopsis cDNA, cloned into pGem-T vector (Promega; http://www.promega.com), and confirmed by sequencing. The constitutively active form of MKK9 was generated by changing both putative phosphorylation sites to Glu residues (S195E and S201E). The mutations resulted in a constitutively active kinase (MKK9-E). The inactive forms of MKK9 and MAPKs were generated by changing the conserved Lys residue in the ATP-binding domain of individual kinases to Arg. These point mutations resulted in kinase-inactive forms (MKK9K76R, MPK1K61R, MPK3K67R, MPK4K72R, MPK6K92R, and MPK7K98R). All mutants were created using a PCR-based site-directed mutagenesis method (Ho et al., 1989).

For in vitro studies, coding regions of MKK9 or individual MPKs was cloned into the Escherichia coli expression vector pMAL-c2 (New England Biolabs; http://www.neb.com) to form a maltose-binding protein (MBP)
The kinase reactions were stopped by adding 4 changes of the buffer. The gel was incubated in reaction buffer (25 mM Tris- HCl, pH 7.5, 1 mM dithiothreitol [DTT], 2 mM MgCl₂, 10 mM MnCl₂, 50 mM ATP, and 2 μCi [γ-32P]ATP) either with or without MBP-MKK9, MBP-MKK9EE, or MBP-MKK9KR at 30 °C for 30 min. The kinase reactions were separated by 10% (w/v) SDS-PAGE gel. For constitutive overexpression of MKK9, MKK9EE, or MKK9KR in plants, individual coding regions were cloned into pGL800, a binary vector derived from pZTP221, so that the gene would be directed by the cauliflower mosaic virus 35S promoter. For DEX-inducible overexpression of MKK9, MKK9EE, or MKK9KR, the coding regions of the respective genes were cloned into the binary vector pGL1152 (Guo and Gan, 2006). For mkk9-1 mutant complementation, a 2,980-bp genomic DNA segment containing the promoter region (1,678 bp upstream from the ATG start codon) and the coding region of MKK9 were cloned into binary vector pZTP221.

**Generation of Transgenic Plants**

The above constructs in binary vectors were introduced into Agrobacterium tumefaciens strain ABI as described previously (He and Gan, 2002). Agrobacterium cells containing the respective constructs were then used to transform Arabidopsis ecotype Col-0 wild-type or mutant plants via vacuum infiltration (Bechtold et al., 1993). Transgenic plants were selected on Murashige and Skoog medium containing kanamycin, gentamycin, or hygromycin, where appropriate.

**RNA Gel-Blot and RT-PCR Analyses**

Total RNA extraction from Arabidopsis leaves and RNA gel blot analysis were performed as described previously (He and Gan, 2002). The hybridization was performed at 65°C. cDNA was reverse transcribed from DNAse-treated total RNA using the M-MLV reverse transcriptase kit (Promega). RT-PCR was performed using the following primers: for MKK9, G2419 and G2420; for MPK6, G2709 and G2270; for SAC12, G10 and G2246; for SAC13, G9 and G116; for SEN4, G2041 and G2042; for WRKY6, G3043 and G3044; for AINAP, G1027 and G1028; for Actin3, G2236 and G2227 (Supplemental Table S1).

**In Vitro Kinase Assay**

E. coli strain TB1 was transformed with expression constructs cloned into the pMAL-c2 vector (New England Biolabs) and expressed as MBP fusion proteins. The kinase reactions were expressed and purified by amylose-affinity chromatography (New England Biolabs) and were subsequently quantitated using Bio-Rad protein assay reagent (Bio-Rad Laboratories; http://www.bio-rad.com). Each MBP-MPK (1 μg) or MBP-MPKKKR protein was incubated in 20 μL of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol [DTT], 10 mM MgCl₂, 10 mM MnCl₂, 50 mM ATP, and 2 μCi [γ-32P]ATP) either with or without MBP-MKK9, MBP-MKK9EE, or MBP-MKK9KR at 30°C for 30 min. The kinase reactions were stopped by adding 4 μL of 6X SDS sample buffer and heating for 5 min at 95°C. Reaction products were separated by 10% (w/v) SDS-PAGE, autoradiographed, and stained with Coomassie Brilliant Blue R 250. A prestained size marker was used to estimate the kinase size (New England Biolabs).

**Preparation of Protein Extracts and Immunoprecipitation**

Preparation of protein extracts was performed as described by Ichimura et al. (2000). The crude extract of whole plants was used for immunoprecipitation as described previously (Zhang and Klessig, 1997).

**In-Gel Kinase Assay**

The in-gel kinase assay was performed as described previously (Zhang and Klessig, 1997) with some modifications. In brief, various proteins men- tioned above were separated on 10% SDS-polyacrylamide gels embedded with 0.5 mg ml⁻¹ myelin basic protein on a separating gel as a kinase substrate. After electrophoresis, the gel was washed three times with washing buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.1 mM Na₂VO₃, 5 mM NaF, 0.5 mg ml⁻¹ bovine serum albumin, and 0.1% Triten X-100) to remove SDS. The proteins were then renatured in a renaturing buffer (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mM Na₂VO₃, and 5 mM NaF) overnight at 4°C with three changes of the buffer. The gel was incubated in reaction buffer (25 mM Tris-HCl, pH 7.5, 2 mM EGTA, 12 mM MgCl₂, 1 mM DTT, and 0.1 mM Na₂VO₃ at room temperature for 30 min, then phosphorylated in 20 mL of the same reaction buffer containing 0.5 μm ATP and 50 μCi [γ-32P]ATP at room temperature for 1 h. The reaction was stopped by transferring the gel into stop solution (5% trichloroacetic acid and 1% sodium pyrophosphate). Gels were washed with stop solution for 5 h at room temperature with four changes of solution, dried on 3MM Whatman paper, and then autoradiographed.

**Protoplast Isolation and Transient Expression Assays**

Transient expression assays were performed with isolated protoplasts from Arabidopsis mesophyll cells. The isolation, transformation, and cultivation of protoplasts were performed according to protocol described by Sheen (http://genetics.mgh.harvard.edu/sheenweb/). mkk9-1 null mutant pro- toplasts were transiently transformed via polyethylene glycol with 10 μg of plasmid. Twelve hours after transformation, the protoplasts were collected by centrifugation and were resuspended in protein extraction buffer for further analysis.

**Gel Mobility Shift Assay**

Gel mobility shift assays were performed as described previously (Peck, 2006) with some modifications. Transformed protoplasts were homogenized in extraction buffer (50 mM HEPES, pH 7.5, 0.1 mM NaCl, EDTA, 5 mM DTT, 0.01% Brij 35, 2 mM MnCl₂, and 0.1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma) and divided into three aliquots. λ-PPase (New England Biolabs) was added to one of the three aliquots (10 units μL⁻¹). Another aliquot was treated with λ-PPase and phosphatase inhibitor (0.1 mM Na₂VO₃). The remaining aliquot and the two treated aliquots were incubated at 30°C for 30 min. The phosphorylation reactions were stopped by adding SDS-PAGE sample buffer and heating for 5 min at 95°C before separation on an SDS-PAGE gel.

**Immunoblotting Assay**

Protein samples were separated on a 10% (w/v) SDS-PAGE minigel and blotted to nitrocellulose membranes using a semidry electrophoresis appara-atus at a constant voltage (200 V) for 90 min. Blots were blocked in phosphate- buffered saline with Tween 20 (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, and 0.1% Tween 20) and 5% (w/v) nonfat dry milk, then probed with a primary antibody (rabbit anti-GFP serum [Invitrogen; http://www.invitrogen.com] or rabbit anti-MPK6 [provided by Dr. Scott Peck, University of Missouri-Columbia], 1:2,000 dilution in PBS/BSA) at 4°C overnight. After washing, blots were incubated for 1 h in blocking buffer and subsequently incubated with the alkaline phosphatase-conjugated goat anti-rabbit IgG ( Sigma; diluted 1:5,000 in PBS) for 2 h at room temperature. Blots were washed three times in PBS and then visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color development sub- strate system (Promega) according to the manufacturer's instructions.

**Measurements of Chlorophyll Content and Fluorescence**

Total chlorophyll was extracted and quantified as described previously (He and Gan, 2002). Fluorescence in leaves was measured using a portable modulated chlorophyll fluorometer (model OS1-FL) according to the manu- facturer's instructions. The F_r/F_m for each leaf was quantified directly using the fluorometer's test mode 1.

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: At1g73500 (MKK9), At1g10210 (MKK9), At2g43790 (MPK6), At1g62300 (MPK7), At4g39430 (MPK3), At4g03370 (MPK4), At2g43790 (MPK6), At2g18170 (MPK7), At4g58980 (SAG12), At2g29350 (SAG13), At4g02070 (SEN4), At1g62300 (WRKY6), At1g69490 (AINAP), and At3g53750 (Actin3).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** MKK9 encodes an active kinase.

**Supplemental Figure S2.** MKK9 is knocked out in a T-DNA line.
LITERATURE CITED

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