

# The PP2C-Type Phosphatase AP2C1, Which Negatively Regulates MPK4 and MPK6, Modulates Innate Immunity, Jasmonic Acid, and Ethylene Levels in *Arabidopsis*

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Wound signaling pathways in plants are mediated by mitogen-activated protein kinases (MAPKs) and stress hormones, such as ethylene and jasmonates. In *Arabidopsis thaliana*, the transmission of wound signals by MAPKs has been the subject of detailed investigations; however, the involvement of specific phosphatases in wound signaling is not known. Here, we show that AP2C1, an *Arabidopsis* Ser/Thr phosphatase of type 2C, is a novel stress signal regulator that inactivates the stress-responsive MAPKs MPK4 and MPK6. Mutant *ap2c1* plants produce significantly higher amounts of jasmonate upon wounding and are more resistant to phytophagous mites (*Tetranychus urticae*). Plants with increased AP2C1 levels display lower wound activation of MAPKs, reduced ethylene production, and compromised innate immunity against the necrotrophic pathogen *Botrytis cinerea*. Our results demonstrate a key role for the AP2C1 phosphatase in regulating stress hormone levels, defense responses, and MAPK activities in *Arabidopsis* and provide evidence that the activity of AP2C1 might control the plant's response to *B. cinerea*.

## INTRODUCTION

Biotic and abiotic stresses in eukaryotes activate mitogen-activated protein kinase (MAPK) cascades that typically consist of a MAPKKK, MAPKK, and MAPK and transmit the signal to generate cellular responses. A MAPK is activated when the conserved TEY motif at the activation loop is phosphorylated by a dual specificity kinase (MAPKK) on both Thr and Tyr (Kiegerl et al., 2000). This activation can be reversed by dephosphorylation through Ser/Thr phosphatases, Tyr phosphatases (PTPs), or dual specificity phosphatases (DSPs; Keyse, 2000; Meskiene et al., 2003). PTPs and DSPs are considered to be primary MAPK phosphatases (MKPs) in yeast and in animals, respectively; however, in some cases, Ser/Thr phosphatases of the PP2C family also regulate MAPK(K) (Tamura et al., 2006). Plant DSPs At MKP1 (Ulm et al., 2001, 2002), Nt MKP1 (Yamakawa et al., 2004; Katou et al., 2005), and PTP At PTP1 (Gupta and Luan, 2003) were suggested to regulate MAPKs. *Arabidopsis thaliana* genomic data show a single PTP and ~23 DSPs, while the PP2C family is expanded to 76 putative members (Kerk et al., 2002;

Schweighofer et al., 2004; Kerk, 2006). PP2Cs are ubiquitous protein phosphatases found in all eukaryotes; however, in plants, their individual functions and substrates await characterization. Several PP2Cs are involved in abscisic acid responses (Leung et al., 1994; Meyer et al., 1994; Sheen, 1998; Tahtiharju and Palva, 2001; Saez et al., 2004), but their substrates have not been identified. The kinase-associated protein phosphatase regulates receptor-like kinases (Stone et al., 1994; Williams et al., 1997; Li et al., 1999). The demonstration that alfalfa (*Medicago sativa*) MP2C regulates MAPKs by dephosphorylation of phospho-Thr in the TEY loop of the MAPK (Meskiene et al., 1998, 2003) suggested that PP2Cs may regulate MAPKs in plants; however, it provided no proof of biological relevance at the whole-plant level.

Wounding induces production of jasmonic acid (JA) and ethylene (ET), which are messengers to activate defense responses. Plants with high constitutive levels of JA are more resistant to herbivores and pathogens (Liechti et al., 2006). Impaired ET perception or signal transduction leads generally to higher susceptibility to necrotrophic pathogens (Knoester et al., 1998; Asai et al., 2002) but may give the opposite phenotype in other pathosystems (Lund et al., 1998; Berrocal-Lobo and Molina, 2004). *Arabidopsis* MAPKs are involved in stress signaling and plant defense at least in part through effects on JA, ET, and salicylic acid (SA) response pathways. An *mpk4* null mutant has constitutively high levels of SA but fails to express the defense marker genes *PDF1.2* and *THI2.1* in response to JA (Petersen et al., 2000). MPK4 is essential for the induction of a subset of ET-responsive

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genes and crucial for the antagonism between SA- and JA/ET-dependent responses (Brodersen et al., 2006). MPK6 mediates flagellin-induced signaling and thus links MAPKs to plant innate immunity (Asai et al., 2002; Menke et al., 2004). However, it is not known which phosphatases regulate wound- and pathogen-activated MAPKs in plants.

Here, it is shown that the *Arabidopsis* PP2C-type phosphatase AP2C1 is a MKP. AP2C1 influences the induction of the defense marker *PDF1.2*. *ap2c1* mutant plants exhibit elevated JA levels in response to wounding and higher resistance to phytophagous mites (*Tetranychus urticae*). AP2C1-overexpressing (AP2C1-oe) plants are compromised in their ability to synthesize wound ET and in their defense response to the necrotrophic pathogen *Botrytis cinerea*.

## RESULTS

### AP2C1 Is a MAPK-Interacting Phosphatase

To identify signaling components regulating stress-activated MAPK pathways in *Arabidopsis*, PP2Cs were selected as likely candidates. Among 76 genes classified as PP2C-type phosphatases according to their amino acid sequences (Kerk et al., 2002; Schweighofer et al., 2004; Kerk, 2006), a protein domain search identified a region matching the consensus for a MAPK interaction motif (named KIM; [K/R]<sub>(3-4)</sub>-X<sub>(1-6)</sub>-[L/I]-X-[L/I]) in four phosphatases of one cluster. A similar KIM is found in yeast and animal MKPs (Tanoue and Nishida, 2003) as well as in plant MAPKKs (Kiegerl et al., 2000). As a result of in silico analysis, AP2C1 (genomic locus: At2g30020; Schweighofer et al., 2004), which is highly similar to alfalfa *MP2C* (Meskiene et al., 1998, 2003), was selected for further study. The AP2C1 protein contains a C-terminal catalytic domain typical to PP2C-type phosphatases (Bork et al., 1996) and an N-terminal extension including the KIM. The full-length coding sequence was isolated from a cDNA library by PCR.

To find proteins interacting with AP2C1, an *Arabidopsis* cDNA library was screened in a yeast two-hybrid approach. Four out of the 26 isolated positive clones contained the cDNA of MPK6. To test the specificity of this interaction, 18 out of the at least 20 predicted MAPKs and 10 MAPKKs in the *Arabidopsis* genome (Ichimura, 2002) were tested for interaction with AP2C1 in yeast. The strongest interaction was found with MPK6 followed by MPK4, but no other MAPKs (Figure 1A) or MAPKKs tested showed interaction (see Supplemental Figure 1 online). To investigate the importance of the putative KIM for phosphatase/MAPK interaction, the missense mutations K98A and K98A,R99Q in the KIM of AP2C1 were created. Both mutant proteins were tested for their interaction with MPK6 and MPK4 in yeast. Compared with wild-type AP2C1, interaction with both MAPKs was weaker for AP2C1-K98A and completely abolished for AP2C1-K98A,R99Q (Figure 1B). These results show that the interaction with MPK4 or MPK6 in yeast depends on an intact KIM in AP2C1.

To find if AP2C1 and MAPKs associate in plant extracts, co-immunoprecipitation assays from plants were performed. Protein gel blot analysis with MPK6-specific antibodies of protein complexes immunoprecipitated from extracts of AP2C1-oe leaves

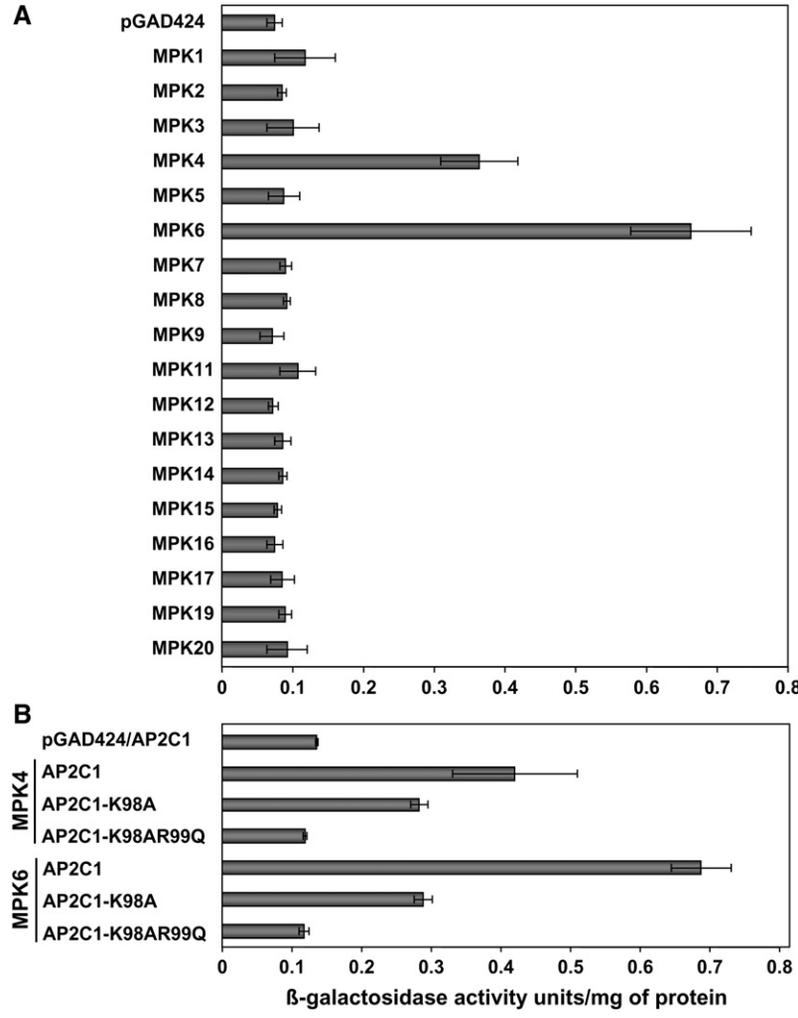
with green fluorescent protein (GFP) antibody identified MPK6 as a protein associated with AP2C1 (AP2C1-oe; Figure 2A). Two MPK6 forms with different electrophoretic mobilities were identified in the immunoprecipitate. More MPK6 was coimmunoprecipitated from wounded leaves, though in crude extracts, MPK6 abundance was not altered. This indicates that more association of AP2C1 with MPK6 occurs in a signal-dependent manner. Efforts to immunoprecipitate a MPK4/AP2C1 complex from leaves were unsuccessful, possibly due to much lower levels of MPK4 protein and/or instability of that association. To see where the AP2C1/MAPK complexes are located in the plant cell, a bimolecular fluorescence complementation (BiFC) assay based on split yellow fluorescent protein (YFP) was performed (Walter et al., 2004). The N- and C-terminal domains of YFP were fused to AP2C1 and either MPK6 or MPK4, respectively, and transiently coexpressed in *Arabidopsis* protoplasts. Fluorescence from reconstituted YFP indicated interaction between AP2C1 and MAPKs. No fluorescence was detectable either in the controls (Figure 2B) or with the combination 35S-YFPctd/35S-YFPntd (data not shown). It was found that AP2C1/MPK4 complexes localize in the nucleus, whereas AP2C1/MPK6 complexes are detectable in the nucleus and in the cytoplasm (Figure 2B). However, this approach did not allow us to identify selectivity between the MAPKs (data not shown). Nevertheless, protein interaction in yeast cells, the BiFC assay, and coimmunoprecipitation results collectively support the model that AP2C1 interacts with MPK4 and MPK6. This prompted us to test whether these interactions may have effects in plant cells.

### Inactivation of MPK4 and MPK6 by AP2C1 in Protoplasts

The constitutively active MAPKKK  $\Delta$ ANP1 can activate MPK6 and MPK3 in *Arabidopsis* mesophyll protoplasts (Kovtun et al., 2000). An activated MAPK pathway was reconstituted in *Arabidopsis* cell culture protoplasts by cotransfection of plasmids expressing HA epitope-tagged MPK3, MPK4, or MPK6, together with  $\Delta$ ANP1. Myelin basic protein-based MAPK assays revealed that in addition to MPK3 and MPK6, MPK4 also can be activated by  $\Delta$ ANP1 (Figure 3A). Increasing amounts of the AP2C1-expressing plasmid cotransformed with different MAPKs resulted in progressively more complete inactivation of MPK4 and MPK6 but not of MPK3 (Figure 3A). However, other PP2Cs, such as ABI2 and HAB1, were not able to inactivate MPK6, suggesting that PP2C-type phosphatases can select for specific substrates. Experiments with recombinant proteins performed in vitro demonstrated that AP2C1 is a Mg<sup>2+</sup>-dependent PP2C-type phosphatase with a dephosphorylation activity similar to ABI2 or HAB1 phosphatases on phosphor-casein (Figure 3B). However, AP2C1 was able to dephosphorylate MPK6 directly in vitro, while ABI2 and HAB1 could not (Figure 3C). Taken together, these data suggest that PP2C-type phosphatases can select for their substrates and that in plant cells, MPK4 and MPK6 activity is regulated by the abundance of AP2C1.

### Stress Activation of MPK4 and MPK6 Is Regulated by AP2C1 in Planta

To test for the biological relevance of the observed interactions, plant lines stably overexpressing AP2C1 (AP2C1-oe) were



**Figure 1.** AP2C1 Interaction with *Arabidopsis* MAPKs in Yeast.

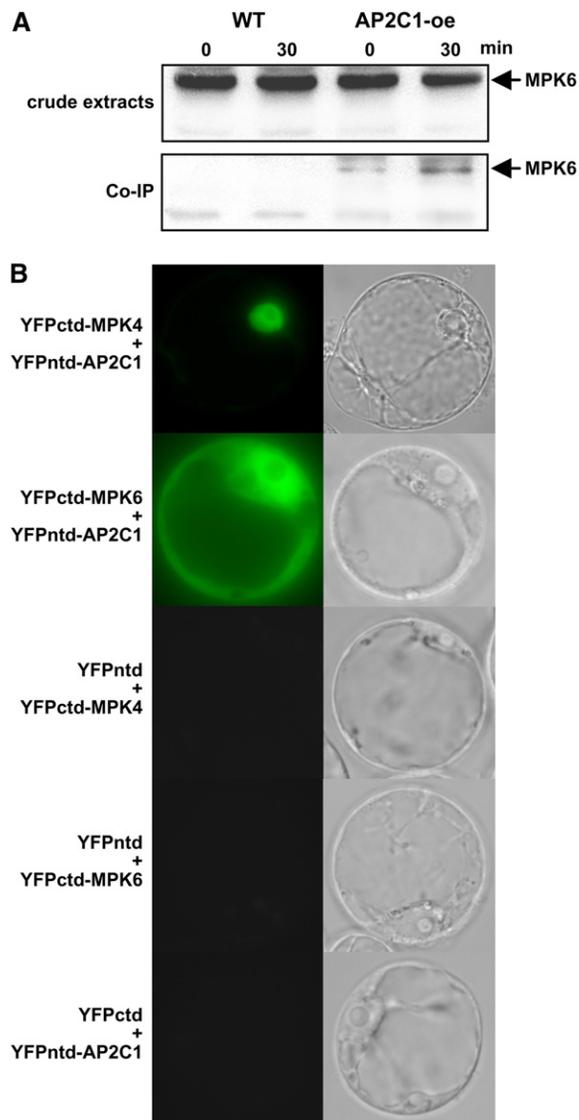
**(A)** AP2C1 interacts specifically with MPK4 and MPK6. Quantitative β-galactosidase measurements in a yeast two-hybrid assay were performed using pBTM116-AP2C1 in combination with 18 MAPKs in the pGAD424 vector.  
**(B)** AP2C1 directs the interaction with MAPKs through its kinase interaction motif (KIM). Quantitative β-galactosidase yeast two-hybrid assay of AP2C1, AP2C1-K98A, or AP2C1-K98A,R99Q in combination with MPK4 or MPK6.

generated. Three independent AP2C1-oe lines were analyzed for wound-induced MAPK activities in each generation (T2-T4) in comparison to the wild type. MPK4 and MPK6 were found to be activated in wild-type plants by wounding as reported earlier (Ichimura et al., 2000); however, they could not be fully activated in AP2C1-oe lines (Figure 4A). At the same time, MPK4 and MPK6 protein amounts remained unaltered, showing that AP2C1 affects their activity posttranslationally.

To test if the inactivation of MAPKs depends on the enzymatic activity of AP2C1, the AP2C1-G178D mutant was created based on the G180D mutation in the ABI1 phosphatase (Bertauche et al., 1996). Activation kinetics of MPK4 and MPK6 upon wounding were not altered in plants constitutively expressing AP2C1-G178D (Figure 4B), indicating that AP2C1 enzymatic activity is essential for the modulation of MPK4 and MPK6 activation in

plants. This molecular phenotype strongly suggests that physical interaction between AP2C1 and MPK4 or MPK6 results in MAPK inactivation in intact plants as well.

A homozygous insertion mutant in AP2C1 (*ap2c1*) was isolated from the SALK collection (<http://signal.salk.edu>) and characterized as carrying a tandem T-DNA insertion in the second exon of the *AP2C1* gene (see Supplemental Figure 2 online). *ap2c1* plants had no obvious phenotype under normal growth conditions. However, the analysis of wound-induced MPK4 and MPK6 activities upon wounding showed more pronounced and/or sustained activation of kinases in *ap2c1* than in wild-type plants (Figure 4A). This enhanced MAPK activation in the mutant line could be abolished by complementation with the cDNA coding for AP2C1 under the control of its own promoter (see Supplemental Figure 3 online).



**Figure 2.** Interaction of AP2C1 with MAPKs in Vivo.

**(A)** AP2C1 coimmunoprecipitates with MPK6 in plant extracts. Leaves of wild-type and AP2C1-GFP overexpressing (AP2C1-oe) plants (line #640.2) were subjected to wounding and harvested at the indicated times. The top panel represents a protein gel blot of crude protein extracts from wild-type and AP2C1-oe plants probed with antibodies raised against native MPK6. The bottom panel shows an identical protein gel blot analysis of protein complexes previously immunoprecipitated with anti-GFP antibodies from the same extracts analyzed in the top panel. An arrow indicates the position of the MPK6 protein after SDS-PAGE. Co-IP, coimmunoprecipitation.

**(B)** Intracellular localization of AP2C1/MPK4 and AP2C1/MPK6 in a split YFP-based BiFC assay in *Arabidopsis* protoplasts. The C-terminal domain of YFP fused to MPK4 or MPK6 was coexpressed under the control of the 35S promoter (YFPctd-MPK4 or YFPctd-MPK6) with a fusion protein of the N-terminal domain of YFP and AP2C1 (YFPntd-AP2C1). The subcellular localization of the interaction is visualized by fluorescence microscopy. Yellow fluorescence is converted to a green signal for better definition in RGB acquisition mode. 35S-YFPntd (YFPntd) and 35S-YFPctd (YFPctd) vectors expressing split YFP domains alone were used as controls.

### AP2C1 Expression Is Pathogen and Wound Induced

The phenotypically normal appearance of AP2C1-oe and *ap2c1* plants suggested that AP2C1 function is restricted to specific conditions. To determine whether pathogen stimuli may influence AP2C1 levels, AP2C1 expression was examined in leaves of transgenic plants carrying a  $\beta$ -glucuronidase (GUS) reporter gene transcriptionally fused to the AP2C1 promoter region. Strong induction of local GUS expression was detected in leaf cells 24 h after inoculation with conidia of *B. cinerea* (Figure 5A). GUS expression also was strongly and locally induced 30 min after wounding (Figure 5B), whereas no GUS expression was detected in wounded control wild-type plants (data not shown). RT-PCR and RNA gel blot analysis confirmed that the AP2C1 RNA levels increase upon wounding in wild-type leaves (see Supplemental Figure 2C online; data not shown). Our observation that fungal inoculation and wounding each increase AP2C1 transcript levels supports the idea that the interaction of AP2C1 with MPK4 or MPK6 may take place downstream of non-self perception and mechanical damage.

### AP2C1 Modulates Pathogen Response in Plants

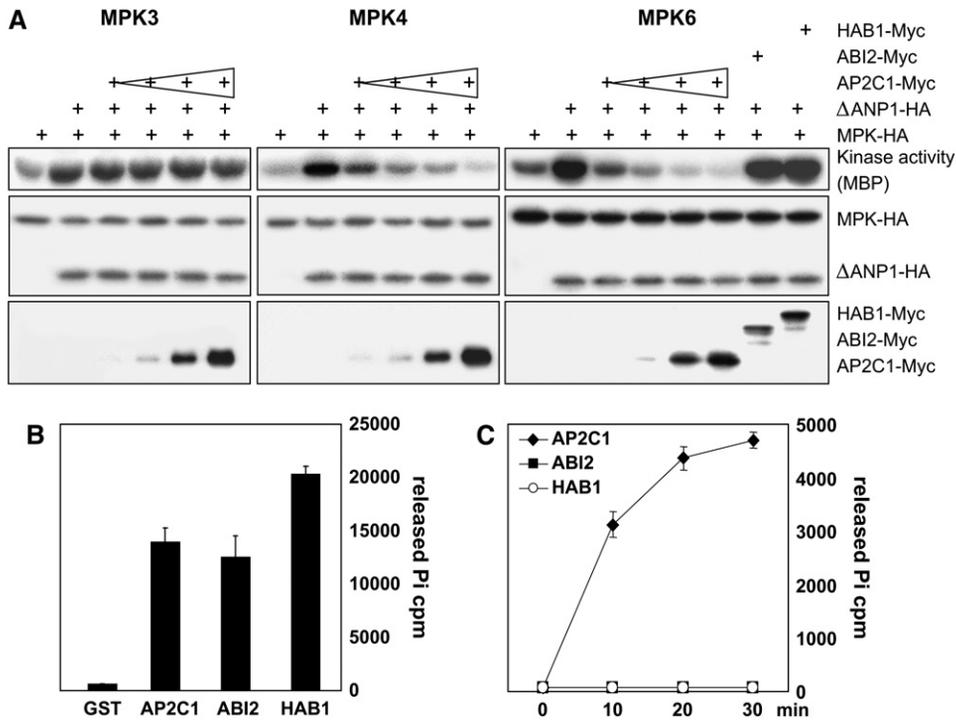
Considering the induction of AP2C1 by *B. cinerea* (Figure 5A) and the fact that a MAPK cascade was implicated in resistance to this pathogen (Asai et al., 2002), the effect of manipulation of AP2C1 expression on resistance against *B. cinerea* was investigated. Figures 6A to 6C show susceptibility of wild-type, AP2C1-oe, and *ap2c1* lines to *B. cinerea*. When leaves were inoculated with fungal conidia, the level of susceptibility of the AP2C1-oe line was similar to that of the camalexin-deficient *pad3-1* mutant (Glazebrook, 2005) and was significantly higher than wild-type or *ap2c1* plants (Figures 6A and 6B). Similar results were obtained using *B. cinerea* mycelium, where the measurement of lesion size confirmed that AP2C1-oe lines are significantly more susceptible ( $P < 0.05$ ) than the wild type or *ap2c1* (Figure 6C; see Supplemental Figure 4 online). On the other hand, the resistance of *ap2c1* plants was only slightly altered compared with the wild type under the same experimental conditions. However, the incidence of aborted infections was higher (20%) in the *ap2c1* line than in either wild-type (10%) or AP2C1-oe plants (8%). The incidence of collapsed leaves followed a reverse trend (AP2C1-oe [24%] > wild type [8%] > *ap2c1* [7%]).

When the same lines were tested for resistance against herbivores, the fecundity of spider mites was reduced on *ap2c1* plants (Figure 6D). The number of eggs laid in a 5-d period on this line was markedly lower than that on the wild type ( $P = 0.04$ ), whereas it did not differ significantly between AP2C1-oe and wild-type plants. Complementation of the *ap2c1* mutation enhanced mites' fecundity (see Supplemental Figure 5 online).

These results establish AP2C1 as a negative regulator of *Arabidopsis* innate immunity against both a fungal pathogen and a herbivorous pest.

### AP2C1 Regulates Plant Stress Hormones and the Expression Pattern of a Stress Marker

MPK6 was shown to phosphorylate and thereby stabilize members of the ACC synthase family, rate-limiting enzymes in ET



**Figure 3.** AP2C1 Inactivates MAPKs in Vivo and in Vitro.

**(A)** AP2C1 inactivates MPK4 and MPK6 in vivo. Protoplasts from an *Arabidopsis* cell suspension were transfected with 5 µg of plasmid DNA coding for MPK3-HA, MPK4-HA, MPK6-HA, ΔANP1-HA (as MAPK activator), and increasing amounts of AP2C1-Myc plasmid DNA (0.05, 0.1, 0.5, and 1 µg) or 1 µg of ABI2-Myc or HAB1-Myc plasmid DNA. Kinase assays were performed on myelin basic protein (MBP). Immunoblots of the MAPKs and ΔANP1 were performed using anti-HA antibodies; AP2C1, HAB1, and ABI2 protein amounts were monitored with anti-Myc antibodies.

**(B)** In vitro phosphatase assay. Phosphatase activities of recombinant AP2C1, ABI2, and HAB1 proteins (0.1 µg) toward [<sup>32</sup>P] phospho-casein (12.5 µg) monitored by measuring the released free phosphate.

**(C)** AP2C1 dephosphorylates MPK6 in vitro. AP2C1, ABI2, and HAB1 proteins (0.1 µg) were incubated with [<sup>32</sup>P] phospho-MPK6 (~1 µg), and the released free phosphate was measured.

biosynthesis (Liu and Zhang, 2004). As our findings suggested that MPK6 is one of the substrates of AP2C1 and that AP2C1 expression is induced locally by wounding (Figure 5B), plant lines with altered AP2C1 content were examined for ET production after wounding. As shown in Figure 7A, AP2C1-oe plants exhibit a significant reduction in ET levels compared with the wild type and *ap2c1* 24 h after leaf wounding.

As wounding also activates the synthesis of JA, basal and wound-induced levels of JA were measured in *ap2c1* and AP2C1-oe plants (Figure 7B). After wounding, JA accumulated to levels severalfold higher in *ap2c1* compared with wild-type or AP2C1-oe plants. AP2C1-oe plants showed reduced JA accumulation that was repeatedly observed. Enhanced JA accumulation due to loss of AP2C1 could be reversed by complementation of the *ap2c1* line with the cDNA coding for AP2C1 under the control of its own promoter (see Supplemental Figure 6 online).

To elucidate whether AP2C1 function affects wound- and pathogen-induced gene expression, semiquantitative RT-PCR was performed for the marker gene defensin (*PDF1.2*), which is induced after pathogen attack and wounding via JA- and ET-dependent signaling pathways (Thomma et al., 2002; Glazebrook, 2005). The kinetics of *PDF1.2* transcript accumulation in re-

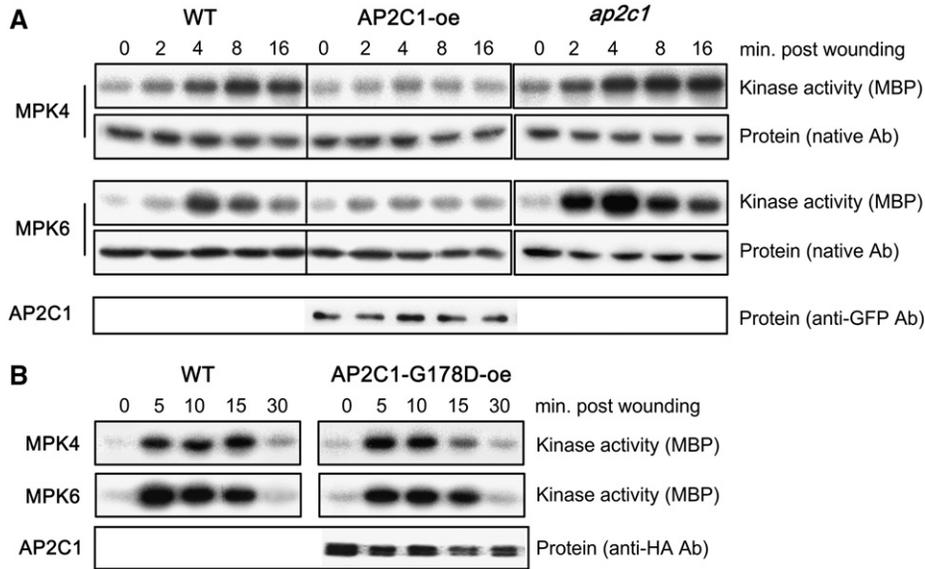
sponse to wounding was analyzed in plants (Figure 8). *PDF1.2* was already expressed at low levels prior to wounding in *ap2c1* plants, as compared with stepwise upregulation in the wild type after wounding. By contrast, accumulation of *PDF1.2* transcript was delayed in AP2C1-oe plants by ~12 h and was generally attenuated compared with the wild type.

Taken together, these data suggest a role for AP2C1 in regulating the levels of ET and JA as well as the expression pattern of *PDF1.2* upon wounding.

## DISCUSSION

### AP2C1 Acts as a MAP Kinase Phosphatase in Planta

AP2C1 contains all features of a PP2C phosphatase, including the conserved catalytic domain with the characteristic 11 subdomains as well as a unique N-terminal domain hosting a putative MAP kinase interaction motif (KIM) (Schweighofer et al., 2004). *Arabidopsis* PP2Cs studied so far have been found to be involved in processes other than MAPK dephosphorylation (Stone et al., 1994; Williams et al., 1997; Li et al., 1999; Merlot et al., 2001; Cherel et al., 2002; Shah et al., 2002; Ohta et al., 2003; Yu



**Figure 4.** Wound Activation of MPK4 and MPK6 in Plants with Altered AP2C1 Expression.

**(A)** MAPKs activities and protein amounts in leaves of wild-type, AP2C1-overexpressing (AP2C1-oe), and *ap2c1* mutant plants in response to wounding. Analysis of line #640.1 is shown (similar data were obtained with lines #640.2 and #591.4). MAPKs were immunoprecipitated for kinase assays or immunodecorated on blots with isoform-specific antisera. AP2C1 amounts were detected with anti-GFP antibodies.

**(B)** MAPKs activities are not altered in plants overexpressing AP2C1-G178D in comparison with the wild type. Analysis of line #789.5 is represented (similar results were obtained on line #789.6). AP2C1 was detected with an anti-HA antibody.

et al., 2003; Saez et al., 2004; Rienties et al., 2005; Song and Clark, 2005). AP2C1 therefore represents an interesting and unusual example of a PP2C phosphatase that bears a KIM and interacts with MAPKs.

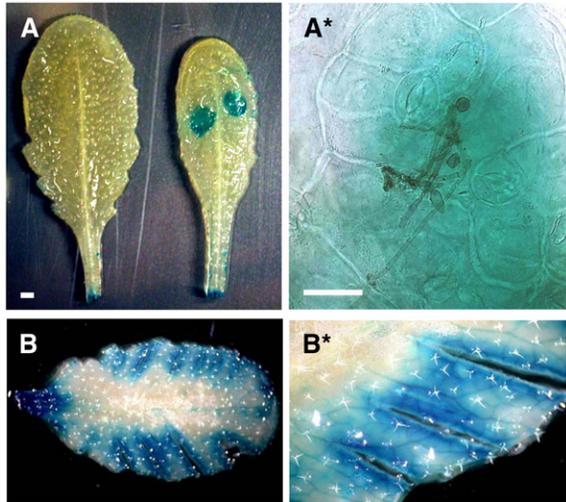
MPK6 and MPK4 but not other MAPK(K)s interaction with AP2C1 in two-hybrid assays (Figure 1A; see Supplemental Figure 1 online) is consistent with AP2C1 inactivation of MPK4/MPK6 but not of MPK3 in transient assays (Figure 3). That these interactions are functional is shown by the formation of complexes involving AP2C1 and MPK6 in plant extracts (Figure 2A) and the strong inhibition of MPK6 and MPK4 activation during wounding of plants overexpressing AP2C1 (AP2C1-oe; Figure 4A). The nucleocytoplasmic localization of the phosphatase-MAPK complexes (Figure 2B) is consistent with the proposed shuffling of the MAPKs to the nucleus upon stress (Ahlfors et al., 2004) and with the involvement of MPK6 in the regulation of gene expression (Asai et al., 2002). Finally, MAPK activities appear to be altered in AP2C1-oe and *ap2c1* plants. The inactivation of the MAPKs requires the catalytic activity of the phosphatase, as the overexpression of a mutated AP2C1 phosphatase (AP2C1-G178D) did not influence the MAPK activities (Figure 4B). Somewhat higher and more sustained wound activation of MAPKs in plants in the absence of AP2C1 suggests that this phosphatase regulates the level and duration of MAPK activation. On the other hand, MAPK inactivation was not completely abolished in *ap2c1* plants, suggesting functional redundancy with other phosphatases, possibly DSPs and/or other KIM-containing members in cluster B of the PP2C family. AP2C1 action is very likely linked to specific environmental conditions because unstressed AP2C1-oe and *ap2c1* plants appear phenotypically normal.

### The *Arabidopsis* PP2C Phosphatase AP2C1 Contains a Functional KIM

Substrate selectivity of mammalian MKPs (both DSPs and PTPs) is determined by specific protein-protein interactions mediated through KIMs (Tanoue and Nishida, 2003). Here, it was found that KIMs are functional elements in plant MAPK/PP2C interactions, as an intact KIM in AP2C1 is essential for protein-protein interaction in yeast cells (Figure 1B). The crystal structure analysis of human PP2C $\alpha$  reveals that the catalytic and noncatalytic regions form two separated domains, and this feature appears to be conserved among PP2Cs (Das et al., 1996), implying that these phosphatases may have evolved by fusion of coding regions for a catalytic phosphatase and various docking extensions. So far, KIM-containing PP2Cs have been found only in plants. Whereas the interaction of MPK6 and MPK4 with AP2C1 in yeast cells required a functional KIM, the KIM-defective AP2C1-K98A,R99Q did appear to interact in protoplasts tested by BiFC (data not shown), possibly due to additional factors facilitating this interaction in plant cells.

### AP2C1 Transcription Is Induced by Stress and Modulates Plant Disease Resistance

AP2C1 may contribute to local crosstalk between abiotic/biotic stress signaling, as AP2C1 is induced early after wounding and infection with the necrotrophic fungus *B. cinerea* (Figure 5). Transcriptomic data showed that AP2C1 is induced after wounding (Cheong et al., 2002), elicitor treatment (Navarro et al.,



**Figure 5.** AP2C1 Induction by Abiotic/Biotic Stress.

(A) Histochemical localization of the activity of the GUS reporter gene transcriptionally fused to 1.3 kb of 5'-upstream sequence of AP2C1 by *B. cinerea* conidia germinating on leaves (mock-treated leaf on the left). (B) Induction of GUS expression in leaves 30 min after wounding. Representative experiments were performed on line #557.2. Bars = 1 mm (images on the left) and 20  $\mu$ m (images on the right).

2004) (therein designated as At ACRE284a), and a series of abiotic and biotic stresses (Zimmermann et al., 2004; <https://www.geneinvestigator.ethz.ch>).

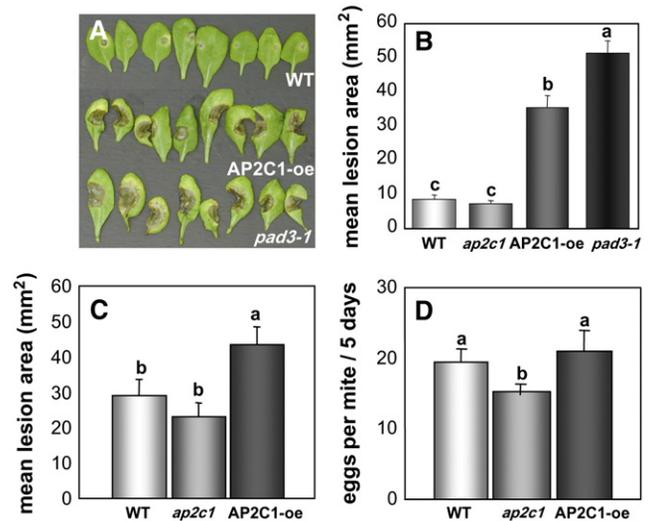
Resistance to *B. cinerea* in *Arabidopsis* is influenced by several defense mechanisms, including some that are MAPK dependent (Thomma et al., 1999; Asai et al., 2002; Alonso et al., 2003; Nandi et al., 2003; Berrocal-Lobo and Molina, 2004). Indeed, there are indications that both of the AP2C1 targets demonstrated here, MPK4 and MPK6, are positively involved in disease resistance toward necrotrophic pathogens, which relies mainly on ET- and JA-regulated defense responses (Asai et al., 2002; Andreasson et al., 2005; Glazebrook, 2005; Brodersen et al., 2006). Overexpression of wild-type AP2C1 enhances susceptibility to *B. cinerea*. Both the severity of disease symptoms (expressed as mean lesion size) and the incidence of disease (expressed as percentage of successful infections) were increased in transgenic plants compared with the wild type and even more when compared with *ap2c1* plants (Figure 6). Thus, AP2C1-oe plants are both more susceptible to penetration and to colonization by *B. cinerea* than wild-type and *ap2c1* plants. The level of acquired susceptibility of AP2C1-oe is similar to that of the *pad3-1* mutant, which is more susceptible to *B. cinerea* infection than wild-type *Arabidopsis* and fails to accumulate the phytoalexin camalexin (Glazebrook, 2005). However, accumulation of camalexin in AP2C1-oe and *ap2c1* plants is similar to the wild type (data not shown).

#### Effect of AP2C1 on Plant Hormone Production and Induction of the Stress Marker PDF1.2

Protein phosphorylation/dephosphorylation controls ET production (Wang et al., 2002; Alonso and Stepanova, 2004; Guo and Ecker, 2004). The MKK4-MPK6 pathway positively regulates

ET (Takahashi et al., 2007), and MPK6 regulates ET biosynthesis through phosphorylation-mediated 1-aminocyclopropane-1-carboxylic acid synthase stabilization (Liu and Zhang, 2004). The data presented here support the model of an initiation of ET synthesis downstream of MAPK activation, as AP2C1-oe plants with reduced MPK6 and MPK4 activities show significant reduction of wound-induced ET levels (Figure 7A). The constant presence of the active phosphatase, preventing normal levels of MAPK activity, could lead to reduced phosphorylation of MAPK targets, among which at least one is a 1-aminocyclopropane-1-carboxylic acid synthase family member.

Wound signal transduction by the octadecanoid pathway and the crucial role for JA in plant responses to wounding is well established (Reymond et al., 2000; Liechti et al., 2006), but little is known about the regulation of JA levels. Here, it was found that a PP2C, which negatively regulates MPK4/6, is also responsible for the regulation of JA levels upon wounding of *Arabidopsis* plants (Figure 7B). MPK4 modulates the antagonism between SA and ET/JA signaling (Petersen et al., 2000; Brodersen et al., 2006) and is a positive regulator of JA-induced signaling (Petersen et al., 2000). In light of the results obtained here on *ap2c1* plants, inactivation of either (or both) MAPKs after wounding by AP2C1, or an effect of AP2C1 on additional targets, seems essential for the regulation of JA synthesis. Recently, Takahashi et al. (2007) showed that MPK6 (but not MPK4) is activated by JA through

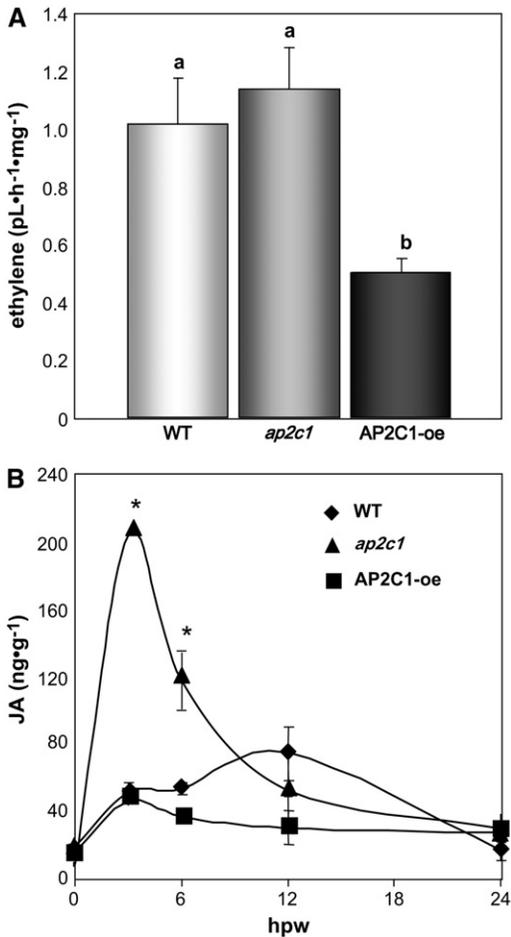


**Figure 6.** Effect of AP2C1 on Disease Resistance.

(A) and (B) Necroses and mean lesion area on leaves 48 h after inoculation with conidia of *B. cinerea*.

(C) Mean necrotic areas 48 h after inoculation with *B. cinerea* culture. Different letters on top of the bars indicate statistically significant differences between means (standard errors are reported). The results are from one representative of three blind experiments and are averages of ~100 leaves from 10 to 15 plants per line (lines #640.1 and #640.2 were analyzed, and the results of #640.2 are presented).

(D) Spider mite reproductive performance. Adult female spider mites (five per plant) were allowed to produce eggs for 5 d on 20 plants per genotype (results from #640.1 are shown). The average number of eggs produced per female on each genotype is shown, along with standard errors. Different letters above bars denote significant differences ( $P < 0.05$ ).

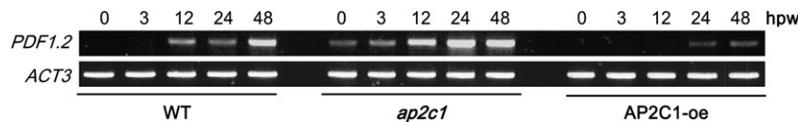


**Figure 7.** Effect of AP2C1 on Wound-Induced Hormone Production.

(A) Production of ET in leaves of AP2C1-oe and *ap2c1* plants after wounding. Approximately 50 leaves from four plants/line were wounded, and accumulated ET was measured after 24 h. Lines #640.1 and #640.2 were analyzed, and results from #640.1 are shown.

(B) JA levels in wounded leaves of wild-type, AP2C1-oe (line #640.2), and *ap2c1* plants. Standard errors are indicated, and asterisks denote significant differences among genotypes for a given time point ( $P < 0.05$ ). hpw, hours post-wounding.

MKK3 and that one of the gene targets upregulated by the MKK3-MPK6 pathway is At LOX2, encoding a chloroplast-targeted lipoxygenase that may be involved in the biosynthesis of JA (Bell et al., 1995). Whether MAPKs participate in the regulation of JA synthesis downstream of JA perception requires further study.



**Figure 8.** Marker Gene Expression in AP2C1-oe and *ap2c1* Lines.

Expression of the stress marker *PDF1.2* in AP2C1-oe (represented by line #640.2) and *ap2c1* plants after wounding in comparison with the wild-type as detected by semiquantitative RT-PCR using gene-specific primers. Actin (*ACT3*) was used as a control.

Increased transcript levels of the plant defensin *PDF1.2a* in the *ap2c1* lines indicate that abnormal expression may occur due to misregulated kinase activities in the absence of the phosphatase (Figure 8). In support of our findings, the JA-activated MKK3-MPK6 module positively regulates *PDF1.2* (Takahashi et al., 2007). At the same time, MPK6 was described as a negative regulator of ET-induced *PDF1.2* transcription (Menke et al., 2004), and MPK4 was reported as a positive regulator of this stress marker after JA treatment (Petersen et al., 2000). Our results indicate that the expression of *PDF1.2* upon wounding is influenced by AP2C1, in agreement with the model proposed by Brodersen et al. (2006).

### Effect of AP2C1 on Resistance to Pathogens

The increased sensitivity to *B. cinerea* correlates well with the delayed *PDF1.2* mRNA accumulation and strong reduction of ET production in AP2C1-oe lines (Figures 6A to 6C and 7A). On the other hand, increased *PDF1.2* transcript and JA accumulation in *ap2c1* plants correlates well with enhanced resistance to spider mites. We could only detect phenotypic differences between AP2C1-oe, *ap2c1* mutant, and wild-type plants under specific stress conditions: wounding, herbivore attack, and challenge with certain pathogens. Importantly, MPK4/MPK6 activation and production of JA were only transiently altered in the *ap2c1* mutant compared with the wild type, in parallel with moderate effects on kinase activity kinetics in AP2C1-oe plants. The observations are consistent with our finding that AP2C1 association with MPK6 is stronger after wounding. These data indicate that AP2C1 is a signal-dependent modulator of MAPK signaling pathways, and under some circumstances, AP2C1 activity may control the amount of damage the plant incurs in response to biotic challenge through its regulation of the MAPK pathway. Compared with the wild type, no variation in growth of virulent and avirulent *Pseudomonas syringae* strains was observed in AP2C1-oe and *ap2c1* plants, in which the levels of free and conjugated SA were not significantly altered (data not shown), suggesting that AP2C1 does not participate in the regulation of SA biosynthesis. However, its putative substrate MPK4 was reported to do so (Petersen et al., 2000). This apparent discrepancy might be linked to different physiological effects in the absence of MPK4 in the *mpk4* null line or to differences between the genetic backgrounds to which the data refer (Landsberg *erecta* ecotype for the *mpk4* null mutant of Petersen et al. [2000] versus Columbia-0 for this study).

Plant-eating insects induce accumulation of defense compounds in a JA-dependent manner, such as secondary metabolites (Kliebenstein et al., 2005) and proteinase inhibitors (Zavala et al., 2004). Constitutively high levels of JA in plants promote

resistance to several herbivores (Li et al., 2002) in contrast with JA biosynthesis mutants, which are highly susceptible (Bell et al., 1995; Li et al., 2002, 2004; Halitschke and Baldwin, 2003; Devoto et al., 2005). *T. urticae*, an extremely polyphagous, herbivorous spider mite, which is recorded from >900 plant species (Egas et al., 2003), causes more feeding damage and has higher reproductive performance on JA mutants than on wild-type plants, with resistance being restored through the addition of exogenous JA (Li et al., 2002; Ament et al., 2004). In our study, the enhancement of wound-induced JA levels in *ap2c1* plants (Figure 7B) correlates well with the marked reduction of *T. urticae* reproductive performance (–25%) on the same mutant (Figure 6D), suggesting a connection between PP2C-regulated stress signaling, JA stress, and plant defense against herbivores.

Taken together, our results demonstrate a role for AP2C1 as a key player in the regulation of MPK4/6 activities upon a stress encounter, in the regulation of wound-induced hormonal and transcriptional pathways and in innate immunity. A deeper understanding of the control of JA levels and of MAPK pathways is a prerequisite for uncovering plant mechanisms of stress perception and their responses and adaptation to the environment and may serve in future improvements of stress tolerance traits in crops. Indeed, normal growth of plants with modified AP2C1 levels highlights the possibility of obtaining desired traits without deleterious consequences to plants.

## METHODS

### Molecular Cloning and Vector Construction

The *AP2C1* cDNA was amplified by PCR from an *Arabidopsis thaliana* cDNA library (Minet et al., 1992) and cloned into pBluescript SK+. For yeast two-hybrid analysis, *AP2C1* cDNA was cloned into pBD-GAL4cam (Stratagene), pBTM116 (Vojtek et al., 1993), or pGAD424 (Clontech). MAPKs and MAPKKs for yeast two-hybrid analysis were cloned as described previously (Teige et al., 2004). AP2C1-K98A, AP2C1-K98A,R99Q, and AP2C1-G178D were created using a site-directed mutagenesis kit (Stratagene). *AP2C1* was cloned into pGreenII0029 (Hellens et al., 2000) downstream of the cauliflower mosaic virus 35S promoter and tagged with a 9-mer c-myc epitope, triple HA, or sGFP(S65T) (Chiu et al., 1996). AP2C1-G178D was tagged with a triple HA epitope. The cDNAs of *ABI2* and *HAB1* (Meskiene et al., 2003) were tagged with a 9-mer c-myc epitope and cloned into the same vector as *AP2C1*. Plasmids expressing HA-tagged MPK3, MPK4, MPK6, and  $\Delta$ ANP1 were described by Asai et al. (2002). The 1.3-kb *AP2C1* promoter region was cloned by PCR using BAC clone f23f1 (ABRC), fused with the *GUS* reporter, and cloned into pGreenII0029. For *ap2c1* mutant complementation, the *AP2C1* promoter region was fused with *AP2C1* cDNA and cloned into pGreenII0029. For in vitro studies, the cDNAs of AP2C1, ABI2, and HAB1 were cloned into pGEX-4T-1. The pGEX-MPK6-K92M,K93R and MKK2-T220E,T226E constructs have been described previously (Teige et al., 2004). For BiFC, the split YFP components were cloned by PCR from pUC-SPYNE and pUC-SPYCE vectors (Walter et al., 2004) and inserted into pRT100-MPK4 and pRT100-MPK6 vectors (Teige et al., 2004). Original pUC-SPYNE/pUC-SPYCE vectors were used as controls.

### Yeast Two-Hybrid Library Screen and Interaction Assays

The yeast two-hybrid screen of an *Arabidopsis* cDNA library (Minet et al., 1992) was performed in the yeast strain PJ-69A (James et al., 1996) with the pBD-GAL4cam-AP2C1 bait plasmid according to Meskiene et al.

(2003). Interaction assays with yeast strain L40 (Vojtek et al., 1993) were done as described (Teige et al., 2004).

### Protoplast Transient Expression and Immunocomplex Assays

Transient expressions using *Arabidopsis* suspension protoplasts were performed according to Meskiene et al. (2003). Immunocomplex assays were performed as described (Cardinale et al., 2002; Meskiene et al., 2003).

### Expression and Purification of Recombinant Proteins

Recombinant MPK6, MKK2, AP2C1, ABI2, and HAB1 were expressed as glutathione *S*-transferase (GST) fusion proteins as previously described (Kiegerl et al., 2000). The protein concentrations of the recombinant proteins were determined with the Bio-Rad detection system using BSA as a standard, and the purity of the protein fractions was determined by Coomassie Brilliant Blue staining after 10% SDS-PAGE.

### Measurement of Phosphatase Activity

Phosphatase activity was measured using  $^{32}$ P-labeled casein (12.5  $\mu$ g/reaction) or  $^{32}$ P-labeled GST-MPK6-K92M,K93R (1  $\mu$ g/reaction) as a substrate and incubated with 0.1  $\mu$ g/reaction of each PP2C at 30°C for 30 min in triplicates (Meskiene et al., 2003). GST-MPK6 K92M,K93R was labeled by GST-MKK2 T220E,T226E (Teige et al., 2004).

### Analysis of the AP2C1 Mutant Line

The putative null line for AP2C1 (SALK\_065126) was analyzed by PCR using a T-DNA primer (LBa1 5'-TGGTTCACGTAGTGGCCATCG-3'; <http://signal.salk.edu>) and gene-specific primers AP1-fwd (5'-CTTGCTCCGTCGCCGTATGTAATTCTCCGG-3') and AP2C1rev (5'-CATCAGACGAGCCTCGTGAAGCAGATAAATCG-3'). Putative homozygous plants were subjected to DNA gel blotting using the 0.74-kb *AspI*-*EcoRI* fragment from the pBI101 vector (Clontech) as a left border-specific probe. Labeling was performed with the Genes Images Labeling Module (Amersham) according to the manufacturer's instructions.

### Plant Material and Generation of Transgenic Lines

*Arabidopsis* ecotype Columbia (Col-0) was used as genetic background. Six-week-old plants grown under short-day conditions (16 h dark/8 h light) were used for the experiments (kinase assays, GUS stainings, pathogen assays, immunoprecipitation, RT-PCR, and ET and JA measurements). pGreen vectors were transferred in *Agrobacterium tumefaciens* strain GV3101::pMP90 (Koncz and Schell, 1986) via electroporation and verified by PCR. Plants were transformed using the floral dipping method (Clough and Bent, 1998) and transformed seeds selected on kanamycin-containing Murashige and Skoog plates (Murashige and Skoog, 1962). Nine independent transgenic AP2C1-overexpressing lines were generated, and three of these lines were analyzed in MAP kinase activity assays (#640.1, #640.2, and #591.4). Two lines were selected (#640.1 and #640.2) and used for further analyses. *ap2c1* plants were complemented with AP2C1 promoter-driven cDNA expression (four lines were isolated, and two of these lines, #ap2c1-644.1 and #ap2c1-644.2, were analyzed). Six lines overexpressing AP2C1-G178D were isolated, and two lines (#789.5 and #789.6) were analyzed. Five lines expressing AP2C1-GUS were generated, and three lines were analyzed for induced GUS expressions (#557.1 to #557.3).

### Wounding of Leaves, Histochemical GUS Assay, and Kinase Assays

Wounding of leaves and kinase assays were performed according to Meskiene et al. (2003). Histochemical GUS reporter assay was performed according to Jefferson et al. (1986).

## RNA Isolation and RT-PCR

Total RNA from leaves was isolated with the RNeasy plant mini kit (Qiagen) according to the manufacturer's instructions. RT-PCR was performed (Teige et al., 2004) using the following primers: ACT3 (At2g37620) fwd, 5'-ATGGTTAAGGCTGGTTTTGC-3'; ACT3 rev, 5'-AGCACAATACCGGTAGTACG-3'; PDF1.2 (At5g44420) fwd, 5'-TTGCTGCTTTGACGACACCGG-3'; PDF1.2 rev, 5'TAGATTTAACATGGGACGTAACAG-3'; AP2C1 (At2g30020)fwd, 5'-TCGGCCGCTGTGGCTGCG-3'; AP2C1rev, 5'-CAT-CAGACGAGCCTCGTGAAGCAGATAAATCG-3'.

## Disease Assays

*Arabidopsis* plants were grown in soil and inoculated by placing an agar plug (3 mm in diameter) on top of fully expanded leaves. Seven-day-old *Botrytis cinerea* cultures grown at room temperature on potato dextrose agar plates were used to this purpose. Microwounding was performed to facilitate fungal infection by poking three holes in the leaf with a needle before inoculation. Noncolonized potato dextrose agar plugs were used as a negative control. A minimum of 10 plants per line and five leaves per plant were inoculated. Disease severity was evaluated 48 h after inoculation by calculating mean areas in mm<sup>2</sup>. Statistical significance was calculated by the least significant difference method using Statistica software (StatSoft) and significance attributed if  $P < 0.05$ . Blind experiments were performed on two independently transformed plant lines (#640.1 and #640.2) three times, with similar results. Leaf infection with conidia of *B. cinerea* was performed as described (Zimmerli et al., 2001).

## Fecundity Experiments

Spider mites (*Tetranychus urticae*) were reared on detached lima bean (*Phaseolus lunatus*) leaves in a climate room with a 16/8-h light regime, 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 60% RH, 22°C. Twenty plants of each *Arabidopsis* genotype were grown in a different climate room under the same conditions. For the fecundity experiment, five adult female spider mites were randomly dispersed over each *Arabidopsis* plant. After 5 d, plants were taken from the soil using tweezers, and each leaf was inspected under a stereomicroscope for eggs and mites. The number of eggs per female was calculated per plant. Data were analyzed by analysis of variance and the Fisher post hoc test.

## ET and JA Measurements

For ET measurements, fully expanded leaves were wounded, transferred into 20-mL vials containing 2 mL of half-strength Murashige and Skoog medium, and air-tight sealed. After 24 h, 250  $\mu\text{L}$  of the gas phase were taken from the headspaces of vials and analyzed by gas chromatography-flame ionization detection. ET production was calculated per hour and milligram of fresh tissue. For JA measurement, 0.5 g (fresh weight) of leaves were harvested at 0 to 24 h after wounding, frozen in liquid nitrogen, and stored at -80°C. JA was extracted and measured by gas chromatography-mass spectrometry as described (Penninckx et al., 1996). For both hormones, statistical significance was evaluated by the least significant difference method and significance attributed if  $P < 0.05$ .

## Accession Number

The *Arabidopsis* Genome Initiative locus identifier for AP2C1 is At2g30020.

## Supplemental Data

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

**Supplemental Figure 1.** Interaction Assay of AP2C1 with At MKKs in the Yeast Two-Hybrid System.

**Supplemental Figure 2.** Analysis of the *ap2c1* Mutant Line.

**Supplemental Figure 3.** Comparison of Wound Activation of MAP Kinases in Wild-Type, *ap2c1*, and *ap2c1* Complemented Plants.

**Supplemental Figure 4.** Effect of AP2C1 on Disease Resistance.

**Supplemental Figure 5.** Complementation of *ap2c1* Plants Enhances Spider Mite Reproductive Performance.

**Supplemental Figure 6.** JA Production in Wild-Type, *ap2c1*, and *ap2c1* Complemented Plants.

## ACKNOWLEDGMENTS

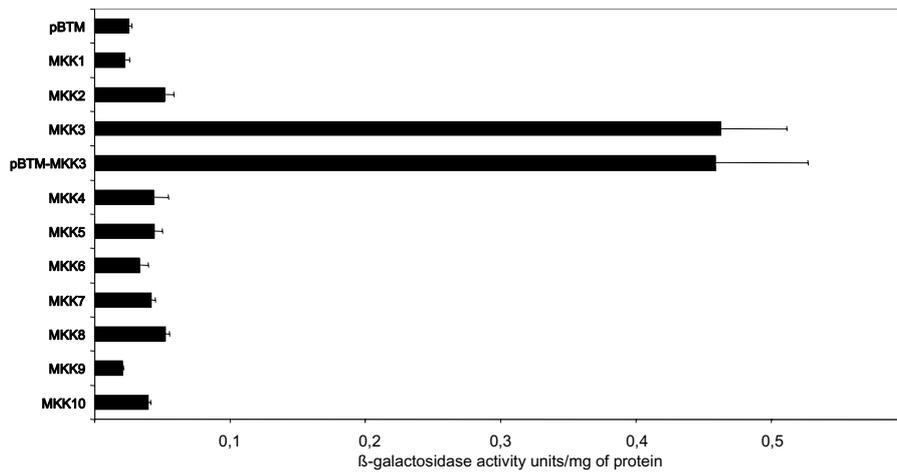
We thank B. van Zaal for the cDNA library, C. Koncz for suspension culture, J. Sheen for ANP1, MPK3, MPK4, and MPK6 expression plasmids, J. Kudla for split YFP constructs, K. Mockaitis for comments on the manuscript, R. Schweyen and A. Barta for lab space for I.M.'s group, M. Baccarini for sharing an office with I.M., A. Auer and A. Belokurov for technical assistance, and the Salk Institute Genomic Analysis Laboratory for the T-DNA insertion mutant. This work was supported by grants from the Austrian Science Fund and the Charlotte Bühler Habilitation fellowship to I.M. and by the European Union project Growth, Vigour, Environment. Further support was from the British Council, Austrian, and Italian Ministries of Education and Science and from the Lithuanian Science Council.

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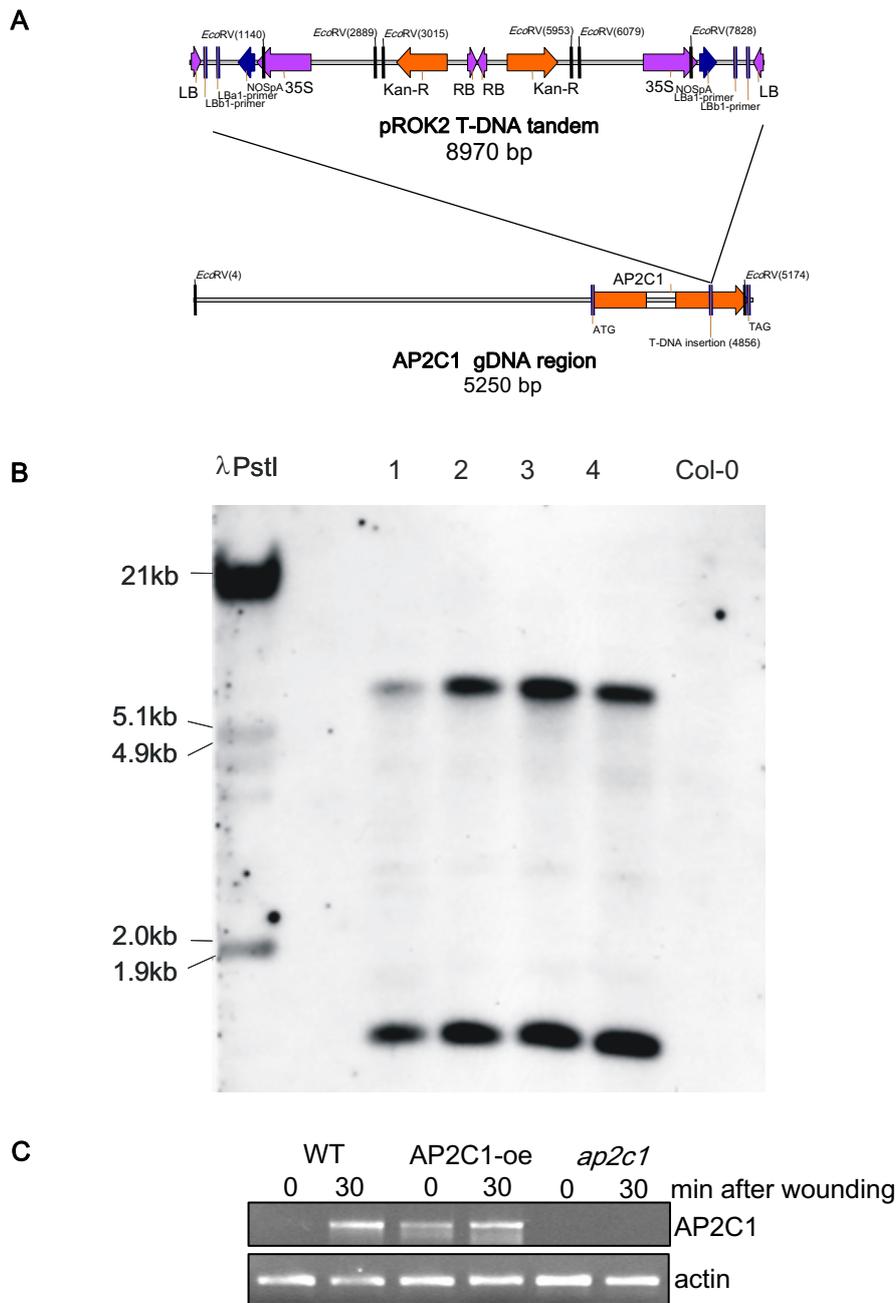
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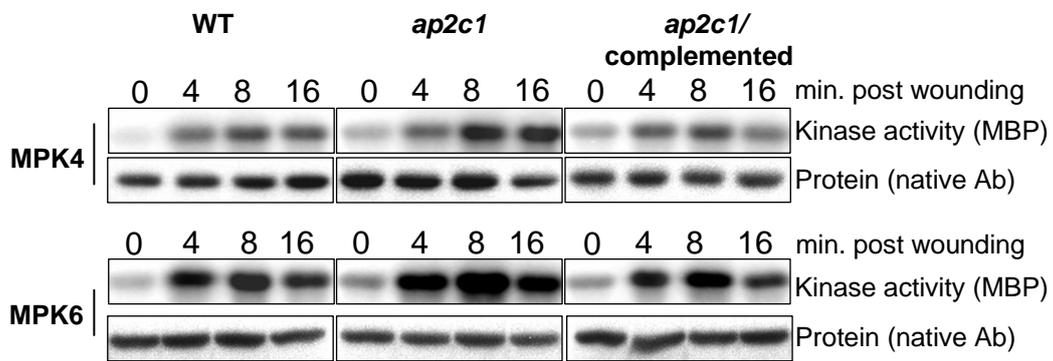
Suppl. Figure 1: **Interaction assay of AP2C1 with AtMKKs in yeast two hybrid system.** pGAD424-AP2C1 and pBTM-MKKs were transformed into the L-40 yeast strain. The activation of *lacZ* reporter gene was analyzed by  $\beta$ -galactosidase assay. MKK3 is autoactive in yeast two hybrid assay. The pBTM-MKK3 bar shows  $\beta$ -galactosidase activation by co-expressing pBTM-MKK3 and empty pGAD vector

Suppl. Figure 1



Suppl. Figure 2: **Analysis of *ap2c1* mutant line**

**(A)** Schematic illustration of T-DNA insertion within AP2C1 gene. Analysis by PCR using left border (LB) and gene-specific primers proved the presence of a tandem T-DNA within exon 2 of AP2C1 gene. **(B)** Analysis of the *ap2c1* mutant line: DNA from four individual plants derived from a putative null line identified in the SALK collection was analyzed by Southern blotting. After *EcoRV* digest and hybridizing with a labeled LB-specific probe two bands of the predicted sizes (5.9 and 1.4 kb) showed the presence of one tandem T-DNA in the genome. **(C)** Detection of AP2C1 transcript after wounding using semi-quantitative RT-PCR.



Suppl. Figure 3: **Comparison of wound activation of MAP kinases in WT, *ap2c1* and *ap2c1* complemented plants**

Wound-induced MPK4 and MPK6 activation in *ap2c1* complemented plants is restored to the level of the WT. Kinase assays and western blot analysis were performed as in Figure 4A. Results shown were obtained on line #*ap2c1*-644.1 and are representative also for the results of line #*ap2c1*-644.2.



WT



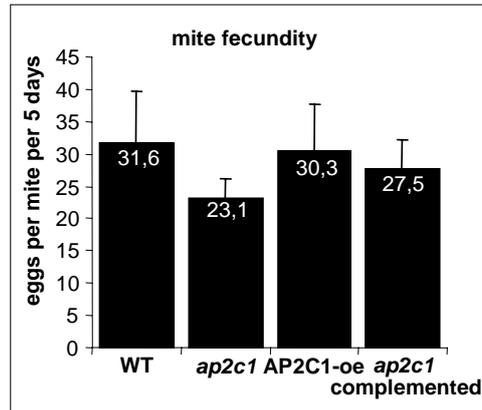
*ap2c1*



AP2C1-oe

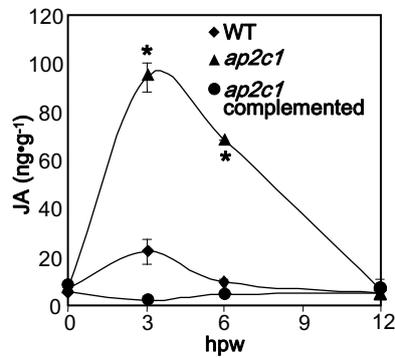
Suppl. Figure 4: **Effect of AP2C1 on disease resistance**

Representative pictures show necroses on leaves of WT, *ap2c1* and AP2C1-oe plants (line #640.2) infected with *B. cinerea* mycelium applied on agar plugs 4 days post inoculation.



Suppl. Figure 5: **Complementation of *ap2c1* plants enhances spider mite reproductive performance**

Spider mite reproductive performance was analyzed as in Figure 6D. Complementation of *ap2c1* mutation enhances the reduced fecundity of spider mites in the *ap2c1* mutant line. Mean values are indicated on corresponding columns; representative results shown were obtained on line #*ap2c1*-644.2 and AP2C1-oe line #640.2.



Suppl. Figure 6: **JA production in WT, *ap2c1* and *ap2c1* complemented plants**  
Increased JA accumulation upon wounding in *ap2c1* plants is abolished by expression of AP2C1 under the control of its own natural promoter. JA levels in wounded leaves of WT, *ap2c1* and *ap2c1* complemented plants (line #ap2c1-644.2) are shown. Standard errors are indicated, statistic analysis was performed by comparing mean values of different lines for any given time-point.