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

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38 **Running title**

39 AP2C1 and MKP1 regulate defence responses

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41 **Highlight**

42 Double MAPK phosphatase mutant plants *ap2c1 mkp1* exhibit constitutive,
43 autoimmune-like stress responses, dependent on their substrate MAPK
44 MPK6.

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61 Abstract

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

82

83 Keywords

84 ANAC, AP2C1, Arabidopsis, DSP, MAPK, MKP1, PP2C, WRKY.

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88 **Introduction**

89 Reversible protein phosphorylation is one of the most commonly used
 90 mechanisms for the molecular transmission of stress signals and
 91 developmental cues. This mechanism is based on the opposing actions of
 92 protein kinases and protein phosphatases. Mitogen-activated protein kinases
 93 (MAPKs) are highly conserved major components of developmental and
 94 stress signalling cascades in eukaryotes. MAPKs are activated by upstream
 95 MAPK kinases via phosphorylation of Thr and Tyr within their activation loop.
 96 This activation eventually leads to the reprogramming of cellular activities,
 97 including the modulation of gene expression, to generate appropriate
 98 responses. The activation of MAPKs does not represent a simple on/off
 99 switch, as both the magnitude and duration of activation are crucial for
 100 determining the signalling outcome (Marshall, 1995). Prolonged or constant
 101 activation of a MAPK cascade can have detrimental effects as illustrated by
 102 the hypersensitive response (HR)-induced cell death in plants expressing a
 103 constitutively active MAPK kinase version (Liu *et al.*, 2007; Ren *et al.*, 2002).
 104 Thus, negative regulation and inactivation mechanisms are important for the
 105 correct cellular response. Specific protein phosphatases can dephosphorylate
 106 and thereby inactivate MAPKs. As dual phosphorylation of the Thr-X-Tyr motif
 107 in the activation loop is required for MAPK activation (Caunt and Keyse,
 108 2013), dephosphorylation of either phospho-amino acid residue inactivates
 109 the MAPK and inhibits downstream signalling. Interestingly, this inactivation
 110 can be accomplished by evolutionarily distant protein phosphatases, including
 111 PP2C-type MAPK phosphatases (Fuchs *et al.*, 2013; Schweighofer *et al.*,
 112 2004; Schweighofer *et al.*, 2007) and PTP-type dual specificity (Tyr and
 113 Ser/Thr) phosphatases (DSPs) (Bartels *et al.*, 2010; Jiang *et al.*, 2018).
 114 However, their interplay is presently unknown.

115 *Arabidopsis thaliana* DSP-type mitogen-activated protein kinase phosphatase
 116 1 (MKP1) interacts with the stress-responsive MAPKs MPK3, MPK4 and
 117 MPK6, and controls their activities (Anderson *et al.*, 2011; Bartels *et al.*, 2009;
 118 Ulm *et al.*, 2002). The *mkp1* knockout mutant is hypersensitive to genotoxic
 119 stress, including UV-B radiation (Gonzalez Besteiro *et al.*, 2011; Gonzalez
 120 Besteiro and Ulm, 2013; Ulm *et al.*, 2002; Ulm *et al.*, 2001), but is more

121 resistant than wild type (WT) to the virulent bacterial pathogen *Pseudomonas*
 122 *syringae* pv. *tomato* (*Pst*) (Anderson *et al.*, 2011; Anderson *et al.*, 2014;
 123 Bartels *et al.*, 2009). Specifically in the Arabidopsis Columbia accession,
 124 *mkp1* shows an autoimmune-like growth phenotype dependent on the disease
 125 resistance gene homologue *SUPPRESSOR OF npr1-1*, *CONSTITUTIVE 1*
 126 (*SNC1*) and is associated with enhanced MAPK activities (Bartels *et al.*,
 127 2009). The phospho-Tyr-specific PTP-type protein phosphatase PTP1 also
 128 interacts with MPK6 and MPK3 in transient assays. The lack of both MKP1
 129 and PTP1 in the *mkp1 ptp1* double mutant leads to upregulation of MPK6-
 130 dependent plant defence responses and a further enhanced autoimmune-like
 131 phenotype (Bartels *et al.*, 2009).

132 A group of PP2C-type phosphatases, including AP2C1, interacts with
 133 MAPKs and controls their activities (Schweighofer *et al.*, 2007; Umbrasaite *et*
 134 *al.*, 2010). AP2C1 is induced by wounding and biotic stress, and functions as
 135 a negative regulator of MPK3, MPK4 and MPK6 controlling levels of wound-
 136 induced jasmonate and ethylene (ET) as well as plant immunity (Galletti *et al.*,
 137 2011; Schweighofer *et al.*, 2007; Shubchynskyy *et al.*, 2017; Sidonskaya *et*
 138 *al.*, 2016). *ap2c1* plants do not display obvious developmental phenotypes
 139 under standard growth conditions (Schweighofer *et al.*, 2007), implying
 140 specific AP2C1 function under stress conditions and contribution of other,
 141 presently unknown, MAPK phosphatases for MAPK control in the absence of
 142 AP2C1.

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

153 Considering the broad spectrum of signals transmitted by the same
 154 MAPKs (Meng and Zhang, 2013; Rodriguez *et al.*, 2010), such as MPK6, it is

155 puzzling how the specificity of the responses for perceived stimuli is
156 generated. The phylogenetic diversity and distinct enzymatic mechanisms of
157 protein phosphatases that are able to inactivate MAPKs support the idea of a
158 contribution of MAPK phosphatases to the versatility and specificity of MAPK
159 networks. Here, we investigate the roles of the phylogenetically distant
160 Arabidopsis MAPK phosphatases AP2C1 and MKP1 and, in particular, their
161 functional redundancies. We show that AP2C1 and MKP1 together repress
162 plant autoimmune-like responses, including salicylic acid (SA) and ET
163 accumulation, and early senescence. These observations in the *ap2c1 mkp1*
164 mutant are underlined by the misexpression of specific transcription factors,
165 including members of the *WRKY*, *AP2/ERF*, and Arabidopsis NAM, ATAF,
166 and CUC (*ANAC*) families whose expression is – at least partially – mediated
167 by MPK6.

168

169 **Materials and methods**

170 **Plant lines, genetic crosses and growth conditions**

171 All plant lines used in this study were in the *Arabidopsis thaliana* accession
172 Columbia (Col-0), with *mkp1* being an introgression line from a Wassilewskija
173 background (Bartels *et al.*, 2009). The T-DNA insertion line *ap2c1*
174 (SALK_065126; (Schweighofer *et al.*, 2007)) was crossed with the T-DNA
175 insertion lines *ptp1* (SALK_118658) and *mkp1*, respectively, to generate the
176 *ap2c1 ptp1* and *ap2c1 mkp1* double mutants. *mpk6-2* (SALK_073907) was
177 used for genetic crosses generating *ap2c1 mkp1 mpk6*. The T-DNA insertion
178 lines *ap2c2* (GABI-Kat_316F11) and *ap2c3* (SALK_109986) (Umbrasaite *et*
179 *al.*, 2010) were crossed with *mkp1* to generate *ap2c2 mkp1* and *ap2c3 mkp1*
180 double mutants. Combinatorial mutants were identified in the F2 generation
181 and also confirmed in subsequent generations by PCR genotyping using T-
182 DNA- and gene-specific primers (Bartels *et al.*, 2009; Schweighofer *et al.*,
183 2007; Umbrasaite *et al.*, 2010). For protein and RNA extraction, as well as for
184 ET and SA measurements, plants were grown on soil for five to seven weeks
185 in a phytotron chamber under short-day conditions (8 h light, 22°C/16 h dark,
186 20°C cycle).

187 For experiments at the seedling stage, seeds were surface sterilized and
188 spread on plates containing half-strength MS (Murashige and Skoog) medium

189 (Duchefa), pH 5.7, 1% (w/v) sucrose and 0.7% plant agar (w/v; Duchefa).
 190 Seedlings were grown in long-day conditions (16 h light/8 h dark) at 22°C. If
 191 indicated, *ap2c1 mkp1* plants were kept at 26°C during day and 22°C at night
 192 with 95% humidity and short-day conditions (8 h light/16 h dark).

193

194 *Ex vivo* kinase activity assay and MAPK immunoblotting

195 Plant protein extraction and the *ex vivo* kinase assay were performed as
 196 described (Schweighofer *et al.*, 2007; Schweighofer *et al.*, 2009) using
 197 polyclonal antibodies for immunoprecipitation and myelin basic protein (MBP)
 198 as *in vitro* substrate of immunoprecipitated MAPKs. MAPK protein amounts
 199 were visualised with Sigma antibodies Anti-AtMPK3 (M8318), Anti-MPK4
 200 (A6979) and Anti-AtMPK6 (A7104).

201

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

203 Total RNA from leaves was isolated with the RNeasy Plant Mini Kit (Qiagen)
 204 and treated with TURBO DNA-free DNaseI (Ambion) according to the
 205 manufacturers' instructions. RNA integrity was checked on 1% (w/v) agarose
 206 gels and the concentration measured before and after DNase I digestion. The
 207 absence of genomic DNA was verified by PCR using primers targeting an
 208 intron of the control gene *At5g65080*. cDNA synthesis was performed using
 209 First Strand cDNA Synthesis Kit (Thermo Scientific). The efficiency of cDNA
 210 synthesis was estimated by RT-qPCR analysis using a primer pair amplifying
 211 the 3' part of the control gene encoding GAPDH and a primer pair amplifying
 212 the 5' part of the same gene. RT-qPCR reactions were performed as
 213 described previously (Balazadeh *et al.*, 2008). *ACTIN2* was selected as a
 214 reference gene for which four replicates were measured in each PCR run, and
 215 their average cycle threshold (CT) was used for relative expression analyses.
 216 TF expression data were normalized by subtracting the mean *ACTIN2* gene
 217 CT value from the CT value (Δ CT) of each gene of interest. The expression
 218 value in the comparison between different genotypes was calculated using the
 219 expression $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ represents ΔCT mutant of interest minus ΔCT
 220 control (wild type, WT). For TF expression profiling, an advanced version of
 221 an expression profiling platform (Balazadeh *et al.*, 2008) that was originally
 222 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 Al₂O₃ 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).

AGI codes

The AGI codes of the genes analysed in this report are:

At1g01010, At1g02220, At1g02250, At1g02340, At1g04240, At1g04370,
At1g07160, At1g08320, At1g13300, At1g18570, At1g18860, At1g18860,
At1g19210, At1g29860, At1g36060, At1g52830, At1g62300, At1g66560,
At1g66560, At1g67100, At1g69600, At1g71520, At1g71860, At1g74080,
At1g75250, At1g80840, At2g01200, At2g30020, At2g33710, At2g37430,
At2g38250, At2g38340, At2g38470, At2g40180, At2g40470, At2g40740,
At2g40740, At2g43000, At2g43140, At2g43790, At2g47520, At3g01970,
At3g04070, At3g13840, At3g15320, At3g15500, At3g21330, At3g23230,
At3g23240, At3g23250, At3g26790, At3g26830, At3g44350, At3g45640,
At3g46080, At3g46090, At3g50510, At3g53600, At3g55270, At4g01370,
At4g01720, At4g01720, At4g18170, At4g18870, At4g23810, At4g32280,
At4g36990, At5g01900, At5g04390, At5g13080, At5g13330, At5g15160,
At5g19520, At5g20240, At5g22570, At5g23000, At5g24110, At5g27810,
At5g39860, At5g43290, At5g43650, At5g44260, At5g45890, At5g46350,

At5g51790, At5g56840, At5g56960, At5g59820, At5g64750, At5g64810,
At5g67450.

258

259

260 Results

261 Double *ap2c1 mkp1* mutant plants show growth and development 262 defects which are at least partially mediated by MPK6

263 To investigate the specific and/or overlapping roles of the MAPK
264 phosphatases AP2C1 and MKP1, we took advantage of the Arabidopsis T-
265 DNA insertion knock-out mutants *ap2c1* and *mkp1*, respectively (Bartels *et al.*,
266 2009; Schweighofer *et al.*, 2007). Phenotypically, *ap2c1* and *mkp1* mutant
267 plants did not show any difference compared to WT when grown for up to five
268 weeks under short-day conditions (Figure 1A, 1C, 1E). However, long-day
269 *mkp1* plants demonstrated altered morphology, such as aberrant leaf
270 development and early senescence, which appeared approximately three
271 weeks after germination (Supplementary Figure 1A), as described previously
272 (Bartels *et al.*, 2009).

273 To further analyse AP2C1 and MKP1 functions in plants, we generated a
274 double mutant by genetic crossing. *ap2c1 mkp1* plants showed phenotypic
275 differences compared to WT and single mutants. These appeared two weeks
276 after germination under standard growth conditions in soil; first their sizes
277 started differing, and during further growth *ap2c1 mkp1* plants revealed more
278 pronounced multiple defects, including severe dwarfism and aberrant leaf
279 development (Figures 1A, 1B, 1C). Four weeks after germination, phenotypic
280 abnormalities became even more evident as early senescence, spontaneous
281 macroscopic lesions and abnormal leaf morphology. Interestingly, these
282 developmental defects were suppressed when plants were grown under
283 conditions of elevated humidity and increased temperature (Figure 1D),
284 indicating a dependency on environmental cues. However, during flowering,
285 misshaped inflorescences and strongly reduced fertility were always observed
286 (Supplementary Figure 2D, 2E). These phenotypes were specific for *ap2c1*
287 *mkp1* plants, as crossing *ap2c1* with *ptp1* did not lead to phenotypic
288 alterations (Figure 1E) compared with *mkp1 ptp1* (Bartels *et al.*, 2009).
289 Crossing *mkp1* with either of two other clade B AP2C mutants, *ap2c2* or

290 *ap2c3* (Umbrasaite *et al.*, 2010), led to mild phenotypes compared with the
291 strong defects of *ap2c1 mkp1* plants (Supplementary Figure 1B, C).

292 Since MPK6 is a documented target of action by AP2C1 and MKP1
293 (Schweighofer *et al.*, 2007; Ulm *et al.*, 2002) we set to address the impact of
294 MPK6 on phenotypic aberrations detected in *ap2c1 mkp1* plants. To this goal,
295 triple mutant plants *ap2c1 mkp1 mpk6* were created and their phenotype was
296 compared with that of *ap2c1 mkp1*. The phenotype of *ap2c1 mkp1 mpk6*
297 plants was more similar to WT than to *ap2c1 mkp1*. The loss of MPK6
298 suppressed most phenotypic defects observed in *ap2c1 mkp1*, such as
299 extreme dwarfism, aberrant leaf shapes, premature leaf senescence and
300 impaired fertility (Figure 1C). However, at later developmental stages, *ap2c1*
301 *mkp1 mpk6* plants appeared overall smaller than WT and displayed
302 senescence in the older leaves (Supplementary Figures 1C, 2G).

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

307

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

309 Our previous work has revealed the involvement of AP2C1 in the regulation of
310 MAPK activities induced by pathogen-associated molecular patterns (PAMP),
311 wounding, and nematodes (Schweighofer *et al.*, 2007; Shubchynskyy *et al.*,
312 2017; Sidonskaya *et al.*, 2016). To check for a potential overlapping role by
313 MKP1, we firstly analysed MPK3, MPK4 and MPK6 activities after wounding
314 the leaves of WT and of the single mutant plants *ap2c1* and *mkp1*. Kinase
315 activities were assayed after immunoprecipitation from total protein extracts
316 using specific antibodies. In agreement with our previous findings
317 (Schweighofer *et al.*, 2007), *ap2c1* plants showed higher and sustained
318 wound-induced activities of MPK3, MPK4 and MPK6 compared to WT (Figure
319 2). Interestingly, MPK4 activity was more intense and sustained in *ap2c1*
320 compared to the *mkp1* plants, indicating a specific role of AP2C1 in the
321 regulation of MPK4 during wounding. MPK3 activity in *ap2c1* plants was
322 slightly enhanced and more sustained in comparison to WT and *mkp1*. In
323 *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* (Figure 2). The MPK3, MPK4, and MPK6 protein levels were comparable in both mutants and WT (Figure 2).

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 (Figure 3), 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 (Ju and Chang, 2012; Wang *et al.*, 2002). 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 (Li *et al.*, 2012; Liu and Zhang, 2004), 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 (Figure 4A). However, significantly higher levels of ET accumulated in wounded *mkp1* plants and even more so in the *ap2c1 mkp1* double mutant (Figure 4A). 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 (Figure 4A).

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 (Figure 4B). 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 *ap2c1*, *mkp1* or *ap2c1 mkp1* plants when compared to WT. We identified three genes encoding TFs that were deregulated in *ap2c1*, but not in *mkp1* (Supplementary Table I), while 25 genes were deregulated in *mkp1*, but not in *ap2c1* (Supplementary Table II), and four genes concomitantly regulated by AP2C1 and MKP1 (Supplementary Table III). Figure 5 shows the number of genes whose expression levels were changed in *ap2c1*, *mkp1* or *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 *ap2c1 mkip1* double mutant plants. Among them, genes encoding members of the WRKY family were most abundant: 15 WRKY genes were upregulated (Figure 6, Supplementary Table IV) and one downregulated (Supplementary Table IV). A further prevalent group of TF-encoding genes affected in *ap2c1 mkip1* plants includes AP2/ERF described for their involvement in development, including RAP2.6L (Yang *et al.*, 2018) and WIND3 (Smit *et al.*, 2020) (Figure 7). ANAC TF family members are implicated in senescence and stress-related processes (Bu *et al.*, 2008; Jensen *et al.*, 2010; Saga *et al.*, 2012; Wu *et al.*, 2012). Our results show that several ANAC TF-encoding genes are upregulated in *ap2c1 mkip1* plants (Figure 8). 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 *ap2c1 mkip1 mpk6* plants are phenotypically much less affected than *ap2c1 mkip1* double mutants suggested that severe phenotypic aberrations in the latter are mediated by MPK6. This prompted us to investigate *ap2c1 mkip1 mpk6* plants for the expression of TFs misregulated in *ap2c1 mkip1*. Indeed, 50 of the 76 TF genes strongly affected in *ap2c1 mkip1* plants were not altered in their expression in *ap2c1 mkip1 mpk6*, linking MPK6 over-activation to their misexpression. Among these, expression of WRKY6, WRKY28, WRKY33 and WRKY45 (Figure 6B), the AP2/ERF family members RAP2.6L, WIND3, ERF1, DREB19, ERF14, and ERF17 (Figure 7), and of several ANAC TF-encoding genes, such as ANAC005, ANAC042, ANAC003, ANAC047, and ANAC055—(Figure 8) are dependent on the presence of MPK6. The expression of WRKY75, WRKY71, WRKY38 and WRKY30 (Figure 6), and of the AP2/ERF genes At2g33710, At1g71520 and At3g23230 remained upregulated more than 5-fold in the absence of MPK6 in *ap2c1 mkip1 mpk6* plants (Figure 7), suggesting that regulation of these TFs is controlled by other factors (possibly other MAPKs).

Defence responses, camalexin, SA and the senescence marker gene *SENESCENCE-ASSOCIATED GENE12* (*SAG12*) are upregulated in *ap2c1 mkip1* plants

426 It has been shown previously that *mkp1* plants accumulate higher levels of the
427 phytoalexin camalexin (Bartels *et al.*, 2009). To investigate if the expression of
428 genes encoding camalexin biosynthesis enzymes was affected in *ap2c1 mkp1*
429 plants, we studied the expression of a key gene in the pathway,
430 *CYP71B15/PAD3*. A strong upregulation (more than 300-fold, respectively)
431 was detected in *ap2c1 mkp1* plants compared to WT (Figure 9). Moreover,
432 *ap2c1 mkp1 mpk6* plants still had remarkably high transcript levels
433 (upregulation ca. 10-fold) of *CYP71B15/PAD3*, showing that MPK6 is an
434 important, but not the sole factor behind its upregulation in *ap2c1 mkp1*
435 plants. Also, *mkp1* single mutant plants showed a >10-fold upregulation of the
436 gene (Figure 9A).

437 To investigate if the increased *CYP71B15/PAD3* expression level correlates
438 with camalexin accumulation, total camalexin was quantified in WT and
439 mutant plants. Indeed, in agreement with previous findings (Bartels *et al.*,
440 2009) we found increased camalexin levels in *mkp1* plants and very high
441 camalexin accumulation in the *ap2c1 mkp1* mutant, which was not solely
442 dependent on MPK6 (Figure 9B).

443 Upregulation of MAPK activities and macroscopic lesion formation in
444 leaves of *ap2c1 mkp1* indicated the possible activation of a hypersensitive-like
445 response in these plants. Since this is associated with the accumulation of the
446 stress hormone SA, we measured SA in leaves of *ap2c1 mkp1*, *ap2c1 mkp1*
447 *mpk6* as well as in WT and single mutants. Indeed, we found a 35-fold
448 increase of SA in *ap2c1 mkp1* plants compared to WT (Figure 10), whereas
449 *ap2c1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants showed SA amounts similar to
450 the WT. In agreement with previous data (Bartels *et al.*, 2009), we detected
451 enhanced total SA amounts (>2-fold) also in *mkp1* plants compared to WT
452 (Figure 10).

453 Leaf necrosis observed in *ap2c1 mkp1* leaves (Figure 1B) and the
454 upregulation of *WRKY6* (Figure 6B), which is a senescence-related marker
455 gene (Rushton *et al.*, 2010), suggested that early senescence was induced in
456 these plants. Thus, we investigated the expression of the senescence-specific
457 marker gene *SAG12* (Noh and Amasino, 1999) and found that it strongly
458 upregulated in *ap2c1 mkp1* plants, dependent on MPK6 (Figure 11). This data

along with the upregulation of *WRKY6* (Figure 6B) indicates 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; Rasmussen *et al.*, 2012; Rodriguez *et al.*, 2010). 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; Meng and Zhang, 2013; Rodriguez *et al.*, 2010). 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 (Bigeard and Hirt, 2018; Peng *et al.*, 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 (Anderson *et al.*, 2011; Bartels *et al.*, 2009; Galletti *et al.*, 2011; Schweighofer *et al.*, 2007; Shubchynskyy *et al.*, 2017; Sidonskaya *et al.*, 2016; Ulm *et al.*, 2002). Enhanced activation of MAPKs by wounding 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 compared to *ap2c1* *versus* WT suggests that the contribution of MKP1 to inactivating MAPKs is already set before wounding, or during a very early stage of signalling. 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 demonstrated 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 (Schweighofer *et al.*, 2014; Umbrasaite *et al.*, 2010; Umbrasaite *et al.*, 2011) 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 MKK1/2 (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 mkip1*-related phenotypes described in this work (see Results).

The single *mkip1* and the double *mkip1 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 mkip1* compared to *mkip1* plants suggests a collaborative action of both AP2C1 and MKP1 as negative regulators of SA and camalexin production (this work). This accumulation is probably MPK6-dependent, as the introduction of the *mpk6* mutation in *ap2c1 mkip1 mpk6* plants restores SA and camalexin levels similar to those of WT and *mkip1 ptp1 mpk6* mutant (Bartels *et al.*, 2009). Notably, rescue of the severe *ap2c1 mkip1* growth phenotypes by elevated temperature is in accordance with the observed temperature dependency of SA-related phenotypes (Ichimura *et al.*, 2006; Su *et al.*, 2007; Suarez-Rodriguez *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 *mkip1* in the Col-0 accession, where partial rescue of *mkip1* and *mkip1 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).

561 Previous findings that SA acts together with ET to regulate cell death
562 (Rao *et al.*, 2002), the requirement of ET biosynthesis for H₂O₂ accumulation
563 and subsequent cell death (Overmyer *et al.*, 2003), and the induction of cell
564 death in Arabidopsis leaves by persistent activation of MAPKs with gain-of-
565 function MKK4 and MKK5 (Ren *et al.*, 2002) all correlate with the cell death
566 phenotype observed in the *ap2c1 mkp1* mutant, where MAPKs - and other
567 stress-related factors - may be (hyper)-activated. Therefore, we conclude that
568 the majority of the phenotypes observed in *ap2c1 mkp1* plants, both visible
569 and molecular, are due to the misregulation of MAPK pathways, even in the
570 absence of stress.

571

572 **AP2C1 and MKP1 affect MAPK-regulated ET biosynthesis**

573 Activated MPK6 controls ET levels by both inducing the transcription of ACS
574 family genes and by phosphorylating ACS proteins, the rate-limiting enzymes
575 in ET biosynthesis. Phosphorylated ACSs become more stable and, thus, ET
576 synthesis is enhanced by elevated MPK6 activity (Kim *et al.*, 2003; Li *et al.*,
577 2012; Liu and Zhang, 2004; Xu *et al.*, 2008). In *ap2c1 mkp1*, the enhanced ET
578 production is certainly due to, at least in part, the highly increased expression
579 of ACS6 compared to WT. A considerable additive effect on ET
580 overproduction by the double *ap2c1 mkp1* mutation suggests that even
581 though MKP1 is a determining MAPK phosphatase affecting ET production,
582 there are overlapping and non-redundant functions of AP2C1 and MKP1 in
583 the regulation of stress-induced ET biosynthesis. Detection of enhanced and
584 MPK6-dependent expression of WRKY33, encoding a TF that binds to the
585 promoter of ACS genes and is a substrate of MPK3/MPK6, suggests an
586 involvement of WKRY33 itself in ACS overexpression in *ap2c1 mkp1* plants
587 (this work and (Li *et al.*, 2012)). The identification of genes encoding TFs of
588 the AP2/ERF family members (ET-responsive element-binding proteins)
589 among the uppermost induced ones in *ap2c1 mkp1* plants suggests a path to
590 enhanced ET amounts in these plants.

591

592 **AP2C1 and MKP1 control the expression of stress-responsive TF-** 593 **encoding genes, predominantly via MPK6**

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 *WRKY*s indicate that stress responses are constitutively active in these plants. This correlates with reports demonstrating an involvement of *WRKY*s 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*, *WRKY42*), and defence- and senescence-related processes (*WRKY6*) (Rushton *et al.*, 2010).

Direct feedback mechanisms among *WRKY*s themselves have been shown (Mao *et al.*, 2011) and are generally proposed, where *WRKY*s positively auto-regulate their own gene expression and/or cross-regulate expression of other *WRKY* genes (Birkenbihl *et al.*, 2017; Mao *et al.*, 2011; Pandey and Somssich, 2009). 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 seems to be a major player responsible for mediating the upregulation of several *WRKY*s, *AP2/ERFs*, *ANACs* and other TF-encoding genes. MPK6 controls the expression of several *WRKY*s to different extents, as shown in *ap2c1 mkp1* plants compared to *ap2c1 mkp1 mpk6* (Figure 6A). This data demonstrates that not only MPK6 but also other factor(s) affect *WRKY* gene expression. MPK3, as the closest paralogue of MPK6, may be a possible candidate, since MPK3 is also more strongly activated in *ap2c1 mkp1* than in the respective single mutant plants. We confirmed MPK6-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 (Birkenbihl *et al.*, 2017; Qiu *et al.*, 2008). Thus, our data suggest that AP2C1 and MKP1 may play a dual role in regulating camalexin biosynthesis, on the one hand by controlling MPK6 activity, which positively regulates *WRKY33* expression, and on the other by

controlling MPK4 activity, which in turn stimulates WRKY33 leading to transactivation of *CYP71B15/PAD3*.

A PAMP-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, show that for *WRKY22/29* overexpression the 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 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; He *et al.*, 2001; John *et al.*, 2001; Lim *et al.*, 2007; Miller *et al.*, 1999). 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* (Guo and Gan, 2005; Noh and Amasino, 1999). 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 MPK6-dependent induction of *ANAC005*, *JUB1/ANAC042* (Saga *et al.*, 2012; Shahnejat-Bushehri *et al.*, 2016; Wu *et al.*, 2012), *ANAC003/XVP* (Yang *et al.*, 2020), *ANAC047* (Mito *et al.*, 2011), and *ANAC055* (Bu *et al.*, 2008; Hickman *et al.*, 2013; Schweizer *et al.*, 2013; Tran *et al.*, 2004) in *ap2c1*

661 *mkp1* plants. This induction of senescence-related TFs reveals a novel link
662 between senescence-related processes and MAPK signalling.

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

668 Taken together, our results show that two evolutionarily unrelated MAPK
669 phosphatases, AP2C1 and MKP1, perform both distinct and overlapping
670 functions in the regulation of stress-induced MPK3, MPK4 and MPK6
671 activities. Our genetic dissection indicates that the known role of MPK6 in
672 mediating cell death, ET-, SA- and senescence-related phenotypes is
673 attenuated by both AP2C1 and MKP1. It also demonstrates that the
674 expression of specific TF-encoding genes is affected by MAPK(s)
675 hyperactivation due to the lack of these two MAPK phosphatases *in planta*,
676 revealing potential new target genes downstream of MPK6 signalling.
677 Additionally, our data suggest new roles for MPK3 or MPK4 in the regulation
678 of cell signalling. In the future, the study of individual and combinatorial
679 mutants will allow us to genetically disentangle the contribution of specific
680 protein kinases and phosphatases to complex signalling networks and
681 downstream cell responses.

682

683 **Supplementary data**

684 **Supplementary Figure 1:** Phenotypes of Arabidopsis single, double and
685 triple mutant plants.

686 **Supplementary Figure 2:** Loss of both AP2C1 and MKP1 leads to severe
687 phenotypes in growth and development, which are mediated by MPK6.

688 **Supplementary Table I.** Expression of TF-encoding genes modulated by the
689 absence of AP2C1 but not MKP1.

690 **Supplementary Table II.** Expression of TF-encoding genes modulated by the
691 absence of MKP1 but not of AP2C1.

692 **Supplementary Table III.** Expression of TF-encoding genes modulated by
693 the absence of both, MKP1 and AP2C1.

694 **Supplementary Table IV.** TF-encoding genes deregulated in *ap2c1 mkp1*
695 plants.

696

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709

710 **Author contributions**

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

714

715 **Dedication**

716 The authors dedicate this article to the memories of:

717

718 Irute Meskiene (1956-2017)

719 (Paškauskas *et al.*, 2017)

720

721 Manfred Schwanninger (1963-2013)

722 (Meder, 2014)

723

724 **Data availability statement**

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

727

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Figure legends

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

A. Phenotypes of WT, *ap2c1*, *mkp1* and *ap2c1 mkp1* plants grown for 8 weeks in short-day conditions. Scale bars = 1 cm. **B.** Formation of macroscopic lesions in leaves of *ap2c1 mkp1* plant shown in Figure 1A. Scale bars = 0.5 cm. **C.** Phenotypes of five-week-old WT, *ap2c1*, *mkp1*, *mpk6*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* plants grown in standard short-day conditions. Scale bars = 1 cm. **D.** Phenotypes of five-week-old WT, *ap2c1*, *mkp1*, *mpk6*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* plants grown in conditions with increased humidity and elevated temperature. Scale bars = 1 cm. **E.** Phenotypes of WT, *ap2c1*, *ptp1* and *ap2c1 ptp1* plants grown for 6 weeks in short-day conditions. Scale bars = 1 cm.

Figure 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. MAPK activities were determined after immunoprecipitation by phosphorylation of myelin basic protein detected by autoradiography. Loading is demonstrated by Coomassie Blue staining (CBS); representative lanes are shown. 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.

Figure 3. Detection of MKP1 and AP2C1 expression levels in *ap2c1* and *mkp1* mutants.

Levels of *MKP1* and *AP2C1* transcripts were quantified by RT-qPCR in 14-day-old seedlings grown in long-day conditions, and compared with levels in WT plants. Transcript amounts of *MKP1* and *AP2C1* in WT were taken as 100%. The relative transcript amounts were normalized to the reference gene,

ACTIN2. Results are the mean of two biological and two technical replicates for each experiment, * $p < 0.05$, Student's *t*-test.

Figure 4. *ap2c1 mkp1* plants have elevated *ASC6* expression and produce more ET upon wounding than WT, mainly mediated by MPK6.

A. ET levels produced by four-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, and *ap2c1 mkp1 mpk6* plants grown in standard long-day conditions. Values are expressed as ET amounts produced per mg plant fresh weight (FW) per hour. Bars represent mean values of three biological replicates $\pm$ SD, * $p < 0.05$ Student's *t*-test. **B.** RT-qPCR analysis of *ASC6* expression in leaves of six-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, and *ap2c1 mkp1 mpk6* plants grown in short-day conditions, where expression levels in WT are set to 1. Bars represent mean values of three biological replicates $\pm$ SD, * $p < 0.05$ Student's *t*-test.

Figure 5. Venn diagram of TFs differentially expressed in 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.

Figure 6. Expression of WRKY-encoding (W) genes.

The transcript levels of WRKY-encoding (W) genes were quantified by RT-qPCR in *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants, and compared to WT (values set to 1). Bars represent mean values of three replicates $\pm$ SD. **A.** Upregulation of *WRKY75*, *WRKY30*, *WRKY71*, and *WRKY38* transcript levels in *ap2c1 mkp1* is not solely dependent on MPK6. Data are expressed on a $\log_{10}$ scale after normalisation over WT values. **B.** Upregulation of *WRKY61*, *WRKY64*, *WRKY62*, *WRKY51*, *WRKY45*, *WRKY55*, *WRKY40*, *WRKY6*, *WRKY47*, *WRKY28* and *WRKY33* transcript levels depends on MPK6, * $p < 0.05$, ** $p < 0.01$ Mann-Whitney *U* test.

Figure 7. 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 *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants and compared to WT, where expression levels were set to 1. Bars represent mean values of at least three replicates $\pm$ SD. Strong upregulation of *At2g33710*, *At1g71520* and *At3g23230* (*ERF98/TDR1*) in *ap2c1 mkp1* is not solely dependent on MPK6, while upregulation of *RAP2.6L*, *WIND3*, *ERF1*, *DREB19*, *ERF14*, and *ERF17* in *ap2c1 mkp1* is almost completely dependent on MPK6, * $p < 0.05$, ** $p < 0.01$ Mann-Whitney *U* test.

Figure 8. Genes encoding members of the ANAC TF family are highly upregulated in *ap2c1 mkp1* plants.

Transcript levels were quantified by RT-qPCR in *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants and compared to WT, where expression levels were set to 1. Bars represent mean values of at least three replicates $\pm$ SD. *ANAC005*, *ANAC042*, *ANAC003*, *ANAC047*, and *ANAC055* are upregulated in *ap2c1 mkp1* in a MPK6-dependent manner, while in *ap2c1 mkp1 mpk6* plants their levels are similar to WT, * $p < 0.05$, Mann-Whitney *U* test.

Figure 9. Upregulation of camalexin biosynthetic gene *CYP71B15/PAD3* and camalexin accumulation in *ap2c1 mkp1* plants are mostly mediated by MPK6.

A. Transcript level of *CYP71B15/PAD3*, an enzyme required for camalexin biosynthesis, was quantified by RT-qPCR in *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants and compared to WT, where expression levels were set to 1. Bars represent mean values of at least three replicates $\pm$ SD, expressed on a $\log_{10}$ scale. **B.** Levels of total camalexin determined by HPLC in leaves of 4-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6* and *mpk6* plants. Results shown are mean with SE ($n=4$), n.d. = not detected, * $p < 0.05$, ** $p < 0.01$, Student's *t*-test.

Figure 10. *ap2c1 mkp1* plants accumulate high levels of SA in a MPK6-dependent manner.

Total SA levels of five-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6*, and *mpk6* plants grown in standard short-day conditions, 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.

Figure 11. Upregulation of the senescence-marker gene *SAG12* in *ap2c1 mkp1* plants is mainly mediated by MPK6.

RT-qPCR quantification of *SAG12* transcript level in leaves of six-week-old *ap2c1*, *mkp1*, *ap2c1 mkp1*, and *ap2c1 mkp1 mpk6* mutant plants compared to WT plants grown in standard short-day conditions. The *ap2c1 mkp1* mutant displays more than 160-times higher *SAG12* transcript level than WT. Of note, *SAG12* upregulation was attenuated in the *ap2c1 mkp1 mpk6* triple mutant (14-times upregulation compared to WT). Error bars represent SD of three biological replicates, * $p < 0.05$, Student's *t*-test.

Supplementary information

Supplementary Table I. Expression of TF-encoding genes modulated by the absence of AP2C1 but not MKP1. Transcript levels were quantified by RT-qPCR and expressed as $\log_2$ of fold change (FC) for each mutant compared to WT. Genes affected by at least 1.56-fold only in *ap2c1* while not more than 0.9-fold in *mkp1* are listed. Data are from two independent biological replicates and are reported with SE.

Supplementary Table II. Expression of TF-encoding genes modulated by the absence of MKP1 but not of AP2C1. Transcript levels were quantified by RT-qPCR and are expressed $\log_2$ of fold change (FC) for each mutant compared to WT. Genes affected by at least 1.56-fold only in *mkp1* while not more than 0.9-fold in *ap2c1* are listed. Data are from two independent biological replicates and are reported with SE.

Supplementary Table III. Expression of TF-encoding genes modulated by the absence of both MKP1 and AP2C1. Transcript levels were quantified by RT-qPCR and are expressed as fold change (FC) in log₂ scale for each of the mutants compared to WT. Genes affected by at least 1.56-fold both in *ap2c1* and *mkp1* are listed. Data are from two independent biological replicates and are reported with SE.

Supplementary Table IV. TF-encoding genes deregulated in *ap2c1 mkp1* plants. Gene transcript levels were quantified by RT-qPCR and expressed as fold change in log₂ scale for each of the mutants compared to WT. Genes affected in *ap2c1 mkp1* compared to WT by at least 1.56-fold in log₂ scale are listed. Data are from three independent biological replicates and are reported with SD.

Supplementary Figure 1. Phenotypes of Arabidopsis single, double and triple mutant plants.

A. Phenotypes of WT, *ap2c1*, and *mkp1* plants grown for three weeks in long-day conditions. **B.** Phenotypes of WT, *ap2c1 mkp1*, *ap2c2 mkp1*, and *ap2c3 mkp1* plants grown for four weeks in short-day conditions. **C.** Phenotypes of WT, *ap2c1*, *ap2c2*, *ap2c3*, *mkp1*, *ap2c1 mkp1*, *ap2c2 mkp1*, *ap2c3 mkp1*, *mpk6*, and *ap2c1 mkp1 mpk6* plants grown for four weeks in short-day condition, followed by three weeks in long-day condition.

Supplementary Figure 2. Loss of both AP2C1 and MKP1 leads to severe phenotypes in growth and development, which are mediated by MPK6.

A. Phenotypes of WT (left) and *ap2c1 mkp1* (right, indicated by arrow) grown for 2.5 weeks in standard long-day conditions. During the first 18 days of growth, the phenotypic differences of *ap2c1 mkp1* compared to WT plants are visibly manifested as a difference in plant size. **B.** After approximately 3.5 weeks (26 days), premature death of leaf tissue as well as abnormal leaf growth and morphology in *ap2c1 mkp1* plants (right, indicated by arrow) became apparent. Scale bars in **A** and **B** = 1 cm. **C.** Close-up of *ap2c1 mkp1* plant grown for 3.5 weeks in standard long-day condition. **D.** Phenotype of seven-week-old *ap2c1 mkp1* plant grown in standard long-day condition. **E.**

Close-up of seven-week-old *ap2c1 mkp1* plant showing misshaped inflorescence. **F, G.** Phenotypes of eight-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, and *ap2c1 mkp1 mpk6* plants grown for the first six weeks in short-day and for a further two weeks in long-day conditions. The *ap2c1 mkp1* double mutant displays a severe dwarf phenotype, premature leaf decay, lack of normal shoot development, and strongly impaired inflorescence growth. The inset picture above shows a close-up of the *ap2c1 mkp1* plant shown. In *ap2c1 mkp1 mpk6* triple mutant plants these phenotypes were rescued.

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

A. Phenotypes of WT, *ap2c1*, *mkp1* and *ap2c1 mkp1* plants grown for 8 weeks in short-day conditions. Scale bars = 1 cm.

B. Formation of macroscopic lesions in leaves of *ap2c1 mkp1* plant shown in Figure 1A. Scale bars = 0.5 cm.

C. Phenotypes of five weeks old WT, *ap2c1*, *mkp1*, *mpk6*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* plants grown in standard short-day conditions. Scale bars = 1 cm.

D. Phenotypes of five-week-old WT, *ap2c1*, *mkp1*, *mpk6*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* plants grown in conditions with increased humidity and elevated temperature. Scale bars = 1 cm.

E. Phenotypes of WT, *ap2c1*, *ptp1* and *ap2c1 ptp1* plants grown for 6 weeks in short-day conditions. Scale bars = 1 cm.

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Levels of *MKP1* and *AP2C1* transcripts were quantified by RT-qPCR in 14-day-old seedlings grown in long-day conditions and compared with levels in WT plants. Transcript amounts of *MKP1* and *AP2C1* in WT were taken as 100%. The relative transcript amounts were normalized to the reference gene, *ACTIN2*. Error bars indicate SD. Results are the mean of two biological and two technical replicates for each experiment, *p < 0.05 Student's *t*-test.

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B. RT-qPCR analysis of ASC6 expression in leaves of six-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* plants grown in short-day conditions, where expression levels in WT are set to 1. Bars represent mean values of three biological replicates with SD, * $p < 0.05$, Student's *t*-test.

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Figure 8. Genes encoding members of the ANAC TF family are highly upregulated in *ap2c1 mkp1* plants. Transcript levels were quantified by RT-qPCR in *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6* and *mpk6* plants and compared to WT, where expression levels in were set to 1. Bars represent mean values of at least three replicates $\pm$ SD. *ANAC005*, *ANAC042*, *ANAC003*, *ANAC047*, and *ANAC055* are upregulated in *ap2c1 mkp1* in a MPK6-dependent manner, while in *ap2c1 mkp1 mpk6* plants their levels are similar to WT, * $p < 0.05$, Mann-Whitney *U* test.

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A. Transcript level of *CYP71B15/PAD3*, an enzyme required for camalexin biosynthesis, was quantified by RT-qPCR in *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6* and *mpk6* plants and compared to WT, where expression levels of were set to 1. Bars represent mean values of at least three replicates $\pm$ SD, expressed on a log₁₀ scale. **B.** Levels of total camalexin determined by HPLC in leaves of 4-week-old WT, *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6* and *mpk6* plants. Results shown are mean with SE (n=4), n.d. = not detected, *p < 0.05, **p < 0.01, Student's *t*-test.

Figure 10. *ap2c1 mkp1* accumulate prominently higher levels of SA in a MPK6-dependent manner.

Total SA levels of five-week-old wt, *ap2c1*, *mkp1*, *ap2c1 mkp1*, *ap2c1 mkp1 mpk6* and *mpk6* and plants 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.

Figure 11. Upregulation of senescence marker gene *SAG12* in *ap2c1 mkp1* plants is mainly mediated by MPK6.

RT-qPCR quantification of *SAG12* transcript level in leaves of six-week-old *ap2c1*, *mkp1*, *ap2c1 mkp1* and *ap2c1 mkp1 mpk6* mutant plants compared to WT plants grown in standard short-day conditions. *ap2c1 mkp1* mutants display more than 160-times higher *SAG12* transcript level than WT. Of note, *SAG12* upregulation was attenuated in the *ap2c1 mkp1 mpk6* triple mutant (14-times upregulation compared to WT). Error bars represent SD of three biological replicates, * $p < 0.05$, Student's *t*-test.