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MKK4/MKK5-MPK1/MPK2 cascade mediates SA-activated leaf senescence via phosphorylation of NPR1 in Arabidopsis

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Abstract

The mechanism by which endogenous salicylic acid (SA) regulates leaf senescence remains elusive. Here we provide direct evidence that an enhancement of endogenous SA level, via chemical-induced upregulation of *ISOCHORISMATE SYNTHASE 1* (*ICS1*), could significantly accelerate the senescence process of old leaves through mediation of the key SA signaling component NON EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1) in *Arabidopsis*. Importantly, by taking advantage of this chemically induced leaf senescence system, we identified a mitogen-activated protein kinase (MAPK) cascade MKK4/5-MPK1/2 that is required for the SA/NPR1-mediated leaf senescence. Both MKK4/5 and MPK1/2 exhibited SA-induced kinase activities, with MPK1/2 being the immediate targets of MKK4/5. Double mutants of *mkk4 mkk5* and *mpk1 mpk2* displayed delayed leaf senescence, while constitutive overexpression of the kinase genes led to premature leaf senescence. Such premature leaf senescence was suppressed when they were overexpressed in an SA synthesis defective mutant (*sid2*) or signaling detective mutant (*npr1*). We further showed that MPK1, but not MPK2, could directly phosphorylate NPR1. Meanwhile, MPK1 also mediated NPR1 monomerization. Notably, induction of disease resistance was significantly compromised in the single and double mutants of the kinase genes. Taken together, our data demonstrate that the MKK4/5-MPK1/2 cascade plays a critical role in modulating SA signaling through a complex regulatory network in *Arabidopsis*.

Key message

We reveal a MAPK cascade, MKK4/5-MPK1, which not only phosphorylates the key SA signaling component NPR1 specifically, but also regulates its oligomer-to-monomer conformational change somehow via mediation of *TRX-h3/5*.

Keywords Probenazole · SA · NPR1 · MAPKs

Introduction

Leaf senescence is a genetically programmed active process for facilitating plant development via massively remobilizing nutrients from senescing leaves to rapidly developing organs and eventually to reproductive/storage organs (Lim et al. 2007; Masclaux-Daubresse and Chardon 2011). The process is regulated by both developmental and environmental cues, which are mostly mediated by phytohormones. Ethylene (ET), abscisic acid (ABA) and jasmonic acid (JA) are positive regulators, whereas cytokinins and auxins act to delay initiation/progression of leaf senescence (Gan and Amasino 1997; Graaff et al. 2006; Kusaba et al. 2013; Jibran et al. 2013). Salicylic acid (SA) is a key mediator of disease resistance in plants (Malamy et al. 1990; Metraux et al. 1990; Gaffney et al. 1993; Delaney et al. 1994; Wildermuth et al. 2001). However, its role in leaf senescence has been largely unclear (Raskin 1992; Vlot et al. 2009), mainly because that mutants or transgenic plants defective in SA biosynthesis/accumulation or signaling showed only a marginal alteration, if any, in their senescence phenotype, albeit a number of genes showed altered expression patterns (Morris et al. 2000). Recently, Zhang et al. reported that the null mutation

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of *SA 3-HYDROXYLASE* (*S3H*) gene, encoding an enzyme that converts SA to 2,3-dihydroxybenzoic acid, resulted in an elevated level of endogenous SA and precocious leaf senescence, suggesting that SA may positively regulate leaf senescence (Zhang et al. 2013).

SA biosynthesis and its signaling pathway during pathogen infection are well documented. Pathogen-induced SA synthesis undergoes predominantly the two sequential reactions catalyzed by isochorismate synthase (ICS) and putative isochorimate pyruvate lyase (IPL), respectively, with chorismate serving as the initial substrate (Wildermuth et al. 2001). In *Arabidopsis*, there are two isochorismate synthases called ICS1 (also named as SID2) and ICS2, with ICS1 acting as the major enzyme (Wildermuth et al. 2001). Pathogen-induced increase in endogenous SA level or exogenous application of SA leads to the growth-to-defense transition with changes in the expression of thousands of genes. A core component of the SA signaling pathway is NON EXPRESSOR OF PATHOGENESIS RELATED GENES 1 (NPR1), a transcriptional coactivator that plays a pivotal role in the plant systemic acquired resistance (SAR) (Cao et al. 1997; Mark et al. 2000). Under normal growth conditions, NPR1 exists as oligomers and resides in the cytoplasm. Upon pathogen infection or SA treatment, NPR1 oligomers are reduced to monomers, which relocate to the nucleus where they are subject to phosphorylation. Phosphorylation of NPR1 is important for its role in gene activation and also facilitates its nuclear import and turnover (Mou et al. 2003; Lee, et al. 2015). It has been shown that NPR1 phosphorylation occurs at residues Ser11/Ser15 (Spoel et al. 2009); however, the kinase(s) responsible for NPR1 phosphorylation at these residues has yet to be identified.

The mitogen-activated protein kinase (MAPK) cascade represents one of the most highly conserved signaling pathways and is involved in various biotic and abiotic stress responses, hormone signaling, cell proliferation, and other developmental processes (Nakagami et al. 2005). In *Arabidopsis*, there are 60–80 predicted MAP kinase kinase kinases (MAPKKKs), 10 MAP kinase kinases (MAPKKs), and at least 20 MAP kinases (MAPKs), which can theoretically generate twenty to thirty thousand distinct MAPKKK-MAPKK-MAPK modules. A battery of MAPK, including MAPKKK8 (MEKK1), MKK1/2/4/5 and MPK3/4/6, have been characterized to play key roles in plant immune responses, possibly through activation of WRKY transcription factors (TFs) (Asai et al. 2002; Gao et al. 2008). Moreover, genetic analyses have demonstrated that MAPKKK8, MKK9 and MPK6 are involved in regulation of leaf senescence (Zhou et al. 2009; Miao et al. 2007), suggesting the complexity and overlapping functions of the MAPK-signaling network in mediating diverse biological processes.

Probenazole (3-allyloxy-1,2-Benzisothiazole-1,1-dioxide, PBZ), an active ingredient of a widely used agrochemical Oryzemate, can activate SA/NPR1 signaling pathway to efficiently trigger SAR (Yoshioka et al. 2001). We previously showed that PBZ enhances endogenous SA level via ICS1 dependent biosynthesis pathway (Yu et al. 2010). In this study, we demonstrate that PBZ can also induce leaf senescence through the SA signaling pathway. Importantly, we reveal a MAPK signaling module MKK4/5-MPK1/2 that is responsible for NPR1 phosphorylation and monomerization, which is critical for both the PBZ-induced leaf senescence and disease resistance.

Results

PBZ accelerates the senescence of old leaves via *SA biosynthesis and NPR1 signaling in Arabidopsis*

Although SA has been shown to regulate the expression of many genes during leaf senescence, the fact that the mutants defective in SA biosynthesis and signaling have negligible senescence-related phenotypes makes it difficult to evaluate the exact role of SA in regulating leaf senescence (Morris et al. 2000). We have previously shown that PBZ is able to enhance the levels of both free and total endogenous SA via ICS1-mediated SA biosynthesis when applied by root drenching (Yu et al. 2010). Intriguingly, we also detected an accelerated senescence of old leaves about 2 weeks after PBZ treatment (Fig. 1a, b). To examine if the accelerated leaf senescing phenotype was caused by the enhanced SA biosynthesis, we treated Arabidopsis plants grown under short day-growth conditions for 5 weeks with SA of different concentrations through root drenching and indeed observed a similar yellowing phenotype on older leaves three days after treatment when 1.5 mM SA was applied (Supplementary Fig. 1). Considering the ease and a more typical symptom obtained with PBZ, as well as being convinced by the following genetic data, we continued to use PBZ to treat Arabidopsis plants in further experiments.

The 5th through the 8th true leaves were chosen for physiological and molecular analyses as they exhibited the most significant leaf yellowing phenotype (Fig. 1b). In addition to a reduced content of chlorophyll (Chl), other typical changes of senescence-associated parameters, including an increased level of ion leakage, induction of a senescence marker gene (*SAG12*), down-regulation of a photosynthetic gene (*CAB*), and reduced levels of photo-chemical quantum efficiency of photosystem II (Fv/Fm ratio and Fv′/Fm′ ratio), were also observed at 12 DAP (days after 0.5 mM PBZ treatment), demonstrating that PBZ can efficiently promote the senescence process of old leaves in our system (Fig. 1c–h). Importantly, no obvious signs of senescence symptoms were observed in an SA biosynthesis mutant *sid2* (*sid2-2*) and SA signaling mutant *npr1* (*npr1-1*), while constitutive

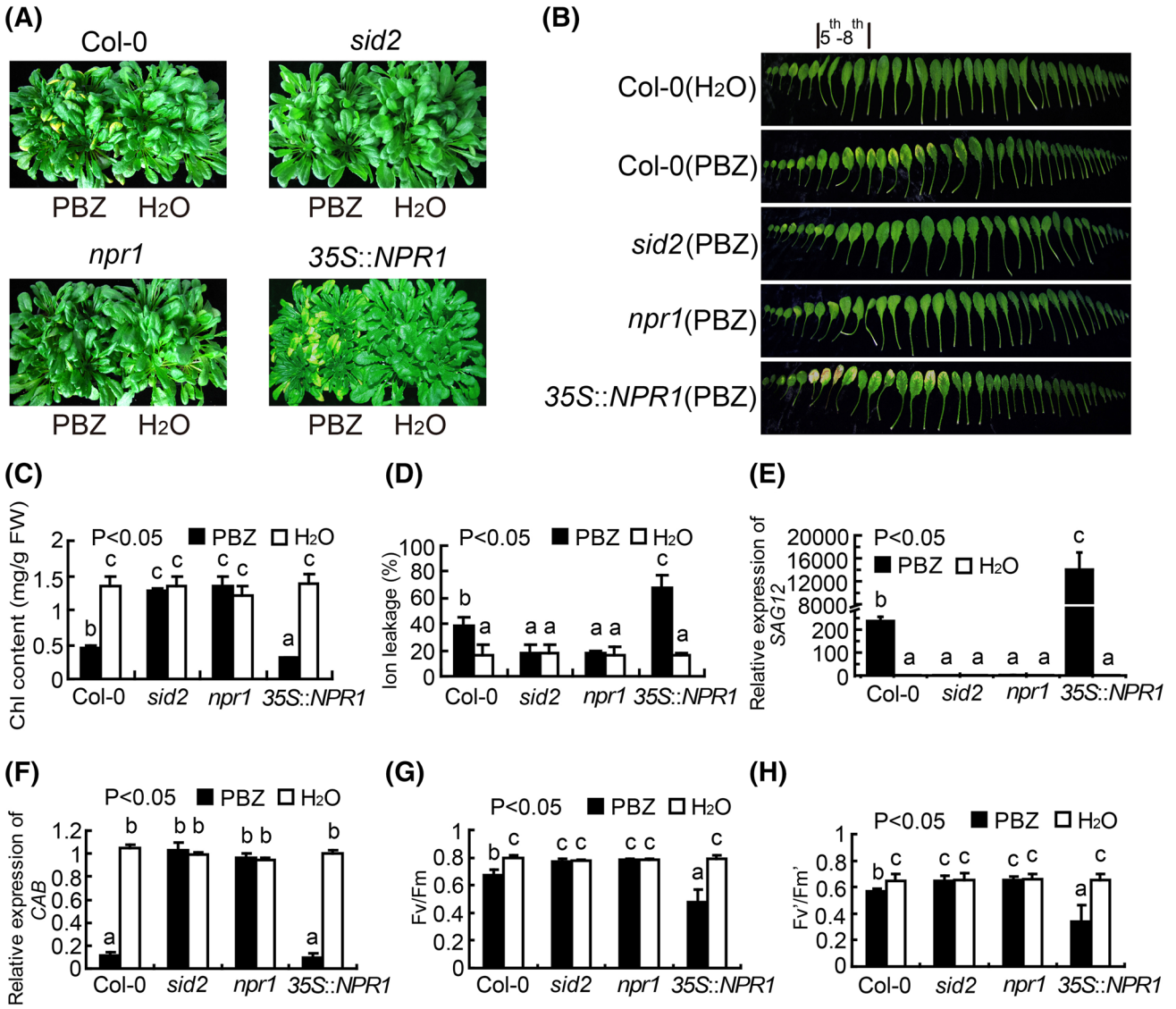

Fig. 1 PBZ promotes the senescence process of old leaves. **a** Plants of indicated genotypes were grown under short day-growth conditions for 5 weeks and treated with or without 0.5 mM PBZ through root drenching. Pictures were taken at 12 DAP (days after PBZ treatment). **b** Leaf phenotypes of indicated genotypes at 12 DAP. Unless stated otherwise, the 5th through the 8th leaves were taken for further physiological and molecular analyses. Chl contents (**c**) and ion leakages (**d**) of Col-0, *sid2*, *npr1*, and *35S*::*NPR1* plants at 12 DAP. Error bars indicate SD (n = 3), Fisher's least significant difference (LSD) ANOVA test, $P < 0.05$. Changes in the expression of a leaf senescence marker gene *SAG12* (**e**) and a photosynthesis marker gene *CAB* (**f**) in the leaves of indicated genotypes at 12 DAP. *ACTIN2* was included as an internal control. Error bars indicate SD (n = 3), LSD ANOVA test, $P < 0.05$. Fv/Fm ratios (**g**) and Fv′/Fm′ ratios (**h**) in the leaves of indicated genotypes at 12 DAP. Error bars indicate SD (n = 3), LSD ANOVA test, $P < 0.05$

overexpression of NPR1 (*35S*::*NPR1*) led to an accelerated leaf yellowing, confirming that PBZ promotes the senescence of old leaves through SA biosynthesis and signaling (Fig. 1).

MKK4/5 and MPK1/2 are required for the SA-accelerated senescence of old leaves

The MAPK cascade signaling plays critical roles in various signal transduction pathways of plants, including disease resistance and leaf senescence (Asai et al. 2002; Zhou et al. 2009; Zhao et al. 2014). Our microarray and qPCR analyses revealed that the expressions of one MAPKKK gene (*MEKK3*), five MAPKK genes (*MKK1*, *MKK2*, *MKK4*, *MKK5* and *MKK9*) and five MAPK genes (*MPK1*, *MPK2*, *MPK4*, *MPK7* and *MPK11*) were significantly up-regulated at 3 DAP, as compared with those by mock treatment (Fig. 2a; Supplementary Table 1). To determine if these genes were involved in mediating the PBZ-accelerated senescence, we identified T-DNA insertion mutants

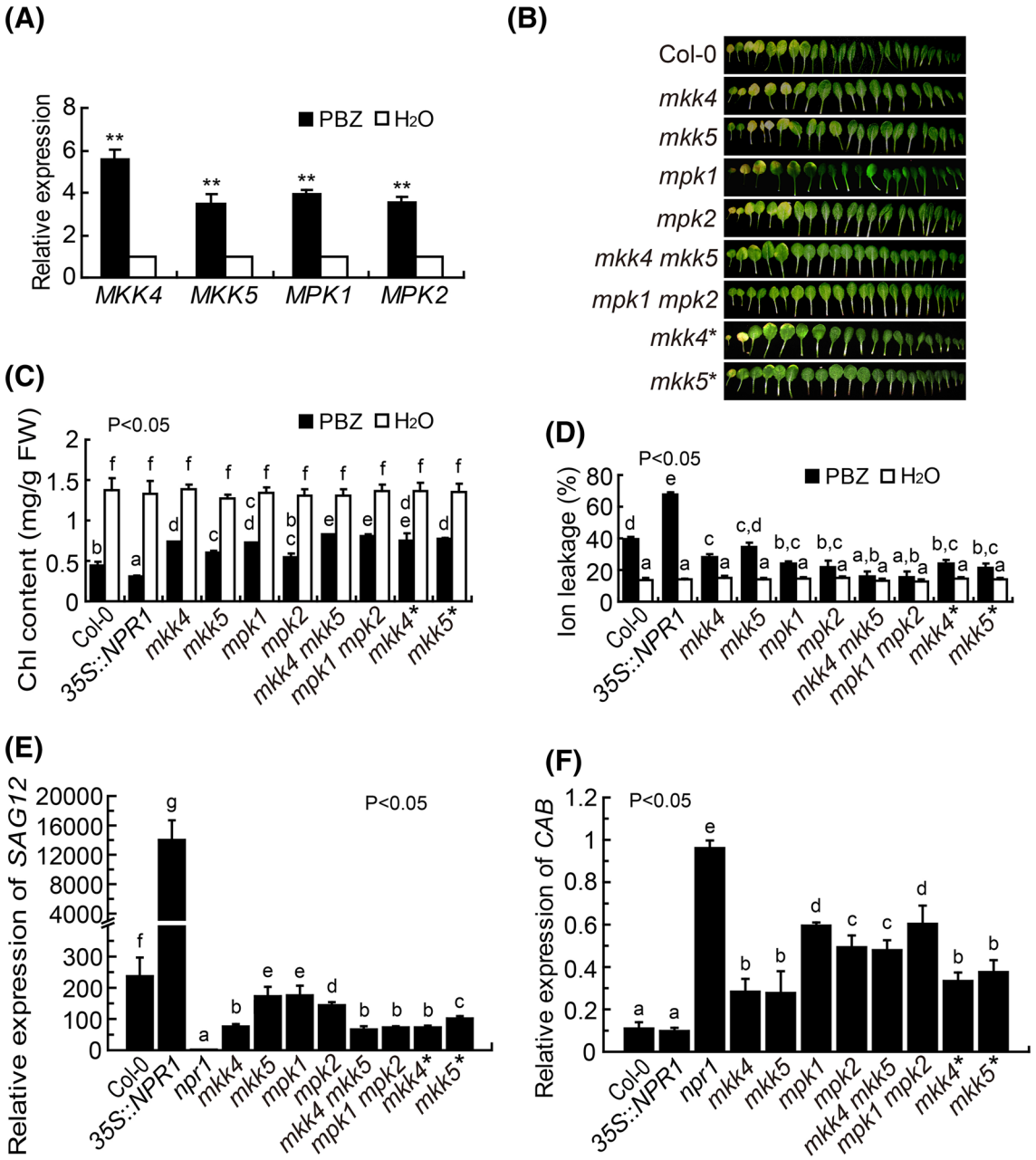

Fig. 2 Involvements of MKK4/5 and MPK1/2 in the regulation of PBZ-induced leaf senescence. **a** Expressions of *MKK*4/5 and *MPK*1/2 in response to PBZ treatment. Error bars indicate SD (n = 4), paired Student's *t* tests, **P < 0.01. **b** Leaf phenotypes of indicated genotypes at 12 DAP. Chl contents (**c**) and ion leakages (**d**) of indicated genotypes at 12 DAP. Error bars indicate SD (n = 4), LSD ANOVA test, P < 0.05. Changes in the expressions of *SAG12* (**e**) and *CAB* (**f**) in the leaves of indicated genotypes at 12 DAP. Expressions of *SAG12* (**e**) and *CAB* (**f**) in water treated plants were arbitrarily set to 1. *ACTIN2* was included as an internal control. Error bars indicate SD (n = 3), LSD ANOVA test, P < 0.05

of these up-regulated genes (Supplementary Fig. 2; Supplementary Table 1). Single knock-down or knock-out mutants of *MKK4*, *MKK5*, *MPK1* and *MPK2* showed moderate to slight compromises in senescence symptom, as compared to wild-type plants (WT) (Fig. 2b–f). Previous studies showed that MKK4 and MKK5 are grouped together, and so are MPK1 and MPK2, based on a phylogenetic analysis, indicating a potential redundancy of their functions (Kazuya et al. 2002). We thus generated a double mutant (*mpk1 mpk2*) of *MPK1* and *MPK2* with their single knock-out mutants (*SALK_063847C*, *SALK_047422C*) and a double mutant (*mkk4 mkk5*) of *MKK4* and *MKK5* with their single knock-down mutants (*SALK_018804C*, *SALK_050700*) since loss-of-function of both MKK4 and MKK5 (*mkk4**

and *mkk5** mutants were kindly provided by Dr. Dingzhong Tang) causes lethality (Wang et al. 2007). In our *mkk4 mkk5*, ~80% and ~60% of their expressions were eliminated for *MKK4* and *MKK5*, respectively (Supplementary Fig. 2). As expected, both *mkk4 mkk5* and *mpk1 mpk2* exhibited more significant reductions in the extent of the PBZ-accelerated senescence (Fig. 2b–f). Notably, loss-of-function mutation of either MKK9 or MPK6, both of which are involved in a MAPK cascade shown to play an important role in natural leaf senescence (Zhou et al. 2009), had no effect on the PBZ-accelerated senescence (Supplementary Fig. 3).

SA biosynthesis is required for the phosphorylation of MKK4/5 and MPK1/2

To understand the action modes of MKK4/5 and MPK1/2 and their molecular links to SA biosynthesis and signaling, we established a protoplast-based transient protein expression system to monitor the dynamics of protein phosphorylation upon SA treatment. As a preliminary experiment, we transiently expressed NPR1-GFP in WT protoplasts, which were subject to 0.04 mM SA treatment 12 h after transfection. Total proteins were extracted at indicated time points and subject to a Phos-tag mobility shift assay. As expected, a quick induction of NPR1-GFP phosphorylation was detected 1 h after SA treatment, which became more prominent 2 h after treatment (Supplementary Fig. 4a). In contrast, no effect on the phosphorylation of NPR1-GFP was observed upon mock treatment, indicating that this system is reliable for monitoring SA dependent MAPK signaling (Yoo et al. 2007) (Supplementary Fig. 4a).

Since phosphorylation of MKKs and MPKs is a prerequisite for their activation, we subsequently examined the phosphorylation status of MKK4/5 and MPK1/2 in response to SA treatment. As in the case of NPR1, a gradual increase of phosphorylated forms of MKK4-4 × MYC/MKK5-4 × MYC and MPK1-4 × FLAG/MPK2-4 × FLAG were obviously observed 2 h after SA treatment (Fig. 3a, b; Supplementary Fig. 4b). We also observed that fractions of MKK5-4 × MYC and MPK1-4 × FLAG were phosphorylated before SA treatment, which could be induced by the background level of SA likely accumulated during protoplast preparation and/or culture or due to that these kinases may also be able to respond to certain signal molecules other than SA (Fig. 3a, b).

To determine whether in vivo SA biosynthesis is required for MKK4/5 and MPK1/2 to mediate PBZ-accelerated senescence, we individually overexpressed *MKK4*, *MKK5*, *MPK1* or *MPK2* under the constitutive control of 35S promoter in WT or in *sid2*. As shown in Fig. 3c, d, transgenic plants overexpressing MKK4/5 or MPK1/2 in WT, but not in *sid2*, exhibited an accelerated leaf yellowing phenotype, suggesting that in vivo SA biosynthesis is critical for the MAPK cascade mediation of PBZ-accelerated senescence (Fig. 3c, d; Supplementary Figs. 5a–d, 6a, b). We subsequently examined the in vivo phosphorylation status of MPK1 and MPK2 in response to PBZ or SA treatment. Indeed, we detected an accumulation of phosphorylated MPK1 at 6 DAP in the WT + *MPK1-4* × *FLAG* but not in the *sid2* + *MPK1-4* × *FLAG* transgenic plants (Fig. 3e, f). However, when we treated the *sid2* + *MPK1-4* × *FLAG* leaves with 0.5 mM SA, we could recover a similar phosphorylated band of MPK1-4 × FLAG (Fig. 3g). Similar results were obtained with MPK2 (Fig. 3e–g). Isochorismate Synthase 1 (ICS1) encodes a key enzyme in SA production. We extracted and quantified total salicylic acid (free and sugar-conjugated) in WT, *mkk4 mkk5* and *mpk1 mpk2* by HPLC as described (Yu et al. 2010), and found there was no significant difference among them (Supplementary Fig. 12). Taken together, these results suggest that PBZ activates the MAPK signaling pathway through biosynthesis of endogenous SA.

Phosphorylation of MPK1/2 requires the full function of MKK4 and MKK5

We then examined whether the activation of MPK1/2 depends on MKK4/5. To this end, MPK1-4 × FLAG or MPK2-4 × FLAG was introduced into the protoplasts prepared from the leaves of *mkk4 mkk5*. As shown in Fig. 4a, b, neither the background- nor exogenous SA-induced phosphorylation could be detected 2 h after treatment, whereas co-introduction of MKK4-4 × MYC and MKK5-4 × MYC with MPK1-4 × FLAG or MPK2-4 × FLAG into *mkk4 mkk5* protoplasts reestablished the phosphorylation of MPK1/2 (Fig. 4a, b), indicating that the phosphorylation of MPK1 and MPK2 depends on the full function of both MKK4 and MKK5. To confirm that the band shift was due to phosphorylation, we treated the protoplasts with λ-PPase, a protein phosphatase that can efficiently remove phosphate groups from phosphorylated residues. As expected, the shifted bands disappeared after λ-PPase treatment (Fig. 4c, d, lane 2), and addition of Na_3VO_4, a phosphatase inhibitor, abolished the effect of λ-PPase action (Fig. 4c, d, lane 3).

MPK1 can phosphorylate NPR1

Phosphorylation of NPR1 is a key step in SA signaling (Spoel et al. 2009). In light of that NPR1 is critical for PBZ-induced leaf senescence and phosphorylation of NPR1 represents a key event of its functionality during SAR, we hypothesized that NPR1 might be a core downstream component of the MKK4/5-MPK1/2 signaling cascade. If this was the case, we would expect that loss-of-function mutation of NPR1 could block the MKK4/5-MPK1/2 signaling in vivo. Indeed, we found that individual overexpressions of *MKK4*, *MKK5*, *MPK1* and *MPK2* in *npr1* failed to exhibit their capability to accelerate leaf senescence upon PBZ

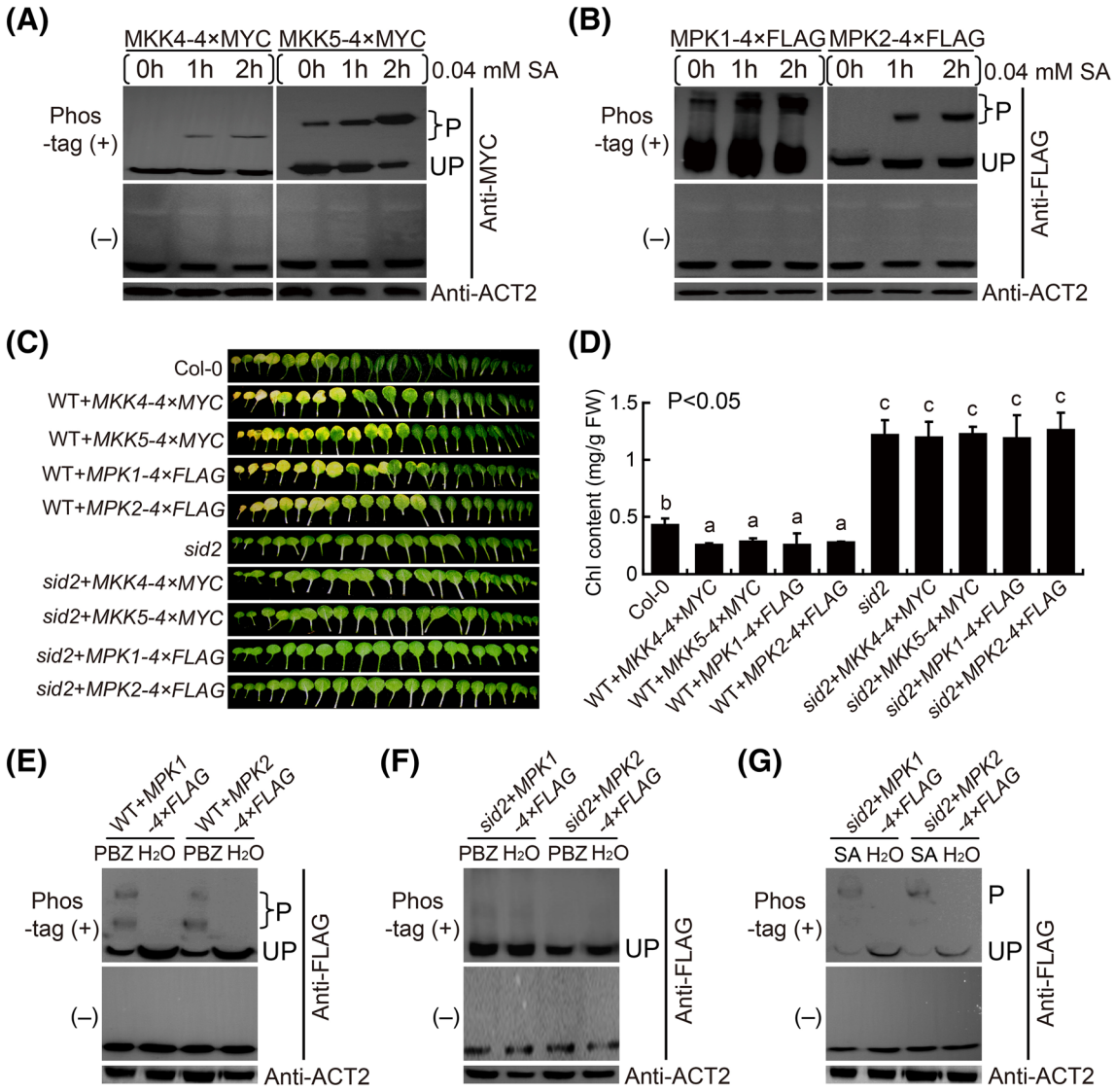

Fig. 3 Phosphorylation of MKK4/5 and MPK1/2 depends on in vivo SA biosynthesis. MKK4-4×MYC/MKK5-4×MYC (**a**) and MPK1-4×FLAG/ MPK2-4×FLAG (**b**) were phosphorylated upon SA treatment in a protoplast-based assay. Respective MYC tagged MKK or FLAG tagged MPK proteins were transiently expressed in WT protoplasts for 12 h and then treated with 0.04 mM SA. Proteins were extracted at indicated time points and subject to immunoblot (IB) analysis by an anti-MYC antibody (**a**) or an anti-FLAG antibody (**b**) with (top panel) or without (middle panel) the addition of the Phos-tag reagent. IB of ACTIN2 (ACT2) (bottom panel) was used as a control. P, phosphorylated; UP, unphosphorylated. **c**, **d** Overexpression of MKK4-4×MYC, MKK5-4×MYC, MPK1-4×FLAG or MPK2-4×FLAG in Col-0 but not in *sid2* caused an obvious leaf yellowing phenotype at 12 DAP. **c** Leaf phenotypes of indicated genotypes at 12 DAP. **d** Chl contents of indicated genotypes at 12 DAP. Error bars indicate SD (n = 3), LSD ANOVA test, $P < 0.05$. **e**, **f**, **g** In vivo phosphorylation of MPK1 or MPK2 in response to PBZ treatment depends on the biosynthesis of endogenous SA. **e** MPK1 and MPK2 were phosphorylated in WT + *MPK1-4×FLAG* and WT + *MPK2-4×FLAG* transgenic plants upon PBZ treatment. Phosphorylation status was examined at 6 DAP. **f** MPK1 and MPK2 were not phosphorylated in *sid2* + *MPK1-4×FLAG* and *sid2* + *MPK2-4×FLAG* transgenic plants upon PBZ treatment. **g** MPK1 and MPK2 were phosphorylated in *sid2* + *MPK1-4×FLAG* and *sid2* + *MPK2-4×FLAG* transgenic plants treated with 0.5 mM SA for 24 h. *P* phosphorylated; *UP* unphosphorylated. Similar results were obtained from three independent experiments

treatment, indicating that MKK4/5 and MPK1/2-mediated leaf senescence requires NPR1 (Fig. 5a, b; Supplementary Fig. 5a–h, 6c).

NPR1, as a transcriptional coactivator, acts together with TGA transcription factors to regulate PR expression (Després et al. 2003). We examined whether MPK1 or MPK2 directly regulates upstream regulators of plant immunity response, such as NPR1. Yeast two-hybrid assays showed that only MPK1 interacted strongly with NPR1 (Supplementary Fig. 11). We then examined whether

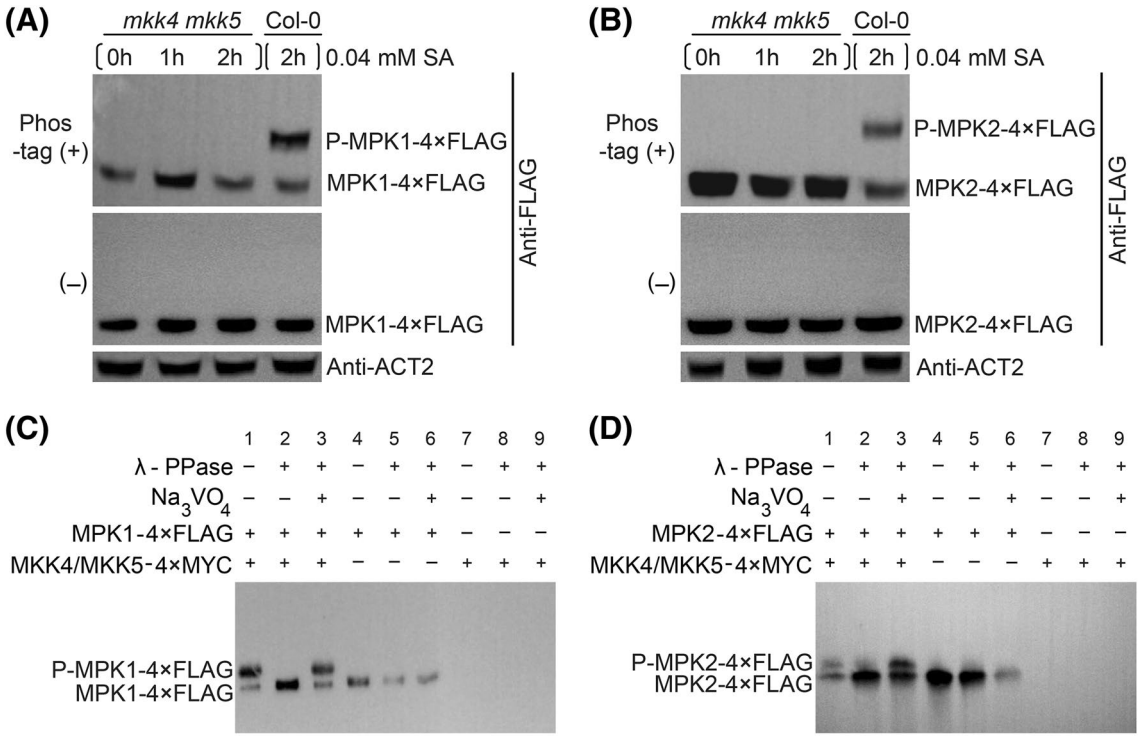

Fig. 4 Phosphorylation of MPK1/2 requires the full function of MKK4 and MKK5. Partial loss-of-functions of MKK4 and MKK5 abolished SA-induced phosphorylation of MPK1 (**a**) and MPK2 (**b**). Presence of MKK4-4×MYC and MKK5-4×MYC recovered the phosphorylation of MPK1-4×FLAG (**c**) or MPK2-4×FLAG (**d**) in vitro. MKK4-4×MYC/MKK5-4×MYC and MPK1-4×FLAG were individually or together introduced into *mkk4 mkk5* protoplasts, which were subsequently subject to phosphorylation analysis. For λ-PPase and Na_3VO_4 treatments, 10 units μl^{-1} λ-PPase and/or 0.1 mM Na_3VO_4 were added to the reaction and incubated at 30 °C for 30 min. Similar results were obtained from three independent experiments

MPK1 or MPK2 is required for phosphorylation of NPR1 in response to SA treatment. Protoplasts were prepared from the leaves of WT + *NPR1-GFP*, *mpk1* + *NPR1-GFP* and *mpk2* + *NPR1-GFP* plants, and treated with 0.04 mM SA. Instead of a quick induction of NPR1 phosphorylation in WT, the SA-induced NPR1 phosphorylation was nearly abolished in *mpk1* background (Fig. 5c). Intriguingly, loss-of-function of MPK2 didn't show an obvious effect on NPR1 phosphorylation. An in vitro phosphorylation assay was carried out with MPK1, MPK2 and NPR1 proteins expressed in and purified from *E.coli*, and it was also shown that NPR1 could be phosphorylated by MPK1 in the presence of 1 mM ATP (Fig. 5d). These results suggest that MPK1, but not MPK2, is critical for NPR1 phosphorylation.

MPK1 mediates NPR1 monomerization

The oligomer-to-monomer transition of NPR1 is an indication of its activation, which leads to its nuclear translocation and phosphorylation, and subsequently the expression of *Pathogenesis-related* (*PR*) genes. Consistent with previous reports (Mou et al. 2003; Spoel et al. 2009), SA treatment decreased the level of NPR1-GFP oligomers, accompanied by an increase of its monomers in WT + *NPR1-GFP* (Fig. 5e). Intriguingly, the monomerization of NPR1 was largely blocked in *mpk1*, indicating that MPK1 is also somehow critical for NPR1 activation (Fig. 5e). Unlike in the case of MPK1, loss-of-function of MPK2 alone had negligible effect on NPR1 monomerization (Fig. 5e). Notably, we also observed a cytoplasmic-to-nucleus translocation of MPK1 in the leaf after SA treatment (Fig. 5f). Furthermore, we found that the expression of both *TRX-h3* and *TRX-h5*, known to regulate cellular redox balance and facilitate NPR1 oligomer-to-monomer transition (Tada et al. 2008), was significantly reduced in *mpk1* (Supplementary Fig. 7).

MKK4/5 and MPK1/2 are involved in induction of disease resistance

SA biosynthesis and signaling have been largely implicated in the mediation of systemically acquired disease resistance in plants (Malamy et al. 1990; Metraux et al. 1990; Gaffney et al. 1993; Delaney et al. 1994; Wildermuth et al. 2001). In *Arabidopsis* (Col-0), SA content at 6 DAP reaches a similar level to that detected at 6 hpi [hours post inoculation with *Pseudomonas syringae pv. maculicola* ES4326

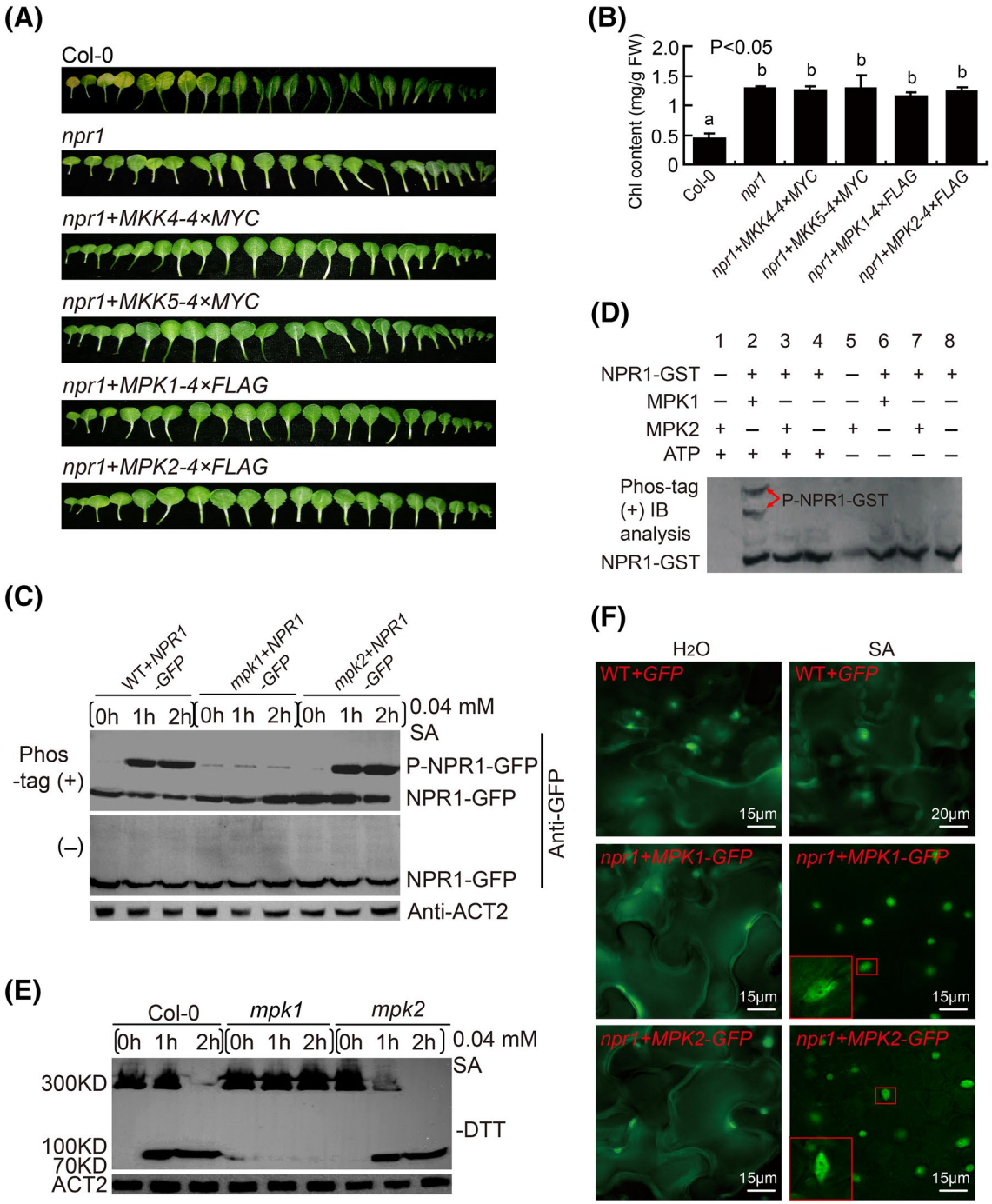

Fig. 5 MPK1 phosphorylates NPR1 and mediates NPR1 monomerization. **a**, **b** Overexpression of MKK4-4×MYC, MKK5-4×MYC, MPK1-4×FLAG or MPK2-4×FLAG in *npr1* didn't cause an obvious leaf yellowing phenotype at 12 DAP. **a** Leaf phenotypes of indicated genotypes at 12 DAP. **b** Chl contents of indicated genotypes at 12 DAP. Error bars indicate SD (n = 3), LSD ANOVA test, $P < 0.05$. **c** Phosphorylation status of NPR1-GFP was examined in the protoplasts prepared from indicated genotypes. **d** NPR1 was phosphorylated in vitro by MPK1 in the presence of ATP. MPK1/MPK2-His and NPR1-GST were expressed in *E. coli* and purified. The plus and minus signs indicated the presence and absence of ATP and respective proteins. Aliquots of the samples were separated by SDS-PAGE with Phos-tag reagent and subject to IB analysis using a GST antibody. **e** Oligomer-to-monomer transition of NPR1-GFP in response to SA treatment was blocked in *mpk1*. For the examination of the oligomer-to-monomer transition of NPR1-GFP, reduced reagent (DTT) was omitted during protein extraction and IB assay. Similar results were obtained from three independent experiments. **f** Cytoplasmic-to-nucleus translocation of MPK1 after SA treatment. Bar = 15 μm. Similar results were obtained from two independent experiments

(*Psm.* ES4326) at an OD_{600} of 0.0001] (Yu et al. 2010; Wang et al. 2015). Notably, NPR1-GFP phosphorylation were also detected in WT + *NPR1-GFP* plants at 6 DAP and 6 hpi (Supplementary Fig. 8). To determine whether MKK4/5 and MPK1/2 are also involved in the induction of disease resistance, we inoculated 5-weeks-old plants with *Psm.* ES4326 at an OD_{600} of 0.001. The leaf chlorosis and growth of *Psm.* ES4326 in *mkk4 mkk5*, *mkk4** and *mpk1 mpk2* were more prominent than those in WT, comparable to those in *npr1*, indicating that their resistance to *Psm.* ES4326 was greatly impaired (Fig. 6a, b). Consistently, the expression of disease resistance marker genes, e.g. *PR1*, *PR2*, *PR5*, and *NPR1*, was more or less compromised in these mutants (Fig. 6c). These results suggest critical roles of MKK4/5 and MPK1/2 in the induction of disease resistance.

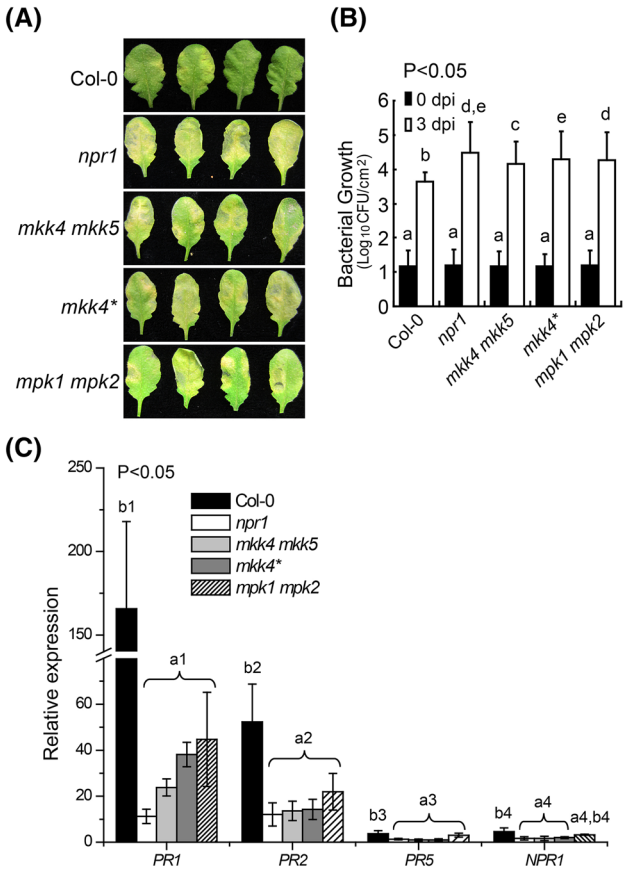

Fig. 6 MKK4/5 and MPK1/2 are involved in the induction of disease resistance. **a** Disease symptoms of Col-0, *npr1*, *mkk4 mkk5*, *mkk4** and *mpk1 mpk2* plants at 3 dpi (days post inoculation). Five-week-old plants grown in short day-growth conditions were pressure-infiltrated with *Psm.*ES4326 at an OD_{600} of 0.001. **b** Measurements of *Psm.*ES4326 growth in the leaves of indicated genotypes at 3 dpi. Error bars indicate SD (n = 8), LSD ANOVA test, $P < 0.05$. **c** Transcript levels of *PR1*, *PR2*, *PR5*, and *NPR1* genes in the leaves of indicated genotypes at 3 dpi. Error bars indicate SD (n = 3), LSD ANOVA test, $P < 0.05$

Discussion

Exogenous SA, ABA, JA and ET have long been shown to be able to induce leaf senescence; but, except in the case of ET, decreases in their levels and/or mutations in their signaling components don't normally cause obvious senescence phenotypes under normal growth conditions (Gan and Amasino 1997; Graaff et al. 2006; Kusaba et al. 2013; Jibran et al. 2013). It therefore remains largely unclear whether and how these hormones are involved in the regulation of leaf senescence (Gan and Amasino 1997; Graaff et al. 2006; Kusaba et al. 2013; Jibran et al. 2013). On the other hand, genetically increasing the level of endogenous hormones often disturb whole plant development and is therefore not an ideal approach to determine the exact role of the major hormones in regulating leaf senescence (Park et al. 1998). In this study, by taking advantage of PBZ-induced biosynthesis of endogenous SA, we demonstrate that increasing the endogenous level of SA can significantly accelerate the senescence of old leaves in *Arabidopsis*. It has been reported that the expression of many senescence related genes is affected in the mutants of SA biosynthesis and signaling genes, but no obvious effects on senescence phenotype are observed in its signaling mutant *npr1* and in *nahG* plants where SA is converted to catechol (Morris et al. 2000). Consistently, we didn't observe any senescence phenotype in mutants defective in SA biosynthesis (*sid2*) and signaling (*npr1*) or in *NPR1* constitutively overexpressed plants (*35S*::*NPR1*) without PBZ treatment. However, upon PBZ treatment, *sid2* and *npr1* plants exhibited a significantly delayed senescence phenotype, whereas *35S*::*NPR1* plants displayed precocious leaf yellowing. All the physiological and molecular parameters examined were consistent with the observed senescence phenotypes. These data may imply that SA-triggered leaf senescence has been evolved to strategically sacrifice parts of the leaf tissue that are severely affected by environmental stresses, e.g. a heavy pathogen infection that induces a dramatic increase in SA level (Pajerowska-Mukhtar et al. 2012). This hypothesis is supported by our observation that basal resistance shares a MAPK cascade with SA acceleration of leaf senescence, as will be discussed below.

MKK4 and MKK5 are widely implicated in plant abiotic and biotic stresses (Asai et al. 2002; Zhao et al. 2014; Kim et al. 2011), whereas MPK1 and MPK2 were reported to be involved in response to wounding (Ortiz-Masia et al. 2007). In this study, we revealed an involvement of MKK4/5-MPK1/2 cascade in SA signaling. We found that single knock-down or knock-out mutants of *MKK4*, *MKK5*, *MPK1* or *MPK2* inhibited PBZ-accelerated leaf senescence to a subtle but detectable degree, with plant growth

and development not being obviously affected under normal conditions. This inhibition was more obvious in their double mutants (*mkk4 mkk5* and *mpk1 mpk2*) (Fig. 2b–f). Consistently, overexpression of *MPK1* or *MPK2* produced a precocious senescence phenotype. Importantly, the precocious senescence was blocked in the mutants of *ICS1* or *NPR1* (Figs. 3c, d, 5a, b). These data suggest that the MKK4/5-MPK1/2 cascade plays an important role in PBZ-accelerated leaf senescence and its functionality requires SA/NPR1. The MKK9-MPK6 cascade, as well as MAPKKK8, has been reported to be involved in the regulation of developmental leaf senescence (Zhou et al. 2009; Miao et al. 2007). However, inconsistent with a recent report (Chai et al. 2014), our data indicate that MKK9-MPK6 cascade may not be involved in SA signaling-dependent leaf senescence (Supplementary Fig. 3). Our results imply that different MAPK cascades may be required to mediate leaf senescence in different scenarios. Meanwhile, we found that MPK1/2 also play an essential role in basal resistance (Fig. 6). These observations suggest that there is a common MAPK cascade regulating both SA-induced disease resistance and leaf senescence.

Remarkably, we showed that MPK1 phosphorylated NPR1, whereas MKK4/5 phosphorylated MPK1/2. NPR1 protein was recently reported to be required for SA-induced senescence of detached leaves in *Arabidopsis* (Chai et al. 2014), and its phosphorylation at Ser589/Thr373 by SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8) mediated SAR induction in distal tissues by facilitating its nuclear import (Lee et al. 2015). Importantly, it was previously shown that NPR1 needs to be phosphorylated at Ser11/Ser15 in the nucleus for full induction and subsequent establishment of SAR (Spoel et al. 2009). However, which kinase(s) phosphorylates NPR1 protein at the residues remains unknown. Chai et al. recently reported that MPK6 could affect the expression of *NPR1* gene via mediation of WRKY6 and TRX-h5 and consequently the monomerization of NPR1 protein during leaf senescence (Chai et al. 2014). Here, by in vitro and in vivo phosphorylation assays, we found that MPK1, but not MPK6, directly phosphorylated NPR1 (Fig. 5c–g; Supplementary Fig. 9). Unexpectedly, MPK2 showed only a trivial, if any, activity of NPR1 phosphorylation, although it was found to regulate SA/NPR1-dependent leaf senescence redundantly with MPK1 (Fig. 5c). It therefore remains unknown for how MPK2 is exactly involved in the regulation of SA/NPR1-dependent leaf senescence. In light of that the function of NPR1 is dependent on W-boxes in its promoter (Yu et al. 2010), and a large number of WRKY TFs have been reported to be involved in the regulation of plant disease resistances and leaf senescence (Wang et al. 2006; Eulgem and Somssich 2007; Ishihama and Yoshioka 2012; Singh et al. 2014), we hypothesize that MPK2 might be responsible for the phosphorylation of some of these WRKY TFs. The hypothesis is supported by the findings that a large number of WRKYs are phosphorylated by MPKs (Popescu et al. 2009; Hiroaki et al. 2015). In addition, we found that MPK2 can interact with WRKYs, which played an important role in leaf senescence signaling pathway by regulating the expression of *ICS1* and *NPR1* in our another article (Gao et al. 2019, under review).

Mou et al. discovered that exogenous application of SA can activate NPR1 conformational change, and, importantly, NPR1 monomerization is both necessary and sufficient for its function (Mou et al. 2003). Interestingly, we found that NPR1 monomerization was also inhibited when *MPK1* was mutated (Fig. 5e), indicating that MPK1 is crucial not only for NPR1 phosphorylation but also for its conformational change. This finding is corroborated by our further detection that the expression of *TRX-h3/5* was significantly reduced in *mpk1* compared with Col-0 plants (Supplementary Fig. 7). Both TRX-h3 and TRX-h5 are required for full induction of *PR* genes, and TRX-h5 is known to facilitate NPR1 oligomer-to-monomer transition via regulating cellular redox balance (Tada et al. 2008). It would therefore be interesting to reveal the molecular link between MPK1 phosphorylation activity and its effect on *TRX-h3/5* expression. These results indicated that MPK1 was involved in regulating the activation of NPR1, possibly by enhancing the expression of *TRX-h3* and *TRX-h5*.

By taking advantage of PBZ-induced leaf senescence, we reveal a MAPK cascade, MKK4/5-MPK1, which not only phosphorylates the key SA signaling component NPR1 specifically, but also regulates its oligomer-to-monomer conformational change somehow via mediation of *TRX-h3/5*. Both of the functions depend on endogenous SA biosynthesis. Our revelation of this novel regulatory module suggests that there exists a hierarchical regulatory network in the NPR1-mediated SA signaling (Supplementary Fig. 10).

Materials and methods

Plant materials and growth conditions

Plants were grown under a short-day photoperiod (8 h light/16 h dark) at 23 ± 2 °C, with approximately 70% relative humidity and 100 μmol m^{-2} s^{-1} light intensity. Five-week-old plants were applied with 0.5 mM probenazole (PBZ) via root drenching to induce endogenous SA biosynthesis. Transgenic plants were obtained via vacuum infiltration (Bechtold et al. 1993) and were selected on ½ Murashige and Skoog medium containing 25 mg l^{-1} kanamycin (for pSKM36), or 25 mg l^{-1} hygromycin (for pCAMBIA1302). Generally, more than ten independent transformants were obtained for each construct, and three lines were

randomly chosen for gene expression and chlorophyll content analyses (Supplementary Figs. 5, 6). T-DNA insertion lines for *mkk4* (SALK_018804C), *mkk5* (SALK_050700), *mpk1* (SALK_063847C) and *mpk2* (SALK_047422C) were obtained from *Arabidopsis* Biological Resource Center (ABRC). *sid2-2* was kindly provided by Dr. Frederick Ausubel, *npr1-1* and *35S*::*NPR1* (35S-NPR1-GFP) by Dr. Xinnian Dong, and *mkk4** (*mkk4-18*) and *mkk5** (*mkk5-18*) by Dr. Dingzhong Tang. A substantial but not complete loss-of-function double mutant of *MKK4* and *MKK5* was generated by crossing *mkk4* with *mkk5* and genotyping accordingly. T-DNA insertion sites were determined by directly sequencing PCR products amplified with T-DNA left border and gene-specific primers. Primers for genotyping were listed in Supplementary Table 2.

Measurements of senescence-associated parameters

For the measurement of senescence-associated physiological parameters, the 5th through the 8th leaves (~ 0.1 g fresh weight) of five-week-old plants were used. Chl contents were quantified with a spectrometer (TU-1900, Persee) according to Benedetti and Arruda (Benedetti and Arruda 2002). Fluorescence in leaves was measured using LI-6400 (LI-COR) under a fixed LED light source (500 mmol m^{-2} s^{-1}) at 25 °C and 300 µmol s^{-1} flow speeds, as specified in the manufacturer's instruction. For measuring ion leakages, leaves were immersed in deionized distilled water, shaken in a 25 °C water bath for 40 min, and the conductivity was measured using a digital conductivity meter (Waterproof ECTestr11 +, MultiRange). Samples were boiled for 15 min and the conductivity then monitored. The ratio of the first measurement over the second measurement was used as an indicator of membrane leakage.

RNA analyses

RNA was extracted from 100 mg of respective leaf samples using Trizol (Invitrogen) and dissolved in 10 µL of DEPC-treated water. cDNA was synthesized using Superscript first-strand synthesis kit. qPCR (quantitative PCR) was performed using SYBR Green I PCR kit (Toyobo) on an iCycler according to the manufacturer's instruction, with *ACTIN2* as a reference (Bio-Rad). The reaction conditions for qPCR were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 30 s, 60 °C for 25 s, and 72 °C for 20 s. For microarray analysis, two biological replicates of each sample were collected before and after 72 h PBZ treatment. Biotinylated cDNAs were prepared according to the standard Affymetrix protocol from 10 µg of total RNA. GeneChips were scanned using the GeneArrayTM scanner 3000. Microarray data were shown in GEO: GSE72636.

Transient expressions of proteins in protoplasts

Transient expression assays were performed with the protoplasts isolated from *Arabidopsis* mesophyll cells. Protoplasts from WT or mutants were transiently transformed by adding 10 mg polyethylene glycol. Twelve hours after transformation, the protoplasts were collected by centrifugation and then re-suspended in protein extraction buffer for further analyses. Details in the isolation, transformation, and cultivation of protoplasts were described by Sheen (https://genetics.mgh.harvard.edu/sheenweb/).

Plasmid construction

To construct overexpressing vectors, coding sequences of *MKK4* and *MKK5* were amplified and inserted into the cloning vector pSKM36 after digestion with AscI and SpeI. A 4 × MYC tag was amplified and inserted into the resultant vectors at the AscI site to generate *MKK4-4* × *MYC* and *MKK5-4* × *MYC* constructs, respectively. Coding sequences of *MPK1* and *MPK2* were amplified and inserted into pCAMBIA1302 to generate *MPK1-4* × *FLAG* and *MPK2-4* × *FLAG* constructs, respectively. For the protein expression in *E. coli*, coding sequences of *NPR1* and *MPKs* were amplified and inserted into pET28 and pGEX-4T-1, respectively. Primers used in the plasmid construction were listed in Supplementary Table 2.

Protein analyses

For Western blot assay, total proteins were extracted from leaf tissues using 1 × SDS sampling buffer, or from protoplasts using protein isolation buffer (50 mM HEPES, pH 7.5; 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij35, 2 mM $MnCl_2$, 0.1% Triton X-100, and 1/100 protease inhibitor cocktail). About 10 µg proteins were separated in a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane. MYC-tagged, FLAG-tagged, and GFP-tagged proteins were detected using anti-MYC (1:2000, M20002M, Abmart), anti-FLAG (1:2000, F1804, Sigma), and anti-GFP antibodies (1:2000, 632569, Clontech), respectively. DTT was omitted for the examination of the oligomer-to-monomer transition of NPR1-GFP. Phosphorylated proteins were separated in a gel with 100 µM Phos-tag reagent (NARD Institute) and 200 µM $MnCl_2$. For λ-PPase and Na_3VO_4 treatments, λ-PPase (10 units ml^{-1}) and/or Na_3VO_4 (0.1 mM) were added, followed by incubating at 30 °C for 30 min. Reactions were stopped by adding ¼ volume of 5 × SDS-PAGE sampling buffer. For the in vitro kinase assay (Peck 2006), 0.5 µg MBP-MPK1 or MBP-MPK2 and 0.25 µg GST-NPR1 were added to kinase assay solution [20 mM Tris (pH 7.4),

100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP]. Recombinant proteins were incubated or co-incubated in kinase reaction solution at 37 °C for 60 min.

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Author contributions J.Z. and J.G. conceived and designed the experiments. J.Z., J.G., Z.Z. and Y.S. performed the experiments, X.W., X.W. and X.Z. contributed new materials, J.G. and J.Z. analyzed the data and wrote the paper. All authors read and improved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have conflict of interest.

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