Dual control of MAPK activities by AP2C1 and MKP1 MAPK phosphatases regulates defence responses in Arabidopsis

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Highlight

Double MAPK phosphatase mutant plants ap2c1 mkp1 exhibit constitutive, autoimmune-like stress responses, dependent on their substrate MAPKs MPK3 and MPK6.

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Abstract
Mitogen-activated protein kinase (MAPK) cascades transmit environmental signals and induce stress and defence responses in plants. These signalling cascades are negatively controlled by specific phosphatases of the type 2C Ser/Thr protein phosphatase (PP2C) and dual-specificity phosphatase (DSP) families that inactivate stress-induced MAPKs; however, the interplay between phosphatases of these different types has remained unknown. Our work reveals that different Arabidopsis MAPK phosphatases, the PP2C-type AP2C1 and the DSP-type MKP1, exhibit both specific and overlapping functions in plant stress responses. Each single mutant and ap2c1 mkp1 double mutant displayed enhanced stress-induced activation of MAPKs MPK3, MPK4, and MPK6, as well as induction of a set of transcription factors. Moreover, ap2c1 mkp1 double mutants show an autoimmune-like response, associated with elevated levels of the stress hormones salicylic acid and ethylene, and of the phytoalexin camalexin. Interestingly, this phenotype is reduced in ap2c1 mkp1 mpk3 and ap2c1 mkp1 mpk6 triple mutants, suggesting that the autoimmune-like response is due to MAPK misregulation. We conclude that the evolutionarily distant MAPK phosphatases AP2C1 and MKP1 contribute crucially to the tight control of MAPK activities, ensuring appropriately balanced stress signalling and suppression of autoimmune-like responses during plant growth and development.

Keywords
ANAC, AP2C1, Arabidopsis, DSP, MAPK, MKP1, PP2C, WRKY.
Introduction
Reversible protein phosphorylation is one of the most commonly used mechanisms for the molecular transmission of stress signals and developmental cues. This mechanism is based on the opposing actions of protein kinases and protein phosphatases. Mitogen-activated protein kinases (MAPKs) are highly conserved major components of developmental and stress signalling cascades in eukaryotes. MAPKs are activated by upstream MAPK kinases via phosphorylation of Thr and Tyr within their activation loop. This activation eventually leads to the reprogramming of cellular activities, including the modulation of gene expression, to generate appropriate responses. The activation of MAPKs does not represent a simple on/off switch, as both the magnitude and duration of activation are crucial for determining the signalling outcome (Marshall, 1995). Prolonged or constant activation of a MAPK cascade can have detrimental effects as illustrated by the hypersensitive response (HR)-induced cell death in plants expressing a constitutively active MAPK kinase version (Ren et al., 2002; Liu et al., 2007). Thus, negative regulation and inactivation mechanisms are important for the correct cellular response. Specific protein phosphatases can dephosphorylate and thereby inactivate MAPKs. As dual phosphorylation of the Thr-X-Tyr motif in the activation loop is required for MAPK activation (Caunt and Keyse, 2013), dephosphorylation of either phospho-amino acid residue inactivates the MAPK and inhibits downstream signalling. Interestingly, this inactivation can be accomplished by evolutionarily distant protein phosphatases, including PP2C-type MAPK phosphatases (Schweighofer et al., 2004; Schweighofer et al., 2007; Fuchs et al., 2013) and PTP-type dual specificity (Tyr and Ser/Thr) phosphatases (DSPs) (Bartels et al., 2010; Jiang et al., 2018). However, their interplay is presently unknown.

*Arabidopsis thaliana* DSP-type MAPK phosphatase 1 (MKP1) interacts with the stress-responsive MAPKs MPK3, MPK4 and MPK6, and controls their activities (Ulm et al., 2002; Bartels et al., 2009; Anderson et al., 2011). The *mkp1* knockout mutant is hypersensitive to genotoxic stress, including UV-B radiation (Gonzalez Besteiro and Ulm, 2013a, b), but is more resistant than wild type (WT) to the virulent bacterial pathogen *Pseudomonas syringae pv. tomato* (*Pto*) (Bartels et al., 2009; Anderson et al., 2011; Anderson et al., 2014; Escudero et al., 2019). Specifically in the Arabidopsis Columbia accession, *mkp1* shows an autoimmune-like growth phenotype dependent on the disease resistance gene homologue *SUPPRESSOR OF npr1-1, CONSTITUTIVE 1* (*SNC1*) and is associated with enhanced...
MAPK activities (Bartels et al., 2009). The phospho-Tyr-specific PTP-type protein phosphatase PTP1 also interacts with MPK6 and MPK3 in transient assays. The mkp1 ptp1 double mutant exhibits upregulation of MPK6-dependent plant defence responses and a further enhanced autoimmune-like phenotype (Bartels et al., 2009).

A group of PP2C-type phosphatases, including AP2C1, interacts with MAPKs and controls their activities (Schweighofer et al., 2007; Umbrasaitė et al., 2010). AP2C1 is induced by wounding and biotic stress, and functions as a negative regulator of MPK3, MPK4 and MPK6 controlling levels of wound-induced jasmonate and ethylene (ET) as well as plant immunity (Schweighofer et al., 2007; Galletti et al., 2011; Sidonskaya et al., 2016; Shubchynskyy et al., 2017). ap2c1 plants do not display obvious developmental phenotypes under standard growth conditions (Schweighofer et al., 2007), implying specific AP2C1 function under stress conditions and contribution of other, presently unknown, MAPK phosphatases for MAPK control in the absence of AP2C1.

Activation of transcription factors (TFs) and changes of gene expression are part of the cellular response to a perceived signal in order to reprogram cellular processes (Rauf et al., 2013). A number of TFs, including WRKY and AP2-domain/ethylene-responsive factor (AP2/ERF) family members, have been suggested or demonstrated to act downstream of MAPKs in plants (Asai et al., 2002; Kim and Zhang, 2004; Menke et al., 2005; Nakano et al., 2006; Popescu et al., 2009; Bethke et al., 2009; Mao et al., 2011; Li et al., 2012; Meng and Zhang, 2013; Guan et al., 2014). Subsequently, these proteins may constitute an important link between pathogen- or wound-induced MAPK signalling and downstream transcriptional reprogramming.

Considering the broad spectrum of signals transmitted by the same MAPKs (Rodriguez et al., 2010; Meng and Zhang, 2013), such as MPK6, it is puzzling how the specificity of the responses for perceived stimuli is generated. The phylogenetic diversity and distinct enzymatic mechanisms of protein phosphatases that are able to inactivate MAPKs support the idea of a contribution of MAPK phosphatases to the versatility and specificity of MAPK networks. Here, we investigate the roles of the phylogenetically distant Arabidopsis MAPK phosphatases AP2C1 and MKP1 and, in particular, their functional redundancies. We show that AP2C1 and MKP1 together repress plant autoimmune-like responses, including salicylic acid (SA) and ET.
accumulation, and early senescence. These observations in the ap2c1 mpk1 mutant are underlined by the misexpression of specific transcription factors, including members of the WRKY, AP2/ERF, and Arabidopsis NAM, ATAF, and CUC (ANAC) families whose expression is – at least partially – mediated by MPK6 and MPK3.

Materials and methods
Plant lines, genetic crosses and growth conditions
All plant lines used in this study were in the Arabidopsis thaliana accession Columbia (Col-0), with mpk1 being an introgression line from a Wassilewskija background (Bartels et al., 2009). The T-DNA insertion line ap2c1 (SALK_065126; Schweighofer et al. (2007)) was crossed with the T-DNA insertion lines ptp1 (SALK_118658) and mpk1, respectively, to generate the ap2c1 ptp1 and ap2c1 mpk1 double mutants. mpk6-2 (SALK_073907) and mpk3-1 (SALK_151594) were used for genetic crosses generating ap2c1 mpk1 mpk6 and ap2c1 mpk1 mpk3. The T-DNA insertion lines ap2c2 (GABI-Kat_316F11) and ap2c3 (SALK_109986) (Umbrasaite et al., 2010) were crossed with mpk1 to generate ap2c2 mpk1 and ap2c3 mpk1 double mutants. Combinatorial mutants were identified in the F2 generation and also confirmed in subsequent generations by PCR genotyping using T-DNA- and gene-specific primers (Schweighofer et al., 2007; Bartels et al., 2009; Umbrasaite et al., 2010; Shubchynskyy et al., 2017). For protein and RNA extraction, as well as for ET and SA measurements, plants were grown on soil for five to seven weeks in a phytotron chamber under short-day conditions (8 h light, 22°C/16 h dark, 20°C cycle).

For experiments at the seedling stage, seeds were surface sterilized and spread on plates containing half-strength MS (Murashige and Skoog) medium (Duchefa), pH 5.7, 1% (w/v) sucrose and 0.7% plant agar (w/v; Duchefa). Seedlings were grown in long-day conditions (16 h light/8 h dark) at 22°C. If indicated, plants were kept in short-day conditions in a tray with closed lid at 24°C with saturating relative humidity.

Ex vivo kinase activity assay and MAPK immunoblotting
Plant protein extraction and the ex vivo kinase assay were performed as described (Schweighofer et al., 2007; Schweighofer et al., 2009) using polyclonal antibodies for immunoprecipitation and myelin basic protein (MBP) as in vitro substrate of immunoprecipitated MAPKs. A master mix containing MBP and γ-ATP was used for
each series of kinase assays to ensure equal substrate distribution to all reactions. Treatment with the pathogen-associated molecular pattern (PAMP) flg22 was performed as described (Shubchynskyy et al., 2017). Signal intensities of phosphorylated MBP were quantified with ImageQuant software (version 5.1; Amersham). MAPK protein amounts were visualised with Sigma antibodies Anti-AtMPK3 (M8318), Anti-MPK4 (A6979) and Anti-AtMPK6 (A7104).

RNA extraction and quantitative reverse-transcription PCR (RT-qPCR)

Total RNA from leaves was isolated with the RNeasy Plant Mini Kit (Qiagen) and treated with TURBO DNA-free DNasel (Ambion) according to the manufacturers’ instructions. RNA integrity was checked on 1% (w/v) agarose gels and the concentration measured before and after DNase I digestion. The absence of genomic DNA was verified by PCR using primers targeting an intron of the control gene At5g65080. cDNA synthesis was performed using First Strand cDNA Synthesis Kit (Thermo Scientific). The efficiency of cDNA synthesis was estimated by RT-qPCR analysis using a primer pair amplifying the 3’ part of the control gene encoding GAPDH and a primer pair amplifying the 5’ part of the same gene. RT-qPCR reactions were performed as described previously (Balazadeh et al., 2008). ACTIN2 was selected as a reference gene for which four replicates were measured in each PCR run, and their average cycle threshold (CT) was used for relative expression analyses. TF expression data were normalized by subtracting the mean ACTIN2 gene CT value from the CT value (ΔCT) of each gene of interest. The expression value in the comparison between different genotypes was calculated using the expression 2^{ΔΔCT}, where ΔΔCT represents ΔCT mutant of interest minus ΔCT control (wild type, WT). For TF expression profiling, an advanced version of an expression profiling platform (Balazadeh et al., 2008) that was originally described by (Czechowski et al., 2004) was used, covering 1,880 Arabidopsis TF genes. Statistical analysis was performed with the JASP software (https://jasp-stats.org; version 0.14.1).

ET measurements, quantification of total SA and camalexin

ET measurements were performed by gas chromatography (Hewlett Packard 5890 Series II) with an Al_{2}O_{3} column (Agilent Technologies). Whole rosettes of 4-week-old plants grown in long-day conditions were taken, leaves wounded, transferred into 20-
mL vials containing 4 mL half-strength MS medium with 0.8% (w/v) plant agar, in order to reduce the volume of the head space, and air-tightly sealed. After 24 h, 100 μL of the gas phase were taken from the vials and analysed by gas chromatography–flame ionization detection (GC-FID). ET production was calculated per hour and milligram of fresh tissue.

Total SA was quantified as described previously (Rozhon et al., 2005) except that 20 μM EDTA was added to the HPLC eluent. Camalexin levels were determined as described previously (Shubchynskyy et al., 2017).

Results

Double ap2c1 mkp1 mutant plants show growth and development defects which are at least partially mediated by MPK6 and MPK3.

To investigate the specific and/or overlapping roles of the MAPK phosphatases AP2C1 and MKP1, we took advantage of the Arabidopsis T-DNA insertion knock-out mutants ap2c1 and mkp1, respectively (Schweighofer et al., 2007; Bartels et al., 2009). Phenotypically, ap2c1 and mkp1 mutant plants did not show any difference compared to WT when grown for up to five weeks under short-day conditions (Fig. 1A). However, long-day mkp1 plants demonstrated altered morphology, such as aberrant leaf development and early senescence, which appeared approximately three weeks after germination (Supplementary Fig. 1A), as described previously (Bartels et al., 2009).

To further analyse AP2C1 and MKP1 functions in plants, we generated a double mutant by genetic crossing. ap2c1 mkp1 plants showed phenotypic differences compared to WT and single mutants. These appeared about two weeks after germination under standard growth conditions in soil in all ap2c1 mkp1 double mutants; first their sizes started differing, and during further growth ap2c1 mkp1 plants revealed more pronounced multiple defects, including severe dwarfism and aberrant leaf development (Fig. 1A and 1B; Supplementary Fig. 1B - G). Four weeks after germination, phenotypic abnormalities became even more evident including early senescence, spontaneous macroscopic lesions and abnormal leaf morphology. These developmental defects were suppressed when plants were grown under conditions of elevated humidity and increased temperature (Supplementary Fig. 2), indicating a dependency on environmental cues. However, during flowering, misshaped inflorescences and strongly reduced fertility were always observed.
(Supplementary Fig. 1E). These phenotypes were specific for \textit{ap2c1 mkp1} plants, as crossing \textit{ap2c1} with \textit{ptp1} did not lead to phenotypic alterations (Fig. 1C) compared with \textit{mkp1 ptp1} (Bartels \textit{et al.}, 2009). Crossing \textit{mkp1} with either of two other clade B AP2C mutants, \textit{ap2c2} or \textit{ap2c3} (Umbrasaite \textit{et al.}, 2010), led to only mild phenotypes compared with the strong defects of \textit{ap2c1 mkp1} plants (Fig. 1B).

Since MPK6 and MPK3 are targets of AP2C1 and MKP1 (Ulm \textit{et al.}, 2002; Schweighofer \textit{et al.}, 2007; Galletti \textit{et al.}, 2011) we addressed the impact of MPK6 and MPK3 on phenotypic aberrations detected in \textit{ap2c1 mkp1} plants. To this goal, triple mutant plants \textit{ap2c1 mkp1 mpk6} and \textit{ap2c1 mkp1 mpk3} were created and their phenotypes were compared with those of \textit{ap2c1 mkp1}. The phenotype of \textit{ap2c1 mkp1 mpk6} plants was more similar to WT than to \textit{ap2c1 mkp1}. The loss of MPK6 suppressed most phenotypic defects observed in \textit{ap2c1 mkp1}, such as extreme dwarfism, aberrant leaf shapes, premature leaf senescence and impaired fertility (Fig. 1A, Supplementary Fig. 3). However, at later developmental stages, \textit{ap2c1 mkp1 mpk6} plants still appeared overall smaller than WT and displayed senescence in the older leaves (Supplementary Fig. 3E and 3M). Similarly, \textit{ap2c1 mkp1 mpk3} plants suppressed the observed \textit{ap2c1 mkp1} phenotypes in early development (Fig. 1A) but to a lesser extent at later developmental stages compared to \textit{ap2c1 mkp1 mpk6} (Supplementary Fig. 3F and 3N). Both, \textit{ap2c1 mkp1 mpk6} and \textit{ap2c1 mkp1 mpk3} developmental phenotypes were suppressed during growth in elevated temperature and increased humidity, as observed with \textit{ap2c1 mkp1} (Supplementary Fig. 2).

Overall, these results suggest that AP2C1 and MKP1 protein phosphatases act partially redundantly and that the presence of at least either gene is necessary for normal plant development. The phenotypes observed in \textit{ap2c1 mkp1} plants are predominantly MPK6- and MPK3-dependent.

**Dual control of stress-induced MAPK activities by AP2C1 and MKP1**

Our previous work has revealed the involvement of AP2C1 in the regulation of MAPK activities induced by wounding, nematode feeding, and PAMPs (Schweighofer \textit{et al.}, 2007; Sidonskaya \textit{et al.}, 2016; Shubchynskyy \textit{et al.}, 2017). To check for a potential overlapping role by MKP1, we firstly analysed MPK3, MPK4 and MPK6 activities after wounding the leaves of WT and of the single mutant plants \textit{ap2c1} and \textit{mkp1}. Kinase activities were assayed after immunoprecipitation from total protein extracts.
using specific antibodies. In agreement with our previous findings (Schweighofer et al., 2007), ap2c1 plants showed higher and sustained wound-induced activities of MPK3, MPK4 and MPK6 compared to WT (Fig. 2). Interestingly, MPK4 activity was more intense and sustained in ap2c1 compared to the mkp1 plants, indicating a specific role of AP2C1 in the regulation of MPK4 during wounding. MPK3 activity in ap2c1 plants was slightly enhanced and more sustained in comparison to WT and mkp1. In mkp1 plants, however, the MPK6 peak activity was shifted to an earlier time point compared to WT. In ap2c1 mkp1 plants we detected strongly and moderately enhanced basal activity of MPK4 and MPK6, respectively, whereas basal activity of MPK3 was not affected in comparison to WT or single mutant lines. The stronger and more sustained wound-induced activation of MAPKs observed in single-mutant plants was additionally enhanced in the double mutant ap2c1 mkp1 (Fig. 2, Supplementary Fig. 4A). The MPK3, MPK4, and MPK6 protein levels were comparable in both mutants and WT (Fig. 2). Treatment of WT, ap2c1 and mkp1 single and double phosphatase mutant plants, as well as ap2c1 mkp1 mpk3 and ap2c1 mkp1 mpk6 triple mutants with the PAMP flg22 led to similar MAPK activation patterns as observed after wounding, with enhanced and prolonged MAPK activities in the ap2c1 mkp1 plants compared to single mutant plants and WT (Supplementary Fig. 4B and 4C).

MKP1 has been reported to be constitutively expressed (Ulm et al., 2002), whereas AP2C1 is transcriptionally responsive to stress (Schweighofer et al., 2007; Sidonskaya et al., 2016). We tested whether reciprocal compensational expression may occur in long-day conditions and thus analysed AP2C1 and MKP1 mRNA levels in mkp1 and ap2c1 mutants, respectively. RT-qPCR analyses showed only very slightly enhanced expression of MKP1 in ap2c1 plants, whereas the expression of AP2C1 was approximately 160% in mkp1 plants compared to WT (Supplementary Fig. 5), suggesting a compensatory transcriptional activation of AP2C1 in the absence of MKP1.

Our results suggest cooperative action and partial redundancy in the regulation of MAPKs by these two evolutionary distant and unrelated MAPK phosphatases.
AP2C1 and MKP1 play partially redundant roles in the control of wound-induced ET synthesis

Enhanced ET production is an early response of plants subjected to biotic/abiotic stresses (Wang et al., 2002; Ju and Chang, 2012). We have previously shown that ectopic expression of AP2C1 suppresses MPK6 activation and wound-induced ET production in plant leaves (Schweighofer et al., 2007). Since both AP2C1 and MKP1 control MPK6 activity, a major determinant in the regulation of ET biosynthesis (Liu and Zhang, 2004; Li et al., 2012), we analysed wound-induced ET amounts in leaves of WT, ap2c1, mkp1, ap2c1 mkp1 and ap2c1 mkp1 mpk6 plants. As reported earlier (Schweighofer et al., 2007), wound-induced ET amounts were similar in ap2c1 and WT (Fig. 3A). However, significantly higher levels of ET accumulated in wounded mkp1 plants and even more so in the ap2c1 mkp1 double mutant (Fig. 3A). Our data suggest a primary role of MKP1 in the control of wound-triggered ET production and that although disruption of AP2C1 alone is not sufficient to alter ET production upon wounding, it contributes significantly to the regulation of ET amounts in the absence of MKP1. Interestingly and in agreement with the overall milder phenotype, wound-induced ET accumulation in ap2c1 mkp1 mpk6 plants was similar to levels detected in WT (Fig. 3A).

The transcriptional regulation of 1-aminocyclopropane-1-carboxylic synthase (ACS) enzymes contributes to control ET production (Li et al., 2012). Therefore, we quantified the transcripts of ACS6, the expression of which is significantly induced after pathogen attack (Li et al., 2012) and wounding (Li et al., 2018). Compared to WT, no changes in ACS6 transcript levels were detected in ap2c1, slightly higher levels in mkp1, and a nine-fold increase in ap2c1 mkp1 which was reduced to WT levels in ap2c1 mkp1 mpk6 plants (Fig. 3B). Thus, our data show that ACS6 is more expressed in ap2c1 mkp1 plants, which likely contributes to the elevated amounts of ET upon wounding, and that both effects are mediated by MPK6.

Taken together, the wound-induced MAPK activities, expression patterns and effects on ET production suggest that AP2C1 and MKP1 have both distinct as well as overlapping functions in wounded leaves.

TF gene expression is de-regulated in ap2c1 mkp1 plants

To investigate if and how AP2C1 and MKP1 influence the regulation of gene expression under standard growth conditions, we used a RT-qPCR platform for high-throughput expression profiling of 1,880 Arabidopsis TF-encoding genes (Balazadeh et al., 2008). We
selected genes showing an at least three-fold mean difference of expression levels in \( \text{ap2c1}, \text{mkp1} \) or \( \text{ap2c1 mkp1} \) plants when compared to WT. We identified three genes encoding TFs that were deregulated in \( \text{ap2c1} \), but not in \( \text{mkp1} \) (Supplementary Table I), while 25 genes were deregulated in \( \text{mkp1} \), but not in \( \text{ap2c1} \) (Supplementary Table II), and four genes concomitantly regulated by AP2C1 and MKP1 (Supplementary Table III). Fig. 4 shows the number of genes whose expression levels were changed in \( \text{ap2c1}, \text{mkp1} \) or \( \text{ap2c1 mkp1} \) plants, compared to the WT. The TF genes dysregulated in the double mutant, and their expression values relative to the WT, are represented in Supplementary Table IV. The deregulation of 76 TF-encoding genes (58 upregulated, 18 downregulated) was found reproducibly in at least three different experiments in \( \text{ap2c1 mkp1} \) double mutant plants. Among them, genes encoding members of the WRKY family were most abundant: 15 WRKY genes were upregulated (Fig. 5, Supplementary Table IV) and one downregulated (Supplementary Table IV). A further prevalent group of TF-encoding genes affected in \( \text{ap2c1 mkp1} \) plants includes AP2/ERF described for their involvement in development, including \( \text{RAP2.6L} \) (Yang et al., 2018) and \( \text{WIND3} \) (Smit et al., 2020). ANAC TF family members are implicated in senescence and stress-related processes (Bu et al., 2008; Jensen et al., 2010; Wu et al., 2012; Saga et al., 2012). Our results show that several ANAC TF-encoding genes are upregulated in \( \text{ap2c1 mkp1} \) plants (Fig. 7). Thus, our data suggest a cooperative function of AP2C1 and MKP1 in the transcriptional regulation of a set of WRKY, AP2/ERF and ANAC genes in the WT.

Our observation that \( \text{ap2c1 mkp1 mpk6} \) and \( \text{ap2c1 mkp1 mpk3} \) plants are phenotypically much less affected than \( \text{ap2c1 mkp1} \) double mutants suggested that severe phenotypic aberrations in the latter are mediated mainly by MPK6 and probably to a lesser extent by MPK3. This prompted us to investigate \( \text{ap2c1 mkp1 mpk6} \) and \( \text{ap2c1 mkp1 mpk3} \) plants for the expression of TFs misregulated in \( \text{ap2c1 mkp1} \). Indeed, most of the TF genes strongly affected in \( \text{ap2c1 mkp1} \) plants (Supplementary Table IV) were not significantly altered in their expression in \( \text{ap2c1 mkp1 mpk6} \) and \( \text{ap2c1 mkp1 mpk3} \), linking MAPK over-activation to the TF misexpression in the double mutant (Fig. 5 – 7). Among these, the expression of \( \text{WRKY75, WRKY71, WRKY64, WRKY40} \) (Fig. 5) and of \( \text{At2g33710} \) (Fig. 6) in the double mutant are more MPK3-independent, while the expression of other identified WRKYs (Fig. 5), AP2/ERF family members \( \text{RAP2.6L, WIND3, DREB19, ERF14, ERF17, ERF71 and ERF98} \) (Fig. 6), and several ANAC TF-encoding genes, such as \( \text{ANAC005, ANAC042, ANAC003, ANAC047, and ANAC055} \)– (Fig. 7) are mainly dependent on the presence of both MPK6 and MPK3.
Defence responses, camalexin, SA and the senescence marker gene
SENESCENCE-ASSOCIATED GENE12 (SAG12) are upregulated in ap2c1 mkp1 plants

It has been shown previously that mkp1 plants accumulate higher levels of the phytoalexin camalexin (Bartels et al., 2009). To investigate if the expression of genes encoding camalexin biosynthesis enzymes was affected in ap2c1 mkp1 plants, we studied the expression of a key gene in the pathway, CYP71B15/PAD3. A strong upregulation (more than 300-fold, respectively) was detected in ap2c1 mkp1 plants compared to WT (Supplementary Fig. 6). Moreover, ap2c1 mkp1 mpk6 plants still had remarkably high transcript levels (upregulation ca. 10-fold) of CYP71B15/PAD3. Also, mkp1 single mutant plants showed a >10-fold upregulation of the gene (Supplementary Fig. 6).

To investigate if the increased CYP71B15/PAD3 expression level correlates with camalexin accumulation, total camalexin was quantified in WT and mutant plants. Indeed, we found increased camalexin levels in mkp1 plants in agreement with previous findings (Bartels et al., 2009), and very high camalexin accumulation in the ap2c1 mkp1 mutant, which was not solely dependent on MPK6 and MPK3 (Fig. 8).

Upregulation of MAPK activities and macroscopic lesion formation in leaves of ap2c1 mkp1 indicated the possible activation of a hypersensitive-like response in these plants. Since this is associated with the accumulation of the stress hormone SA, we measured SA in leaves of ap2c1 mkp1, ap2c1 mkp1 mpk6 as well as in WT and single mutants. Indeed, we found a 35-fold increase of SA in ap2c1 mkp1 plants compared to WT (Fig. 9), whereas ap2c1, ap2c1 mkp1 mpk6, and mpk6 plants showed SA amounts similar to the WT. In agreement with previous data (Bartels et al., 2009), we detected enhanced total SA amounts (>2-fold) also in mkp1 plants compared to WT (Fig. 9).

Leaf necrosis observed in ap2c1 mkp1 leaves (Fig. 1A; Supplementary Fig. 1G) and the upregulation of WRKY6 (Fig. 5), which is a senescence-related marker gene (Rushton et al., 2010), suggested that early senescence was induced in these plants. Thus, we investigated the expression of the senescence-specific marker gene SAG12 (Noh and Amasino, 1999) and found that it strongly upregulated in ap2c1 mkp1 plants, dependent on MPK6 (Fig. 10). These data along with the upregulation of WRKY6 (Fig. 5) indicate aberrant, early induction of senescence-related processes in the double phosphatase mutant.
Discussion

Coordinated control of MAPK activities by AP2C1 and MKP1

Acclimation for survival is a fundamental principle, which relies on intracellular signalling in every organism. Different signals converge at the level of MAPK cascades, and from there diverge into a range of different downstream pathways and responses (Andreasson and Ellis, 2010; Rodriguez et al., 2010; Rasmussen et al., 2012). Considering the broad spectrum of signals transduced by overlapping players of MAPK pathways it is puzzling how response specificity is attained (Lampard et al., 2009; Rodriguez et al., 2010; Meng and Zhang, 2013). Several signalling scenarios have been investigated that could help explain pathway specificity, including activity-dependent kinase distribution and localization, protein complex formation (e.g. interaction with scaffolding proteins), and dephosphorylation by protein phosphatases (Krysan and Colcombet, 2018). Over the last decades mainly the functions of MPK3/MPK4/MPK6 in diverse pathways have been described, indicating them as both points of divergence and integration hubs in cellular signalling (Peng et al., 2018; Bigeard and Hirt, 2018).

Here, we provide evidence that two evolutionarily distinct MAPK phosphatases control stress-related signalling in Arabidopsis by inactivating an overlapping set of target MAPKs that mediate stress and defence responses. The Ser/Thr PP2C phosphatase AP2C1 and the dual-specificity phosphatase MKP1 contribute to ensure appropriate inactivation of MAPKs during stress. Both AP2C1 and MKP1 target MPK3, MPK4 and MPK6 (Ulm et al., 2002; Schweighofer et al., 2007; Bartels et al., 2009; Anderson et al., 2011; Galletti et al., 2011; Sidonskaya et al., 2016; Shubchynskyy et al., 2017). Enhanced activation of MAPKs by wounding, PAMP (flg22), and constitutive stress signalling in the absence of stress in ap2c1 mkp1 plants indicate that the lack of both MAPK phosphatases creates a shortfall downstream of MAPKs, exemplified by deregulated expression of TF-encoding genes.

An enhanced kinase activity in mkp1 plants versus WT at the earlier time points after wounding and flg22 application compared to ap2c1 versus WT suggests that the contribution of MKP1 to inactivating MAPKs is already set before stress treatment, or during a very early stage of signalling. In agreement with this suggested function of MKP1 during early signaling previous work has also shown, that mkp1 and mkp1 ptp1 mutant plants demonstrate equally elevated MAPK activities without stress treatment, underlining the major role of MKP1 in MAPK regulation in ambient conditions (Bartels et al., 2009). On the contrary, AP2C1 adds to MAPK inactivation at later time points. It is possible that AP2C1 is primarily responsible for keeping the stress-induced activation below a certain threshold and controlling the duration
of kinase activation during acute stress acting as an “emergency brake”, while MKP1 is predominantly responsible for suppressing kinase activities under normal conditions, providing a “constitutive brake”. This hypothesis is supported by the induction of AP2C1 expression by a plethora of stresses, while MKP1 shows comparatively marginal changes in expression (https://www.genevestigator.com). These observations are also consistent with a recent comprehensive analysis of the Arabidopsis proteome, which covers more than 14,000 proteins and where in ambient conditions the overall MKP1 abundance outnumbers by far that of AP2C1 (http://athena.proteomics.wzw.tum.de/) (Mergner et al., 2020), underlining the rather specific role of AP2C1 under stress conditions. The AP2C1 paralogues AP2C2 and AP2C3 (Umbrasaite et al., 2010; Umbrasaite et al., 2011; Schweighofer et al., 2014) as well as MKP1 and PTP1 interact with the same MAPKs and dephosphorylate them to various extents (Bartels et al., 2009). However, the rather mild phenotypes of ap2c2 mkp1 and ap2c3 mkp1 plants and the WT-like appearance of ap2c1 ptp1 (this work) compared to ap2c1 mkp1 plants clearly indicate specific genetic interactions and redundant functions of the evolutionary distant AP2C1 and MKP1 phosphatases in the regulation of signalling pathways.

**Manifestation of cell death in ap2c1 mkp1 plants**

The lesions in leaves of ap2c1 mkp1 plants suggest autoimmune-like responses most likely caused by misregulation of MAPKs and/or failed control of guarding resistance (R) proteins (Rodriguez et al., 2016). AP2C1 and MKP1 share the target MAPKs MPK3, MPK4 and MPK6, where MPK4 and some of its upstream MAPK cascade members were originally described as negative regulators of plant immunity based on their mutant plant phenotypes, for example MEKK1 and M KK1/2 (Petersen et al., 2000; Rasmussen et al., 2012). The improper activation of the R-gene SUMM2 is mainly responsible for the phenotypical defects of the mpk4 mutant and of other mutant plants in the pathway, identifying the MEKK1-MKK1/2-MPK4 module as a positive regulator of stress responses (Zhang et al., 2012). Similar observations connecting phosphatase-targeted MAPKs with autoimmune-like phenotypes have been made by ectopically expressing constitutively active MPK3 (Genot et al., 2017) or by inducibly expressing MKK5, which activates MPK3 and MPK6 (Lassowskat et al., 2014). Both approaches led to a plethora of phenotypic and molecular changes including dwarfism, lesion formation, de-repression of defence gene expression, and the accumulation of stress hormones, similar to the ap2c1 mkp1-related phenotypes described in this work (see Results).
The single mkp1 and the double mkp1 ptp1 mutants show constitutive defence responses including increased levels of SA and camalexin, suggesting partially overlapping functions of MKP1 and PTP1 in repressing SA biosynthesis (Bartels et al., 2009). Similarly, the strong accumulation of SA and camalexin in ap2c1 mkp1 compared to mkp1 plants suggests a collaborative action of both AP2C1 and MKP1 as negative regulators of SA and camalexin production (this work). The camalexin accumulation in ap2c1 mkp1 is largely reduced in ap2c1 mkp1 mpk6 and ap2c1 mkp1 mpk3 triple mutants, indicating the dependency of camalexin biosynthesis on partially redundant actions of MPK6 and MPK3, which is in agreement with previous findings (Mao et al., 2011). The SA accumulation in ap2c1 mkp1 plants is probably mainly MPK6-dependent, as the introduction of the mpk6 mutation in ap2c1 mkp1 mpk6 plants restores SA levels similar to those of WT and mkp1 ptp1 mpk6 mutant (Bartels et al., 2009). Notably, rescue of the severe ap2c1 mkp1 growth phenotypes by elevated temperature is in accordance with the observed temperature dependency of SA-related phenotypes (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007; Su et al., 2007), as well as with the suppression of SNC1 expression and reduction of SNC1 activity by high temperature (Yang and Hua, 2004; Zhu et al., 2010). The resistance protein SNC1 is a modifier of mkp1 in the Col-0 accession, where partial rescue of mkp1 and mkp1 ptp1 growth phenotypes by a loss-of-function snc1 mutation indicates a sensitized SNC1 signaling pathway in the absence of MKP1 (Bartels et al., 2009).

Previous findings that SA acts together with ET to regulate cell death (Rao et al., 2002), the requirement of ET biosynthesis for H$_2$O$_2$ accumulation and subsequent cell death (Övermyer et al., 2003), and the induction of cell death in Arabidopsis leaves by persistent activation of MAPKs with gain-of-function MKK4 and MKK5 (Ren et al., 2002) all correlate with the cell death phenotype observed in the ap2c1 mkp1 mutant, where MAPKs - and other stress-related factors - may be (hyper)-activated. Therefore, we conclude that the majority of the phenotypes observed in ap2c1 mkp1 plants, both visible and molecular, are due to the misregulation of MAPK pathways, even in the absence of stress.
AP2C1 and MKP1 affect MAPK-regulated ET biosynthesis

Activated MPK6 controls ET levels by both inducing the transcription of ACS family genes and by phosphorylating ACS proteins, the rate-limiting enzymes in ET biosynthesis. Phosphorylated ACSs become more stable and, thus, ET synthesis is enhanced by elevated MPK6 activity (Kim et al., 2003; Liu and Zhang, 2004; Xu et al., 2008; Li et al., 2012). In ap2c1 mkp1, the enhanced ET production is likely due to, at least in part, the highly increased expression of ACS6 compared to WT. A considerable additive effect on ET overproduction by the double ap2c1 mkp1 mutation suggests that even though MKP1 is a determining MAPK phosphatase affecting ET production, there are overlapping and non-redundant functions of AP2C1 and MKP1 in the regulation of stress-induced ET biosynthesis. Detection of enhanced and MPK6-dependent expression of WRKY33, encoding a TF that binds to the promoter of ACS genes and is a substrate of MPK3 and MPK6, suggests an involvement of WKRY33 itself in ACS overexpression in ap2c1 mkp1 plants (this work and (Li et al., 2012)). The identification of genes encoding TFs of the AP2/ERF family members (ET-responsive element-binding proteins) among the uppermost induced ones in ap2c1 mkp1 plants suggests a path to enhanced ET amounts in these plants.

AP2C1 and MKP1 control the expression of stress-responsive TF-encoding genes, predominantly via partially redundant actions of MPK6 and MPK3

Transcriptional reprogramming in response to activated MAPK signalling suggests an involvement of TFs. Our results indicate that the concomitant lack of the MAPK regulators AP2C1 and MKP1 results in elevated basal MAPK activities and leads to highly enhanced expression of WRKY TF genes, in some cases by more than hundred-fold compared to WT. The ap2c1 mkp1 mutant phenotypes and the described functions of some upregulated WRKYs indicate that stress responses are constitutively active in these plants. This correlates with reports demonstrating an involvement of WRKYs in oxidative stress responses, in the induction of ET and camalexin biosynthesis (WRKY30, WRKY33), in the response to pathogens (WRKY71, WRKY40), in basal defence (WRKY38), and defence- and senescence-related processes (WRKY6) (Rushton et al., 2010).
Direct feedback mechanisms among WRKYs themselves have been shown (Mao et al., 2011) and are generally proposed, where WRKYs positively auto-regulate their own gene expression and/or cross-regulate expression of other WRKY genes (Pandey and Somssich, 2009; Mao et al., 2011; Birkenbihl et al., 2017). Thus, it could be that the enhanced activation of MAPKs in ap2c1 mkp1 plants leads to phosphorylation and thus activation of MAPK target WRKY proteins, which serve as activated TFs for a further series of WRKY genes. In any case, MPK6 and MPK3 seem to be major players responsible for mediating the upregulation of several WRKYs, AP2/ERFs, ANACs and other TF-encoding genes. Both MAPKs control the expression of several WRKYs to different extents, as shown in ap2c1 mkp1 plants compared to ap2c1 mkp1 mpk6 and ap2c1 mkp1 mpk3 (Fig. 5). These data also demonstrate that not only MAPKs but also other factor(s) affect WRKY gene expression. We confirmed MPK6- and MPK3-dependent WRKY33 expression (Mao et al., 2011); however, the higher MPK4 activities in ap2c1 mkp1 may also lead to higher amounts of active WRKY33 protein (Qiu et al., 2008; Birkenbihl et al., 2017). Thus, our data suggest that AP2C1 and MKP1 may play a dual role in regulating camalexin biosynthesis, on the one hand by controlling MPK6 and MPK3 activities, which positively regulate WKRY33 expression, and on the other by controlling MPK4 activity, which in turn stimulates WRKY33 leading to transactivation of CYP71B15/PAD3.

Remarkably, concomitant absence of AP2C1 and MKP1 in unchallenged conditions causes a distinct transcriptional activation of TFs as compared with single ap2c1 and mkp1 mutant plants after stress treatments: Challenging ap2c1 plants with Pseudomonas syringae pv. tomato (Pto) for four hours led to 88 differentially regulated TFs (Shubchynskyy et al., 2017), whereas only four TFs from this set were upregulated in untreated ap2c1 mkp1 plants (this study).

Comparing transcriptional changes of PAMP-treated mkp1 plants (Jiang et al., 2017) with untreated ap2c1 mkp1 double mutant plants (this study) revealed a more MKP1-specific TF induction: A next generation sequencing (RNAseq) transcriptome analysis of mkp1 plants 90 minutes after treatment with the PAMP elf26 revealed that from 67 identified TFs among the 1102 MKP1-dependent transcripts (Jiang et al., 2017) 21 TFs were also changed in untreated ap2c1 mkp1 plants (this study). In accordance with the severe phenotype of mkp1 ptp1 mutant plants and its suppression by elevated growth temperature or crossing with mpk3 and mpk6
mutants (Bartels et al., 2009) the proposed predominant roles of MKP1 in ambient and of AP2C1 during stress conditions to regulate (MAPK) signaling are emphasized.

A PAMP flg22-activated MPK3/MPK6 pathway was previously reported to elevate WRKY22 and WRKY29 expression (Asai et al., 2002). Strongly enhanced MPK3/MPK6 activities, but unaffected expression of either WRKY22 or WRKY29 in untreated ap2c1 mkp1 plants, after PAMP elf26 and Pto treatment (Jiang et al., 2017; Shubchynskyy et al., 2017), show that for flg22-induced WRKY22/29 overexpression the MPK3 and MPK6 hyperactivation is sufficient (Asai et al., 2002) but not necessary (this work) and that other factors (possibly MAPKs) may be playing a role instead of MPK3 and MPK6.

**Senescence is repressed by AP2C1 and MKP1 phosphatases in an MPK6-dependent way**

Several lines of evidence indicate that the ap2c1 mkp1 mutant undergoes precocious senescence. Leaf senescence is a highly regulated process that finally leads to cell death and tissue disintegration, at the same time contributing to the fitness of the whole plant. Senescence is controlled by endogenous and environmental cues, and can be triggered prematurely by different abiotic/biotic stresses due to pathogen attack, wounding, UV light irradiation, and high ozone levels (Hanfrey et al., 1996; Miller et al., 1999; John et al., 2001; He et al., 2001; Lim et al., 2007). The MKK9-MPK6 cascade has been shown to positively regulate leaf senescence in Arabidopsis (Zhou et al., 2009). Hyperactivation of MPK6 and other MAPKs, in addition to autoimmune-like responses, also promotes senescence, which is very evident in older leaves of ap2c1 mkp1 plants and correlates with significant upregulation of the senescence-specific marker gene SAG12 (Noh and Amasino, 1999; Guo and Gan, 2005). Partial suppression of SAG12 overexpression in ap2c1 mkp1 mpk6 suggests an MPK6-dependent regulation (possibly involving other MAPKs) in promoting plant senescence.

Genome-wide transcriptomics previously identified several senescence-related TFs from the ANAC family (Breeze et al., 2011). We could highlight strong MAPK-dependent induction of ANAC005, JUB1/ANAC042 (Wu et al., 2012; Saga et al., 2012; Shahnejat-Bushehri et al., 2016), ANAC003/XVP (Yang et al., 2020), ANAC047 (Mito et al., 2011), and ANAC055 (Tran et al., 2004; Bu et al., 2008;
Hickman et al., 2013; Schweizer et al., 2013) in ap2c1 mkp1 plants. This induction of senescence-related TFs reveals a novel link between senescence-related processes and MAPK signalling.

We conclude that the induction of senescence processes as well as hypersensitive response-like cell death results in premature death of leaves in ap2c1 mkp1 plants. The crosstalk between senescence and abiotic stress or pathogen responses is accentuated in ap2c1 mkp1 plants where upregulation of TFs involved in these processes is happening.

Taken together, our results show that two evolutionarily unrelated MAPK phosphatases, AP2C1 and MKP1, perform both distinct and overlapping functions in the regulation of stress-induced MPK3, MPK4 and MPK6 activities. Our genetic dissection indicates that the known roles of MPK6 and MPK3 in mediating cell death, ET-, SA- and senescence-related phenotypes is attenuated by both AP2C1 and MKP1. It also demonstrates that the expression of specific TF-encoding genes is affected by MAPK(s) hyperactivation due to the lack of these two MAPK phosphatases in planta, revealing potential new signalling target genes downstream of MPK6 and MPK3. In the future, the study of individual and combinatorial mutants will allow us to genetically disentangle the contribution of specific protein kinases and phosphatases to complex signalling networks and downstream cell responses.
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Author contributions

ZA, VK, VS, KK, MaS, WR, MiS, SeB, SaB and AS performed experiments, ZA, VK, VS, KK, FM, RU, SaB, BMR, IM and AS designed experiments; and ZA, SaB, BMR, IM and AS wrote the paper.

Dedication

We wish to dedicate this article to the co-authors, who passed away too early:

Felix Mauch (1955-2021)
Irute Meskiene (1956-2017),
for obituary see Paškauskas et al. (2017).
Manfred Schwanninger (1963-2013),
for obituary see Meder (2014).

Data availability statement

The data supporting the findings of this study are available from the corresponding author (Alois Schweighofer), upon request.
References

Anderson JC, Bartels S, Gonzalez Besteiro MA, Shahollari B, Ulm R, Peck SC. 2011. Arabidopsis MAP Kinase Phosphatase 1 (AtMKP1) negatively regulates MPK6-mediated PAMP responses and resistance against bacteria. Plant Journal 67, 258-268.

Anderson JC, Wan Y, Kim YM, Pasa-Tolic L, Metz TO, Peck SC. 2014. Decreased abundance of type III secretion system-inducing signals in Arabidopsis mkp1 enhances resistance against Pseudomonas syringae. Proceedings of the National Academy of Sciences, USA 111, 6846-6851.

Andreason E, Ellis B. 2010. Convergence and specificity in the Arabidopsis MAPK nexus. Trends in Plant Science 15, 106-113.

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J. 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415, 977-983.

Balazadeh S, Riano-Pachon DM, Mueller-Roeber B. 2008. Transcription factors regulating leaf senescence in Arabidopsis thaliana. Plant Biology 10 Suppl 1, 63-75.

Bartels S, Anderson JC, Gonzalez Besteiro MA, Carreri A, Hirt H, Buchala A, Metraux JP, Peck SC, Ulm R. 2009. MAP KINASE PHOSPHATASE 1 and PROTEIN TYROSINE PHOSPHATASE 1 are repressors of salicylic acid synthesis and SNC1-mediated responses in Arabidopsis. Plant Cell 21, 2884-2897.

Bartels S, Gonzalez Besteiro MA, Lang D, Ulm R. 2010. Emerging functions for plant MAP kinase phosphatases. Trends in Plant Science 15, 322-329.

Bethke G, Unthan T, Uhrig JF, Poschl Y, Gust AA, Scheel D, Lee J. 2009. Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in Arabidopsis thaliana via ethylene signaling. Proceedings of the National Academy of Sciences, USA 106, 8067-8072.

Bigeard J, Hirt H. 2018. Nuclear signaling of plant MAPKs. Frontiers in Plant Science 9, 469.

Birkenbihl RP, Kracher B, Roccaro M, Somssich IE. 2017. Induced genome-wide binding of three arabidopsis WRKY transcription factors during early MAMP-triggered immunity. Plant Cell 29, 20-38.

Breeze E, Harrison E, McHattie S, Hughes L, Hickman R, Hill C, Kiddle S, Kim YS, Penfold CA, Jenkins D, Zhang C, Morris K, Jenner C, Jackson S, Thomas
B, Tabrett A, Legaie R, Moore JD, Wild DL, Ott S, Rand D, Beynon J, Denby K, Mead A, Buchanan-Wollaston V. 2011. High-resolution temporal profiling of transcripts during Arabidopsis leaf senescence reveals a distinct chronology of processes and regulation. Plant Cell 23, 873-894.

Bu Q, Jiang H, Li CB, Zhai Q, Zhang J, Wu X, Sun J, Xie Q, Li C. 2008. Role of the Arabidopsis thaliana NAC transcription factors ANAC019 and ANAC055 in regulating jasmonic acid-signaled defense responses. Cell Research 18, 756-767.

Caunt CJ, Keyse SM. 2013. Dual-specificity MAP kinase phosphatases (MKPs): shaping the outcome of MAP kinase signalling. FEBS Journal 280, 489-504.

Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK. 2004. Real-time RT-PCR profiling of over 1400 Arabidopsis transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. Plant Journal 38, 366-379.

Escudero V, Torres MA, Delgado M, Sopena-Torres S, Swami S, Morales J, Munoz-Barrios A, Melida H, Jones AM, Jorda L, Molina A. 2019. Mitogen-activated protein kinase phosphatase 1 (MKP1) negatively regulates the production of reactive oxygen species during Arabidopsis immune responses. Mol Plant Microbe Interact 32, 464-478.

Fuchs S, Grill E, Meskiene I, Schweighofer A. 2013. Type 2C protein phosphatases in plants. FEBS Journal 280, 681-693.

Galletti R, Ferrari S, De Lorenzo G. 2011. Arabidopsis MPK3 and MPK6 play different roles in basal and oligogalacturonide- or flagellin-induced resistance against Botrytis cinerea. Plant Physiology 157, 804-814.

Genot B, Lang J, Berriri S, Garmier M, Pateyron F, Haustraete K, Van Der Straeten D, Hirt H, Colcombet J. 2017. Constitutively active Arabidopsis MAP kinase 3 triggers defense responses involving salicylic acid and SUMM2 resistance protein. Plant Physiology 174, 1238-1249.

Gonzalez Besteiro MA, Bartels S, Albert A, Ulm R. 2011. Arabidopsis MAP kinase phosphatase 1 and its target MAP kinases 3 and 6 antagonistically determine UV-B stress tolerance, independent of the UVR8 photoreceptor pathway. Plant Journal 68, 727-737.

Gonzalez Besteiro MA, Ulm R. 2013a. ATR and MKP1 play distinct roles in response to UV-B stress in Arabidopsis. Plant Journal 73, 1034-1043
Gonzalez Besteiro MA, Ulm R. 2013b. Phosphorylation and stabilization of Arabidopsis MAP kinase phosphatase 1 in response to UV-B stress. Journal of Biological Chemistry 288, 480-486.

Guan Y, Meng X, Khanna R, LaMontagne E, Liu Y, Zhang S. 2014. Phosphorylation of a WRKY transcription factor by MAPKs is required for pollen development and function in Arabidopsis. PLoS Genetics 10, e1004384.

Guo Y, Gan S. 2005. Leaf senescence: signals, execution, and regulation. Current Topics in Developmental Biology 71, 83-112.

Hanfrey C, Fife M, Buchanan-Wollaston V. 1996. Leaf senescence in Brassica napus: expression of genes encoding pathogenesis-related proteins. Plant Molecular Biology 30, 597-609.

He Y, Tang W, Swain JD, Green AL, Jack TP, Gan S. 2001. Networking senescence-regulating pathways by using Arabidopsis enhancer trap lines. Plant Physiology 126, 707-716.

Hickman R, Hill C, Penfold CA, Breeze E, Bowden L, Moore JD, Zhang P, Jackson A, Cooke E, Bewicke-Copley F, Mead A, Beynon J, Wild DL, Denby KJ, Ott S, Buchanan-Wollaston V. 2013. A local regulatory network around three NAC transcription factors in stress responses and senescence in Arabidopsis leaves. Plant Journal 75, 26-39.

Ichimura K, Casais C, Peck SC, Shinozaki K, Shirasu K. 2006. MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. Journal of Biological Chemistry 281, 36969-36976.

Jensen MK, Kjaersgaard T, Nielsen MM, Galberg P, Petersen K, O'Shea C, Skriver K. 2010. The Arabidopsis thaliana NAC transcription factor family: structure-function relationships and determinants of ANAC019 stress signalling. Biochemical Journal 426, 183-196.

Jiang L, Chen Y, Luo L, Peck SC. 2018. Central roles and regulatory mechanisms of dual-specificity MAPK phosphatases in developmental and stress signaling. Frontiers in Plant Science 9, 1697.

John CF, Morris K, Jordan BR, Thomas B, S AH-M. 2001. Ultraviolet-B exposure leads to up-regulation of senescence-associated genes in Arabidopsis thaliana. Journal of Experimental Botany 52, 1367-1373.
Ju C, Chang C. 2012. Advances in ethylene signalling: protein complexes at the endoplasmic reticulum membrane. AoB Plants 2012, pls031.

Kim CY, Liu Y, Thorne ET, Yang H, Fukushige H, Gassmann W, Hildebrand D, Sharp RE, Zhang S. 2003. Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. Plant Cell 15, 2707-2718.

Kim CY, Zhang S. 2004. Activation of a mitogen-activated protein kinase cascade induces WRKY family of transcription factors and defense genes in tobacco. Plant Journal 38, 142-151.

Krysan PJ, Colcombet J. 2018. Cellular complexity in MAPK signaling in plants: questions and emerging tools to answer them. Frontiers in Plant Science 9, 1674.

Lampard GR, Lukowitz W, Ellis BE, Bergmann DC. 2009. Novel and expanded roles for MAPK signaling in Arabidopsis stomatal cell fate revealed by cell type-specific manipulations. Plant Cell 21, 3506-3517.

Lassowskat I, Bottcher C, Eschen-Lippold L, Scheel D, Lee J. 2014. Sustained mitogen-activated protein kinase activation reprograms defense metabolism and phosphoprotein profile in Arabidopsis thaliana. Frontiers in Plant Science 5, 554.

Li G, Meng X, Wang R, Mao G, Han L, Liu Y, Zhang S. 2012. Dual-level regulation of ACC synthase activity by MPK3/MPK6 cascade and its downstream WRKY transcription factor during ethylene induction in Arabidopsis. PLoS Genetics 8, e1002767.

Li S, Han X, Yang L, Deng X, Wu H, Zhang M, Liu Y, Zhang S, Xu J. 2018. Mitogen-activated protein kinases and calcium-dependent protein kinases are involved in wounding-induced ethylene biosynthesis in Arabidopsis thaliana. Plant, Cell & Environment 41, 134-147.

Lim PO, Kim HJ, Nam HG. 2007. Leaf senescence. Annual Review of Plant Biology 58, 115-136.

Liu Y, Ren D, Pike S, Pallardy S, Gassmann W, Zhang S. 2007. Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. Plant Journal 51, 941-954.

Liu Y, Zhang S. 2004. Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell 16, 3386-3399.
Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S. 2011. Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis. Plant Cell 23, 1639-1653.

Marshall CJ. 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 80, 179-185.

Meder R. 2014. Manfred Schwanninger 4 September 1963–25 December 2013. NIR news 25, 18-19.

Meng X, Zhang S. 2013. MAPK cascades in plant disease resistance signaling. Annual Review of Phytopathology 51, 245-266.

Menke FL, Kang HG, Chen Z, Park JM, Kumar D, Klessig DF. 2005. Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Molecular Plant-Microbe Interactions 18, 1027-1034.

Mergner J, Frejno M, List M, Papacek M, Chen X, Chaudhary A, Samaras P, Richter S, Shikata H, Messerer M, Lang D, Altmann S, Cyprys P, Zolg DP, Mathieson T, Bantscheff M, Hazarika RR, Schmidt T, Dawid C, Dunkel A, Hofmann T, Sprunck S, Falter-Braun P, Johannes F, Mayer KFX, Jurgens G, Wilhelm M, Baumbach J, Grill E, Schneitz K, Schwechheimer C, Kuster B. 2020. Mass-spectrometry-based draft of the Arabidopsis proteome. Nature 579, 409-414.

Miller JD, Arteca RN, Pell EJ. 1999. Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. Plant Physiology 120, 1015-1024.

Mito T, Seki M, Shinozaki K, Ohme-Takagi M, Matsui K. 2011. Generation of chimeric repressors that confer salt tolerance in Arabidopsis and rice. Plant Biotechnology Journal 9, 736-746.

Nakano T, Suzuki K, Fujimura T, Shinshi H. 2006. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiology 140, 411-432.

Noh YS, Amasino RM. 1999. Identification of a promoter region responsible for the senescence-specific expression of SAG12. Plant Molecular Biology 41, 181-194.

Overmyer K, Brosche M, Kangasjarvi J. 2003. Reactive oxygen species and hormonal control of cell death. Trends in Plant Science 8, 335-342.

Pandey SP, Somssich IE. 2009. The role of WRKY transcription factors in plant immunity. Plant Physiology 150, 1648-1655.
Paškauskas R, Schweighofer A, Kvederavičiūtė K. 2017. In Memoriam Irutė Meškienė (1956–2017). Botanica 23, 178-182.

Peng Y, van Wersch R, Zhang Y. 2018. Convergent and divergent signaling in PAMP-triggered immunity and effector-triggered immunity. Molecular Plant-Microbe Interactions 31, 403-409.

Petersen M, Brodersen P, Naested H, Andreasson E, Lindhart U, Johansen B, Nielsen HB, Lacy M, Austin MJ, Parker JE, Sharma SB, Klessig DF, Martienssen R, Mattsson O, Jensen AB, Mundy J. 2000. Arabidopsis MAP kinase 4 negatively regulates systemic acquired resistance. Cell 103, 1111-1120.

Popescu SC, Popescu GV, Bachan S, Zhang Z, Gerstein M, Snyder M, Dinesh-Kumar SP. 2009. MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. Genes & Development 23, 80-92.

Qiu JL, Fiil BK, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Clausen S, Lichota J, Brodersen P, Grasser KD, Mattsson O, Glazebrook J, Mundy J, Petersen M. 2008. Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO Journal 27, 2214-2221.

Rao MV, Lee HI, Davis KR. 2002. Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant Journal 32, 447-456.

Rasmussen MW, Roux M, Petersen M, Mundy J. 2012. MAP kinase cascades in arabidopsis innate immunity. Frontiers in Plant Science 3, 169.

Rauf M, Arif M, Fisahn J, Xue GP, Balazadeh S, Mueller-Roeber B. 2013. NAC transcription factor speedy hyponastic growth regulates flooding-induced leaf movement in Arabidopsis. Plant Cell 25, 4941-4955.

Ren D, Yang H, Zhang S. 2002. Cell death mediated by MAPK is associated with hydrogen peroxide production in Arabidopsis. Journal of Biological Chemistry 277, 559-565.

Rodriguez E, El Ghoul H, Mundy J, Petersen M. 2016. Making sense of plant autoimmunity and ‘negative regulators’. FEBS Journal 283, 1385-1391.

Rodriguez MC, Petersen M, Mundy J. 2010. Mitogen-activated protein kinase signaling in plants. Annual Review of Plant Biology 61, 621-649.

Rozhon W, Petutschnig E, Wrzaczek M, Jonak C. 2005. Quantification of free and total salicylic acid in plants by solid-phase extraction and isocratic high-performance
anion-exchange chromatography. Analytical and Bioanalytical Chemistry **382**, 1620-1627.

Rushton PJ, Somssich IE, Ringler P, Shen QJ. 2010. WRKY transcription factors. Trends in Plant Science **15**, 247-258.

Saga H, Ogawa T, Kai K, Suzuki H, Ogata Y, Sakurai N, Shibata D, Ohta D. 2012. Identification and characterization of ANAC042, a transcription factor family gene involved in the regulation of camalexin biosynthesis in Arabidopsis. Molecular Plant-Microbe Interactions **25**, 684-696.

Schweighofer A, Ayatollahi Z, Meskiene I. 2009. Phosphatase activities analyzed by *in vivo* expressions. Methods in Molecular Biology **479**, 247-260.

Schweighofer A, Hirt H, Meskiene I. 2004. Plant PP2C phosphatases: emerging functions in stress signaling. Trends in Plant Science **9**, 236-243.

Schweighofer A, Kazanaviciute V, Scheikl E, Teige M, Doczi R, Hirt H, Schwanninger M, Kant M, Schuurink R, Mauch F, Buchala A, Cardinale F, Meskiene I. 2007. The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. Plant Cell **19**, 2213-2224.

Schweighofer A, Shubchynskyy V, Kazanaviciute V, Djamei A, Meskiene I. 2014. Bimolecular fluorescent complementation (BiFC) by MAP kinases and MAPK phosphatases. Methods in Molecular Biology **1171**, 147-158.

Schweizer F, Bodenhausen N, Lassueur S, Masclaux FG, Reymond P. 2013. Differential contribution of transcription factors to *Arabidopsis thaliana* defense against *Spodoptera littoralis*. Frontiers in Plant Science **4**, 13.

Shahnejat-Bushehri S, Tarkowska D, Sakuraba Y, Balazadeh S. 2016. Arabidopsis NAC transcription factor JUB1 regulates GA/BR metabolism and signalling. Nature Plants **2**, 16013.

Shubchynskyy V, Boniecka J, Schweighofer A, Simulis J, Kvederaviciute K, Stumpe M, Mauch F, Balazadeh S, Mueller-Roeber B, Boutrot F, Zipfel C, Meskiene I. 2017. Protein phosphatase AP2C1 negatively regulates basal resistance and defense responses to *Pseudomonas syringae*. Journal of Experimental Botany **68**, 1169-1183.

Sidonskaya E, Schweighofer A, Shubchynskyy V, Kammerhofer N, Hofmann J, Wieczorek K, Meskiene I. 2016. Plant resistance against the parasitic nematode *Heterodera schachtii* is mediated by MPK3 and MPK6 kinases, which are controlled...
by the MAPK phosphatase AP2C1 in Arabidopsis. Journal of Experimental Botany 67, 107-118.

Smit ME, McGregor SR, Sun H, Gough C, Bagman AM, Soyars CL, Kroon JT, Gaudinier A, Williams CJ, Yang X, Nimchuk ZL, Weijers D, Turner SR, Brady SM, Etchells JP. 2020. A PXY-mediated transcriptional network integrates signaling mechanisms to control vascular development in Arabidopsis. Plant Cell 32, 319-335.

Su SH, Suarez-Rodriguez MC, Krysan P. 2007. Genetic interaction and phenotypic analysis of the Arabidopsis MAP kinase pathway mutations mekk1 and mpk4 suggests signaling pathway complexity. FEBS Letters 581, 3171-3177.

Suarez-Rodriguez MC, Adams-Phillips L, Liu Y, Wang H, Su SH, Jester PJ, Zhang S, Bent AF, Krysan PJ. 2007. MEKK1 is required for flg22-induced MPK4 activation in Arabidopsis plants. Plant Physiology 143, 661-669.

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K. 2004. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16, 2481-2498.

Ulm R, Ichimura K, Mizoguchi T, Peck SC, Zhu T, Wang X, Shinozaki K, Paszkowski J. 2002. Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. EMBO Journal 21, 6483-6493.

Ulm R, Revenkova E, di Sansebastiano GP, Bechtold N, Paszkowski J. 2001. Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in Arabidopsis. Genes & Development 15, 699-709.

Umbrasaitė J, Schweighofer A, Kazanaviciute V, Magyar Z, Ayatollahi Z, Unterwurzacher V, Choopayak C, Boniecka J, Murray JA, Bogre L, Meskiene I. 2010. MAPK phosphatase AP2C3 induces ectopic proliferation of epidermal cells leading to stomata development in Arabidopsis. PLoS One 5, e15357.

Umbrasaitė J, Schweighofer A, Meskiene I. 2011. Substrate analysis of Arabidopsis PP2C-type protein phosphatases. Methods in Molecular Biology 779, 149-161.

Wang KL, Li H, Ecker JR. 2002. Ethylene biosynthesis and signaling networks. Plant Cell 14 Suppl, S131-151.

Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, Asensi-Fabado MA, Munne-Bosch S, Antonio C, Tohge T, Fernie AR, Kaufmann K, Xue GP, Mueller-
Roeber B, Balazadeh S. 2012. JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. Plant Cell 24, 482-506.

Xu J, Li Y, Wang Y, Liu H, Lei L, Yang H, Liu G, Ren D. 2008. Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in Arabidopsis. Journal of Biological Chemistry 283, 26996-27006.

Yang JH, Lee KH, Du Q, Yang S, Yuan B, Qi L, Wang H. 2020. A membrane-associated NAC domain transcription factor XVP interacts with TDIF co-receptor and regulates vascular meristem activity. New Phytologist 226, 59-74.

Yang S, Hua J. 2004. A haplotype-specific Resistance gene regulated by BONZAI mediates temperature-dependent growth control in Arabidopsis. Plant Cell 16, 1060-1071.

Yang S, Poretska O, Sieberer T. 2018. ALTERED MERISTEM PROGRAM1 restricts shoot meristem proliferation and regeneration by limiting HD-ZIP III-mediated expression of RAP2.6L. Plant Physiology 177, 1580-1594.

Zhang Z, Wu Y, Gao M, Zhang J, Kong Q, Liu Y, Ba H, Zhou J, Zhang Y. 2012. Disruption of PAMP-induced MAP kinase cascade by a Pseudomonas syringae effector activates plant immunity mediated by the NB-LRR protein SUMM2. Cell Host Microbe 11, 253-263.

Zhou C, Cai Z, Guo Y, Gan S. 2009. An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. Plant Physiology 150, 167-177.

Zhu Y, Qian W, Hua J. 2010. Temperature modulates plant defense responses through NB-LRR proteins. PLoS Pathogens 6, e1000844.
Figure legends

Fig. 1. Loss of both AP2C1 and MKP1 causes developmental defects and precocious cell death, mediated by MPK6 and MPK3.

(A) Phenotypes of five-week-old plants of the indicated genotypes grown in standard short-day conditions.

(B) Phenotypes of plants of the indicated genotypes grown for four weeks in short-day conditions, followed by three weeks in long-day conditions.

(C) Phenotypes of plants of the indicated genotypes grown for 6 weeks in short-day conditions. (A-C) Scale bars = 1 cm.

Fig. 2. AP2C1 and MKP1 control wound-induced MAPK activities.

Analysis of wound-induced MPK6, MPK4 and MPK3 kinase activities and protein amounts of leaves from six-week-old WT, ap2c1, mkp1, and ap2c1 mkp1 plants grown in short-day conditions.

(A) MAPK activities were determined after immunoprecipitation by phosphorylation of MBP detected by autoradiography. The entire kinase assay is based on one common master mix containing MBP and γ-ATP. Loading is demonstrated by Coomassie Blue staining (CBS); representative lanes are shown. The experiment was repeated two times with similar results.

(B) MAPK protein amounts before and after wounding are demonstrated by immunoblotting of MPK3, MPK4, and MPK6 from total protein extract using specific antibodies. Loading is demonstrated by Ponceau S staining (Rubisco protein). mpw: minutes post wounding.

Fig. 3. ap2c1 mkp1 plants have elevated ACS6 expression and produce more ET upon wounding than WT, mainly mediated by MPK6.

(A) ET levels produced by four-week-old plants of the indicated genotypes grown in standard long-day conditions. ET amounts are shown as pL per mg plant fresh weight (FW) per hour. Bars represent mean values of three biological replicates ± SD, *p < 0.05 Student’s t-test.
(B) RT-qPCR analysis of ACS6 expression in leaves of six-week-old plants of the indicated genotypes grown in short-day conditions, where expression levels in WT are set to 1. Bars represent mean values of three biological replicates ± SD, *p < 0.05 Student’s t-test.

Fig. 4. Venn diagram of TFs differentially expressed in the MAPK phosphatase mutant plants. The number of genes at least three-fold up- or downregulated in ap2c1, in mkp1 and in ap2c1 mkp1 plants compared to WT in three biological replicates is indicated. The expression of 1,880 TF-encoding genes was analysed.

Fig. 5. Expression of WRKY-encoding (W) genes.

The transcript levels of WRKY-encoding (W) genes were quantified by RT-qPCR in plants of the indicated genotypes and compared to WT (values set to 1). Bars represent mean values of three replicates ± SD. Data are plotted on a log10 scale after normalisation over WT values, *p < 0.05, **p < 0.01 Mann-Whitney U test.

Fig. 6. Genes encoding members of the AP2/ERF TF family are highly upregulated in ap2c1 mkp1 plants.

Transcript levels of AP2/ERF encoding genes were quantified by RT-qPCR in plants of the indicated genotypes and compared to WT, where expression levels in WT were set to 1. Bars represent mean values of at least three replicates ± SD, *p < 0.05, **p < 0.01 Mann-Whitney U test.

Fig. 7. Genes encoding members of the ANAC TF family are highly upregulated in ap2c1 mkp1 plants.

Transcript levels were quantified by RT-qPCR in plants of the indicated genotypes and compared to WT, where expression levels were set to 1. Bars represent mean values of at least three replicates ± SD, *p < 0.05, Mann-Whitney U test.
Fig. 8. Camalexin accumulation in *ap2c1 mkp1* plants is mainly mediated by MPK6 and MPK3.

Levels of total camalexin determined by HPLC in leaves of 4-week-old plants of the indicated genotypes. Results shown are mean with SE (n=4), n.d. = not detected, **p < 0.01, Student’s *t*-test.

Fig. 9. *ap2c1 mkp1* plants accumulate high levels of SA in a MPK6-dependent manner.

Total SA levels of five-week-old plants of the indicated genotypes grown in standard short-day conditions were determined by HPLC and expressed as ng per g FW. Error bars represent SD of four biological replicates, ***p < 0.001, Student’s *t*-test.

Fig. 10. Upregulation of the senescence-marker gene *SAG12* in *ap2c1 mkp1* plants is mainly mediated by MPK6.

RT-qPCR quantification of *SAG12* transcript level in plants of the indicated genotypes compared to WT plants grown in standard short-day conditions. Error bars represent SD of three biological replicates, *p < 0.05, Student’s *t*-test.
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