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The Calcium-Dependent Protein Kinase CPK28 Regulates Development by Inducing Growth Phase-Specific, Spatially Restricted Alterations in Jasmonic Acid Levels Independent of Defense Responses in Arabidopsis[OPEN]

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Phytohormones play an important role in development and stress adaptations in plants, and several interacting hormonal pathways have been suggested to accomplish fine-tuning of stress responses at the expense of growth. This work describes the role played by the CALCIUM-DEPENDENT PROTEIN KINASE CPK28 in balancing phytohormone-mediated development in *Arabidopsis thaliana*, specifically during generative growth. *cpk28* mutants exhibit growth reduction solely as adult plants, coinciding with altered balance of the phytohormones jasmonic acid (JA) and gibberellic acid (GA). JA-dependent gene expression and the levels of several JA metabolites were elevated in a growth phase-dependent manner in *cpk28*, and accumulation of JA metabolites was confined locally to the central rosette tissue. No elevated resistance toward herbivores or necrotrophic pathogens was detected for *cpk28* plants, either on the whole-plant level or specifically within the tissue displaying elevated JA levels. Abolishment of JA biosynthesis or JA signaling led to a full reversion of the *cpk28* growth phenotype, while modification of GA signaling did not. Our data identify CPK28 as a growth phase-dependent key negative regulator of distinct processes: While in seedlings, CPK28 regulates reactive oxygen species-mediated defense signaling; in adult plants, CPK28 confers developmental processes by the tissue-specific balance of JA and GA without affecting JA-mediated defense responses.

INTRODUCTION

Plant development and the resulting morphological variability are determined by species-specific, genetically encoded predispositions and are modified by specific abiotic and biotic environmental cues. Integrating these elements, plants have to carefully regulate growth processes in order to ensure survival and reproduction. Both developmental cues and perception of environmental challenges result in the generation of second messenger signals, such as transient, stimulus-specific and dynamic alterations of calcium (Ca^{2+}) levels in the cytoplasm (Webb et al., 1996; Kudla et al., 2010). These Ca^{2+} alterations are decoded via a complex calcium-dependent signaling network including protein-protein interactions and phosphorylation cascades to trigger subsequent downstream transcriptional reprogramming as well as changes in protein composition and metabolic content (McAinsh and Pittman, 2009; Dodd et al., 2010; Kudla et al., 2010). Calcium-dependent protein kinases (CDPKs), restricted to the plant kingdom and some protists, combine a calcium-sensing protein domain and a protein kinase effector domain within a single molecule and represent potential Ca^{2+} decoders to translate developmental and environmental stress cues (Liese and Romeis, 2013). So far, members of the CDPK gene family with 34 isoforms in *Arabidopsis thaliana*, also denominated CPKs in this species, have generally been identified as positive regulators in the activation of abiotic and biotic stress responses triggering enhanced plant tolerance or resistance (Asano et al., 2012; Boudsocq and Sheen, 2013; Romeis and Herde, 2014). Even more, single isoforms, for example, CPK21 in abscisic acid signaling or CPK5 in pathogen-associated molecular pattern (PAMP)-triggered innate immunity, were shown to play a dual role in rapid stress signal transduction and propagation on the one hand and in long-term, phytohormone-mediated adaptive (and in last consequence developmental) processes on the other hand. However, much less is known about CPKs regulating plant development a priori, with the only exception of CPKs characterized in mediating single cell growth of pollen tubes (Myers et al., 2009; Gutermuth et al., 2013; Zhao et al., 2013; Zhou et al., 2014).

We have previously identified CPK28 as a negative regulator of plant developmental processes. Remarkably, only upon the transition from the vegetative to the generative phase does the *cpk28* loss-of-function mutant become compromised in leaf development and stem elongation, resulting in a unique and robust

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growth phenotype independent of any stress stimulus (Matschi et al., 2013). Analysis of stem cross sections revealed an altered vascular anatomy in *cpk28*, characterized by reduced xylem development and ectopic lignification, concomitant with increased gene transcription of central inducers of secondary cell wall synthesis, *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST3*. The developmental phenotype is accompanied by stage-specific reduction in the expression of marker genes for gibberellic acid (GA) biosynthesis in *cpk28*.

Interestingly, the observed phenotypic features, such as growth inhibition, stunted shoots, or reduced rosette diameter, have been also attributed to elevated levels of the phytohormone jasmonic acid (JA), induced either by wounding or herbivory or by exogenously applied JA, which was also observed in mutant lines showing constitutively elevated endogenous levels of JA (Turner et al., 2002; Zhang and Turner, 2008). Well-regulated crosstalk between JA and GA is a crucial component of plant development, regulating energy investment in either defense mechanisms or plant growth (Kazan and Manners, 2012; Huot et al., 2014). The antagonistic effect has been observed, for instance, in mutants of the DELLA repressors of the GA signaling pathway, exhibiting reduced JA-dependent defense responses, whereas constitutively active DELLA repressors showed stimulus-independent enhanced JA marker gene expression (Navarro et al., 2008). An underlying mechanism has been suggested for the direct interaction between respective DELLA and JASMONATE-ZIM DOMAIN (JAZ) repressor proteins (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012b). Interestingly, virus-induced gene silencing (VIGS)-induced co-suppression of *Nicotiana attenuata CDPK4* and *CDPK5*, two close homologs of At-CPK28, resulted in reduced gene expression of GA biosynthetic enzymes and decreased GA phytohormone content, correlating with the observed plant growth reduction on the one hand and elevated JA levels mediating subsequent resistance toward larval feeding on the other hand (Yang et al., 2012a; Heinrich et al., 2013). However, these experiments do not indicate whether developmental phenotypes are directly caused by JA signaling or simply reflect resource reallocation from growth to defense mediated by JA.

In the context of environmental stress signaling, elevated JA biosynthesis, bioactive JA-Ile perception, and JA-Ile-mediated transcriptional reprogramming has been well described in response to mechanical wounding and herbivory as well as attack by necrotrophic microbes (Memelink, 2009; Wu and Baldwin, 2010; Wasternack and Hause, 2013). A rise in the intracellular calcium levels was reported upon wounding and herbivory (Maffei et al., 2004). Also, members of the glutamate-like receptor family, which may function as potential plasma membrane-located calcium channels, were identified as crucial elements of wound signal propagation for distal JA-mediated response activation (Mousavi et al., 2013). Likewise, pharmacological inhibition of wound-induced calcium influx negatively affected the expression of JA-induced genes (León et al., 1998; Sun et al., 2006). In maize (*Zea mays*), a rapid increase in biochemical activity of CDPK11 was detected within 10 min after wounding both in local (wounded) as well as in distal (untreated) leaves (Szczegielniak et al., 2012). Also, Arabidopsis mutant lines *cpk3* and *cpk6* were reported to display reduced marker gene expression after herbivore feeding (Kanchiswamy et al., 2010). These data demonstrate a role of CDPKs in positive regulation of early wound signaling by mediating signal propagation as well as transcriptional reprogramming, similar to what has been described for At-CPK5 in the innate immune response to microbial pathogen attack (Dubiella et al., 2013; Romeis and Herde, 2014). Taken together, these data point toward a functional relevance of stress-induced calcium-regulated signaling, which is a prerequisite to subsequent JA-mediated stress responses and defense. The nature of the calcium sensor involved, and whether CDPKs may participate in the direct regulation of environmental stress-induced JA responses, is yet unknown.

Interestingly, CPK28 has recently been described as a negative regulator of early innate immune signaling in a suppressor screen for enhanced PAMP-triggered production of reactive oxygen species (ROS) in seedlings (Monaghan et al., 2014). CPK28 was shown to directly phosphorylate BOTRYTIS-INDUCED KINASE1 (BIK1), a kinase required for PAMP-induced defense signaling initiation, resulting in BIK1 degradation, and it has been discussed that, as a consequence, BIK1-activated ROS production via NADPH-oxidase RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) is compromised. CPK28 displays calcium-dependent protein kinase activity in vitro (Matschi et al., 2013). However, a functional link between an elevation of cytoplasmic calcium and CPK28 activation as a negative regulator has neither been confirmed in environmental stress-induced signaling in seedlings nor in developmental phytohormone-mediated processes upon the plant's transition from the vegetative to the generative growth phase.

Here, we report on the role of calcium-dependent kinase CPK28 as a key regulator of phytohormone-mediated plant development during the generative growth phase in Arabidopsis. Growth reduction of *cpk28* loss-of-function mutants correlates with an altered balance of phytohormones JA and GA, whereby elevated JA-dependent gene expression and JA phytohormone levels revealed not only a growth phase dependent but also a local, spatially defined accumulation in the central rosette tissue. Importantly, the *cpk28* growth phenotype was suppressed in JA biosynthesis (*aos*) or signaling (*coi1-16*) mutants, but not in the absence of defense-related ROS production (*rbohd*). Furthermore, *cpk28* mutant plants do not show altered resistance to a necrotrophic fungal pathogen or to herbivore feeding. Our data identify CPK28 as negative regulator displaying a plant growth phase-dependent dual function: In addition to resetting PAMP-induced defense signaling in seedlings, CPK28 regulates, upon the transition to the generative growth phase, JA-dependent developmental processes in stem elongation and vasculature, importantly, independent of JA-mediated stress and defense signaling responses.

RESULTS

Expression of JA Marker Genes Is Stage-Specifically Enhanced in *cpk28*

cpk28 was previously identified based on its stage-specific growth phenotype, with severe reduction of stem elongation, changes in leaf morphology and anthocyanin accumulation, and

altered vascular architecture (Matschi et al., 2013). Reduced growth but also elevated anthocyanin levels have been correlated with plants accumulating JA, either in response to repeated mechanical wounding (Zhang and Turner, 2008) or in mutants (Ellis and Turner, 2001; Turner et al., 2002; Bonaventure et al., 2007a; Qi et al., 2011). Based on these morphological similarities, we investigated the role of CPK28 in JA metabolism and signaling. Transcriptional analysis of JA-responsive marker genes comparing the wild type and *cpk28-1* by quantitative RT-PCR (qRT-PCR) was conducted in aerial parts of plants harvested at various developmental stages (10, 20, 27, and 31 d after germination [dag] in long-day [LD] conditions). Expression of the JA-responsive marker plant defensin gene *PDF1.2* was found to be stage-specifically enhanced in *cpk28-1* compared with the wild type (Figure 1A). Only in later stages of plant development, when plants start to flower (starting around day 27) and induce bolting (around day 30; Matschi et al., 2013), did the gene expression of *PDF1.2* in *cpk28-1* increase, showing a 45-fold induction at day 27 and 100-fold induction at 31 dag compared with the wild type. Stage-specific transcript elevation of the marker gene *CORONATINE INDUCED1* (*CORI3*) was also observed at 31 dag for *cpk28-1*. Focusing on the last time point, we confirmed increased gene expression of JA-responsive marker genes *PDF1.2* and *VEGETATIVE STORAGE PROTEIN2* (*VSP2*), as well as of JA signal transduction components *JAZ1* and *JAZ10*, in *cpk28-1* and *cpk28-2* (Figures 1B and 1C). Reversion of the enhanced gene transcription to wild-type levels could be achieved by expression of active CPK28 kinase in the mutant background in *35S:CPK28-YFP* (*YELLOW FLUORESCENT PROTEIN*) or *pCPK28:CPK28-YFP* (Figures 1B and 1C). No significant changes in gene expression could be observed for JA biosynthesis genes *ALLENE OXIDE SYNTHASE* (*AOS*) or *12-OXOPHYTODIENOATE REDUCTASE3* (*OPR3*) in *cpk28* mutants, overexpression line *35S:CPK28-YFP*, or complementation line *pCPK28:CPK28-YFP* (Figure 1D).

To exclude a specific role for CPK28 in early JA-influenced developmental processes such as root growth, we analyzed root growth of the wild type, *cpk28* mutant lines, and *pCPK28:CPK28-YFP* (Supplemental Figure 1). Monitoring root elongation on agar plates did not reveal any growth differences between the tested genotypes, either under control conditions or upon jasmonic acid methyl ester (MeJA) treatment (Supplemental Figure 1); MeJA has been described to induce JA-dependent root growth inhibition (Staswick et al., 1992). Taken together, CPK28 appears crucial to the regulation of JA metabolism or signaling, specifically during the generative phase of plant development.

JA and JA Signal Transduction Are Required for the Growth Reduction of *cpk28*

High levels of JA marker gene expression suggested accumulation of JA metabolites in *cpk28*. Therefore, we tested whether the presence of JA biosynthesis or JA signaling is a prerequisite for realization of the *cpk28* growth phenotype by genetic analysis. *cpk28* was crossed with either *aos*, defective in JA biosynthesis (Park et al., 2002), or the JA signaling mutant *coi1-16* (Ellis and Turner, 2002; Noir et al., 2013), with severely reduced functionality of the CORONATINE INSENSITIVE1 (COI1) coreceptor responsible for perception of the bioactive metabolite (+)-7-*iso*-JA-Ile (Fonseca et al., 2009).

Phenotypic evaluation of the double mutant lines revealed a full reversion of the *cpk28* mutant phenotype when either JA biosynthesis or JA signaling was abolished (Figure 2). The overall plant morphology of *cpk28* × *aos* and *cpk28* × *coi1-16* at the full flowering stage (47 dag) resembled the parental lines *aos* or *coi1-16*, respectively, exhibiting normal shoot elongation and rosette growth (Supplemental Figure 2A). *cpk28* properties such as rosette and leaf shape (Figure 2A) and phenotypic parameters were restored to wild-type levels in the double mutants. While *cpk28* displayed a reduction of rosette diameter (Figure 2B) and plant height at 47 dag (Supplemental Figure 2B), no significant differences compared with wild-type levels were detected in *cpk28* × *aos* and *cpk28* × *coi1-16*. Anthocyanin content of *cpk28* was elevated at 31 dag, whereas single and double mutants of *aos* or *coi1-16* showed significantly reduced levels compared with the wild type (Figure 2C). Remarkably, the vascular phenotype of the *cpk28* stem, such as reduced development of xylem tracheary elements and ectopic lignification of phloem fiber cells, was reverted when JA biosynthesis or signal transduction was disturbed (Figure 2D).

Further characterization of *cpk28* × *aos* and *cpk28* × *coi1-16* was accomplished by gene expression analysis of wild-type, single, and double mutants 31 dag by qRT-PCR (Supplemental Figure 3). Expression of the JA-responsive genes *PDF1.2* and *VSP2* was significantly reduced for both genes in *cpk28* × *aos* and *cpk28* × *coi1-16* as well as in the parent lines *aos* and *coi1-16* compared with the wild type (Supplemental Figure 3A). JA-regulated marker genes *PATHOGENESIS-RELATED3* (*PR3*) and *PR4* both showed elevated expression in *cpk28*, while gene transcription in *cpk28* × *aos* and *cpk28* × *coi1-16* as well as in the parental lines *aos* or *coi1-16* did not differ from wild-type levels. Transcript levels of the signaling components *JAZ1* and *JAZ10* were elevated 3-fold in *cpk28*, while the double mutants showed reduced expression of *JAZ1* and no significant differences in expression of *JAZ10* compared with the wild type (Supplemental Figure 3B). Furthermore, consistent with the abolishment of the vascular *cpk28* phenotype in *cpk28* × *aos* and *cpk28* × *coi1-16* (Figure 2D), reversion of gene expression to wild-type levels was detected for NAC transcription factor *NST1* in the double mutants, shown to be elevated in *cpk28* (Matschi et al., 2013). Expression of a marker gene of GA biosynthesis, *GA3-oxidase1* (*GA3ox1*), previously reported to be reduced in *cpk28* (Matschi et al., 2013), was reverted to wild-type levels by depletion of JA or JA signaling in *cpk28*. Transcript levels in *cpk28* × *aos* and *cpk28* × *coi1-16* mutant lines as well as in the parental lines *aos* and *coi1-16* were not statistically different from wild-type levels (Supplemental Figure 3C).

The genetic analysis and phenotypic and transcriptional characterization demonstrate that JA or JA-Ile biosynthesis and function is a prerequisite of the *cpk28* growth phenotype.

DELLA Abundance Has No Direct Influence on the JA-Mediated *cpk28* Phenotype

A mutual antagonistic effect of GA and JA in plant development, balancing JA-induced defense mechanisms with GA-mediated

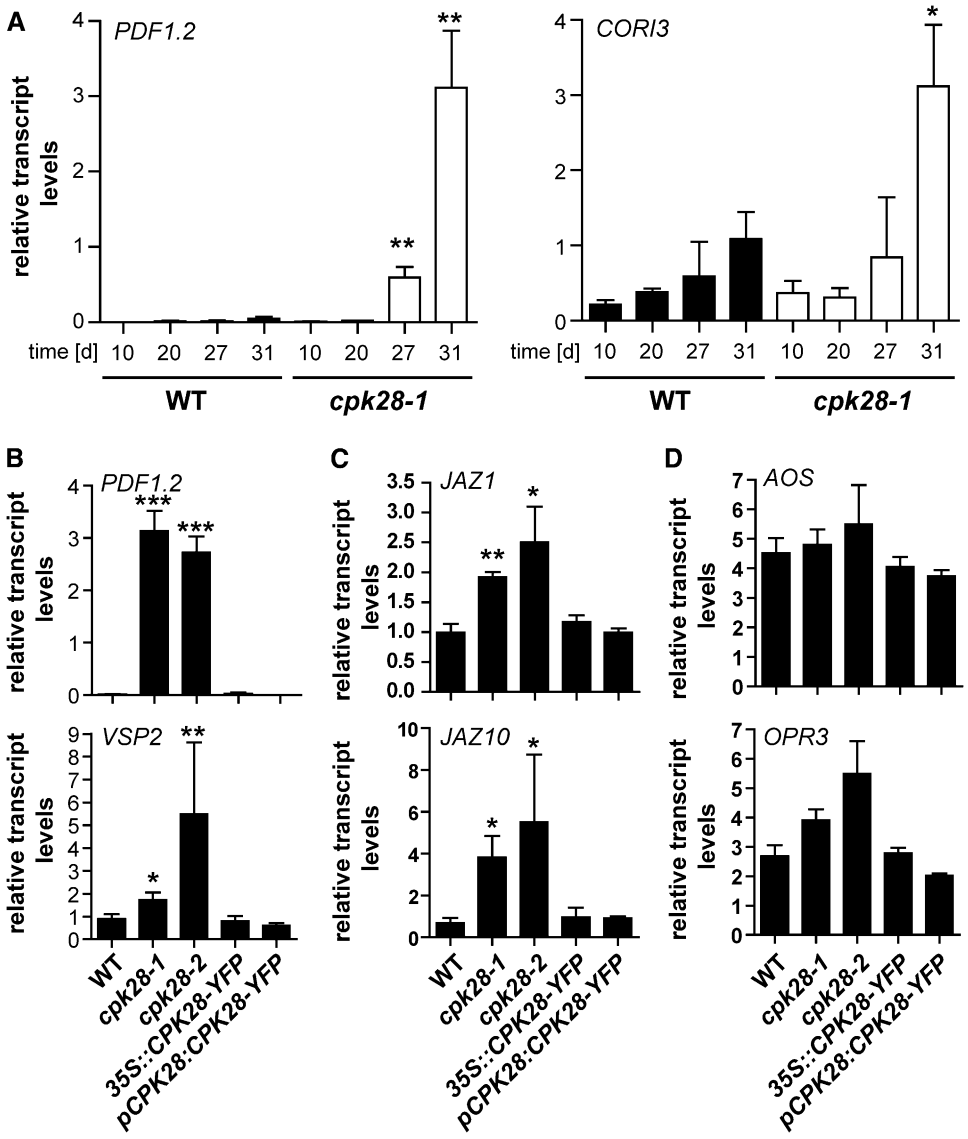

Figure 1. *cpk28* Mutants Show Stage-Dependent Enhanced Expression of JA Marker Genes.

(A) Time-dependent expression of JA-responsive genes *PDF1.2* and *CORI3* in the wild type and *cpk28-1* was analyzed by qRT-PCR. After growth in LD conditions for the respective days (d), aerial parts of plants were subjected to qRT-PCR and quantification was normalized to the expression level of *ACTIN2.* Values represent means of three biological replicates with two pooled plants each ± SE (two technical replicates per biological replicate). Repetition of the experiment showed similar results.

(B) to **(D)** Expression of JA-responsive marker genes *PDF1.2* and *VSP2* **(B)**, JA signal transduction components *JAZ1* and *JAZ10* **(C)**, and JA biosynthesis genes *AOS* and *OPR3* **(D)** in the wild type, *cpk28-1*, *cpk28-2*, and transgenic lines *35S:CPK28-YFP* and *pCPK28:CPK28-YFP* (31 dag) (Student's *t* test; significance levels of *P < 0.05, **P < 0.01, and ***P < 0.001).

growth responses, is discussed in the literature (Hou et al., 2010; Kazan and Manners, 2012; Yang et al., 2012b). *cpk28* shows enhanced JA (Figure 1) but reduced GA marker gene expression (Matschi et al., 2013), suggesting a disturbance in the GA/JA balance as cause for the *cpk28* growth alterations. We therefore studied the impact of GA application on JA marker gene expression. Treatment with GA_3 partially restored the growth phenotype of *cpk28* regarding plant height or anthocyanin content, but not the secondary growth phenotype (Matschi et al., 2013). Gene expression analysis of mock (−) and GA-treated (+) plants revealed only a minor influence of exogenously applied GA on the elevated JA marker gene transcription in *cpk28* mutants (Figure 3). With the exception of *CORI3*, whose expression could be reverted to wild-type levels by GA treatment, GA-treated *cpk28* plants still showed a significantly enhanced transcript accumulation of analyzed JA marker gene *PDF1.2* and JA signaling components *JAZ1* or *JAZ10* compared with respective wild-type expression. Therefore, GA supplementation does not appear to

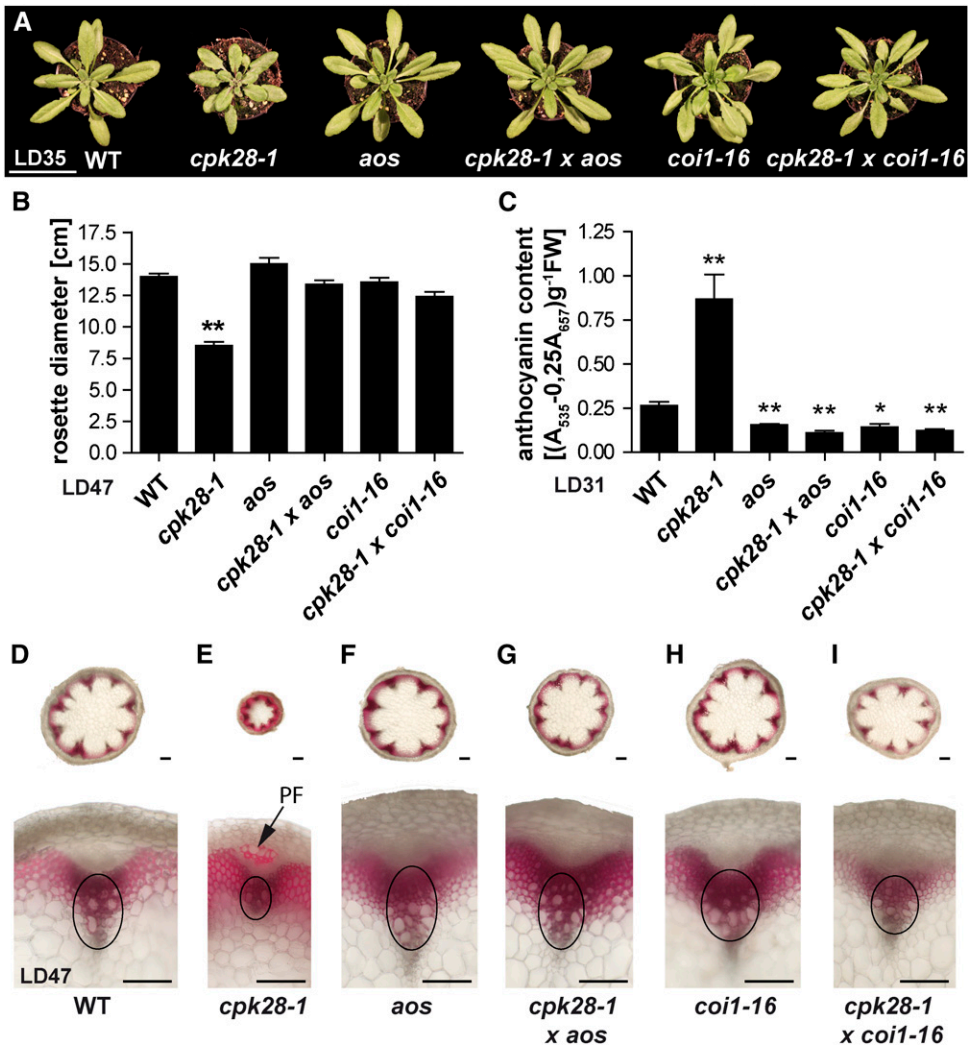

Figure 2. JA Biosynthesis or JA Perception Is Required for the *cpk28* Growth Phenotype.

cpk28-1 was crossed with *aos* or *coi1-16* to generate *cpk28-1* × *aos* or *cpk28-1* × *coi1-16*, respectively, and double mutants were analyzed for *cpk28* phenotypic features.

(A) Rosette morphology of the wild type, *cpk28-1*, *aos*, *cpk28-1* × *aos*, *coi1-16*, and *cpk28-1* × *coi1-16* at day 35 (LD35). Bar = 5 cm.

(B) Rosette diameter in the wild type and different single and double mutants at day 47 (n = 15).

(C) Anthocyanin content of the wild type and single and double mutants at day 31 (n = 3). Whole plants were harvested and anthocyanins were extracted and determined photometrically. Values are given as mean ± SE of n biological samples, representative of three independent experiments (Student's t test; significance levels *P < 0.05 and **P < 0.01).

(D) to **(I)** Inflorescence stem anatomy of the wild type and single and double mutants (47 dag). Hand-cut cross sections show the basal internode of the wild type and mutants. Plant material was stained for lignin with 2% phloroglucinol and acidified with 37% HCl. Circles indicate xylem tissue of vascular bundles with fewer developed lignified xylem tracheary elements in *cpk28*. PF, lignified phloem fibers. Bars = 100 μm.

be sufficient to completely suppress JA-mediated transcript elevation in *cpk28*.

Based on the model of directly interacting DELLA and JAZ transcriptional regulators mutually repressing each other, a molecular mechanism balancing JA and GA signaling by the respective JAZ/DELLA protein abundance has been proposed (Hou et al., 2010; Yang et al., 2012b). We therefore conducted an epistasis analysis with *cpk28* and the two main DELLA repressors regulating shoot elongation, RGA (REPRESSOR OF *ga1-3*) and GAI (GA INSENSITIVE) (Dill and Sun, 2001; Achard and Genschik, 2009). Double mutants *cpk28-1* × *rga28* and *cpk28-1* × *gai* did not reveal any morphological differences compared with *cpk28* mutants (Supplemental Figures 4A and 4B). Consistently, no differences in RGA protein levels were detected in wild-type and *cpk28* plants (31 dag; Supplemental Figure 4C), suggesting that DELLA protein abundance is unaltered and, therefore, not crucial for GA/JA balance in the context of CPK28-mediated growth processes.

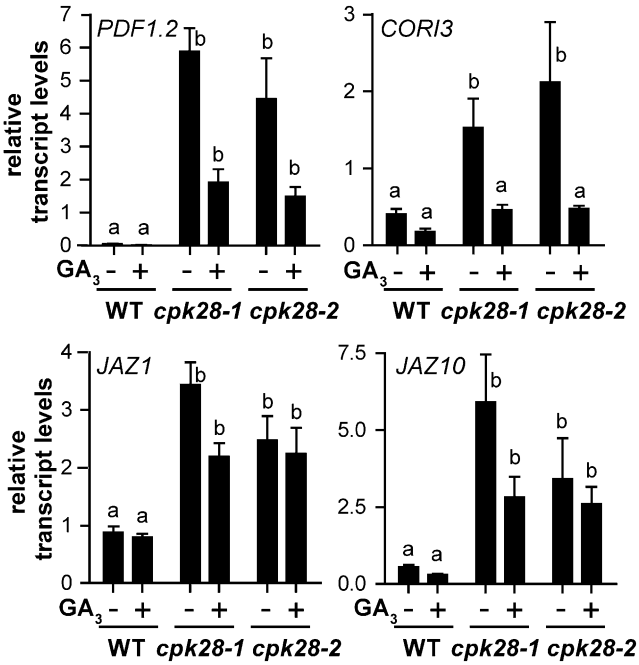

Figure 3. GA Treatment Does Not Revert Elevated JA Marker Gene Expression in *cpk28*.

Gene expression levels of *PDF1.2*, *CORI3*, *JAZ1*, and *JAZ10* in wild-type and *cpk28* plants (31 dag) treated with 100 μM gibberellic acid (GA_3) (+) or 0.95% ethanol (−) every 3rd to 4th day from germination onwards. Whole rosettes were subjected to qRT-PCR, and quantification was normalized to the expression level of *ACTIN2*. Values represent means of three biological replicates (pool of two plants per replicate) ± SE with two technical replicates per each biological replicate (one-way ANOVA, means with the same letter are not significantly different from each other; Tukey's post-test, $P < 0.05$).

Loss of Function of CPK28 Does Not Confer Increased JA-Dependent Resistance

JA signaling was shown to be essential for resistance against insects or necrotrophic fungi (Wasternack, 2007; Wu and Baldwin, 2010). Enhanced JA-dependent gene expression in *cpk28* let us investigate whether plants show altered resistance toward these attackers. No-choice feeding assays with larvae of the generalist lepidopterans *Trichoplusia ni* and *Spodoptera littoralis* did not result in differences in larval weight and therefore increased resistance toward larval feeding in *cpk28* mutant plants exposed to herbivores after the transition to the generative phase (31 dag) (Figures 4A and 4B). In line with these assays, infection with the necrotrophic fungus *Alternaria brassicicola* did not lead to differences in disease symptoms after drop inoculation of the wild type or *cpk28* mutants during the generative phase (31 dag), while in comparison, the JA-deficient *aos* mutant showed clearly enhanced fungal infection (Figures 4C and 4D). Additionally, infection of *cpk28* × *aos* and *cpk28* × *coi1-16* leaves resulted in a suppressed defense response similar as seen for the parent genotypes *aos* and *coi1-16*, respectively (Supplemental Figure 5), confirming the independence of CPK28 and JA-mediated defense activation on the whole plant level in Arabidopsis.

cpk28 Mutants Display a Unique Stage- and Tissue-Specific Elevation of Different JA Metabolites

Aiming to align the contradictory results of JA-dependent expression and biotic stress assays, we measured the levels of several jasmonates at different developmental stages (20 and 27 dag) in the wild type, *cpk28*, *35S:CPK28-YFP*, and *pCPK28-CPK28-YFP* by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In whole unstressed rosettes, 12-oxo-phytodienoic acid (OPDA), JA, JA-Ile, and 12-OH-JA contents were similar between all genotypes (Supplemental Figure 6). Mechanical wounding of leaves resulted in a rapid accumulation of these metabolites, but levels did not significantly differ between the tested genotypes.

Because *CPK28* expression and the *cpk28* phenotype are associated with the central area of the plant (shoot, meristem, and petioles; Matschi et al., 2013), we tested the hypothesis of elevated JA metabolite levels being solely localized to this tissue and diluted beyond detection in whole plant rosettes (Supplemental Figure 7A). We therefore applied a different harvesting scheme (Supplemental Figure 7B) and could indeed detect significantly higher levels of OPDA, JA, JA-Ile, and 12-OH-JA specifically in the inner part of the *cpk28* rosette compared with the wild type (Figure 5, 31 dag). Expression of the active CPK28 kinase in *35S:CPK28-YFP* reverted this elevation to wild-type levels. In line with all observed phenotypes and the gene expression data, no differences in JA metabolite levels were detectable in vegetative plants (20 dag). Altogether, our data show not only a stage-dependent increase in JA metabolite levels in *cpk28*, restricted to the generative development of the plant, but even more importantly, they demonstrate a defined spatial distribution of JA accumulation during this growth phase.

CPK28 Confers a Specific Distribution of JA- and GA-Dependent Gene Expression

The spatial restriction of JA accumulation in *cpk28* raised the question of whether expression levels of JA marker gene exhibit a similar distribution pattern within the plant. To investigate this, the harvesting scheme of Supplemental Figure 7B was applied to plants at 31 dag and gene expression of the central rosette tissue (I) and leaf tissue (L) was compared (Figure 6). Both tissues of *cpk28* mutant plants showed enhanced expression levels of JA-responsive *PDF1.2* and *CORI3* compared with the wild type (Figure 6A). Gene expression of the two genes differed in the different tissues of *cpk28* mutants: While *PDF1.2* expression was significantly higher in leaves than in the inner plant part, *CORI3* expression was drastically enhanced in the central plant tissue (Figure 6A) where JA accumulation was detected. No significant difference in transcript levels was detected between the inner plant part and leaf tissue of the wild type, although *PDF1.2* showed a clear trend of increased gene expression in leaves. For the JA signaling component *JAZ10*, transcript elevation was determined exclusively for the inner rosette of *cpk28.* Gene expression in *cpk28* leaf tissue was not different

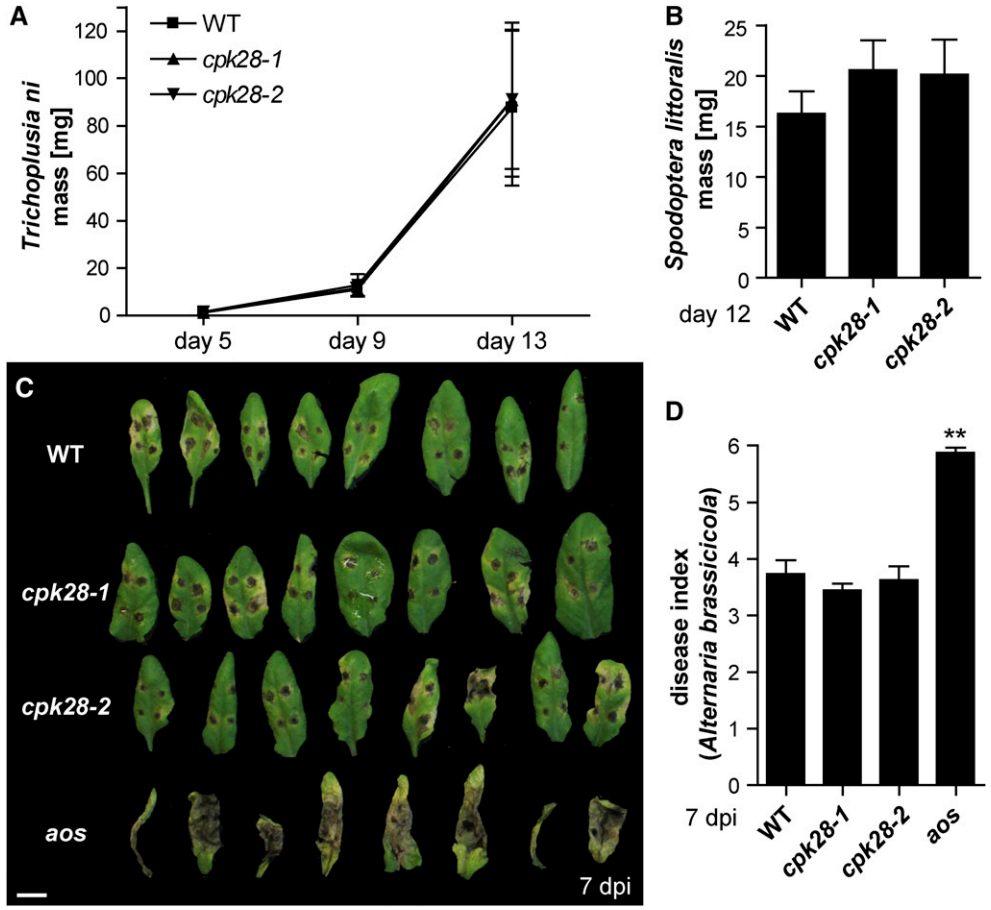

Figure 4. Loss of CPK28 Function Does Not Confer JA-Mediated Resistance.

(A) and **(B)** Weight gain assays of *T. ni* **(A)** or *S. littoralis* **(B)** on 27-d-old Arabidopsis plants grown in long days (16 h light). Data show mean ± SE of fresh weight gain of newly hatched *T. ni* larvae feeding for 5, 9, and 13 d on the wild type and *cpk28* **(A)** (n = 20) or *S. littoralis* larvae for 12 d **(B)** (n = 20).
(C) and **(D)** *A. brassicicola* infection assay. Leaves of 27-d-old wild-type, *cpk28*, and *aos* plants were drop-inoculated with spores of *A. brassicicola*.
(C) Disease symptoms were monitored 7 d after inoculation. Bar = 1 cm.
(D) Mean values of disease indices are presented ± SE for at least 15 replicates. Asterisks represent significant differences from the wild type (Student's t test; **P < 0.01).

from transcript levels in wild-type inner rosettes or leaves (Figure 6A), whereas comparison of both wild-type tissues showed a tendency for higher expression in the central rosette.

We also compared the expression of *cpk28*-characterizing marker genes *NST1* (Figure 6B) and *GA3ox1* (Figure 6C). While there was a tendency for higher expression in central rosette tissue, *NST1* transcript levels were not significantly different in wild-type inner rosettes versus leaves. The previously reported enhanced gene expression of *NST1* in *cpk28* (Matschi et al., 2013) was apparently solely based on the enhanced expression of *NST1* in the central plant parts, as transcript levels in *cpk28* leaves were similar to the wild type (Figure 6B). Expression of *GA3ox1* was shown to be concentrated in the plant center rather than leaf tissue in wild-type plants (Figure 6C). *GA3ox1* gene expression in *cpk28* was significantly reduced compared with both wild-type tissues, while a difference in spatial concentration between leaves and the central rosette parts could not be detected in *cpk28*.

CPK28-Dependent Tissue-Specific JA Accumulation Does Not Contribute to JA-Mediated Disease Resistance

To investigate the function of the temporally and spatially restricted JA elevation in the context of JA-mediated disease resistance, we performed herbivore feeding and fungal pathogen infection assays restricted to the inner rosette of the plant. Because standard clip cages used for larval feeding assays were not appropriate for confining caterpillars to the central rosette tissue, we designed and built split-cage devices allowing feeding only on the central part of the rosette. Plants were grown through a hole in the lower disc part of the cage on soil, and larvae of *T. ni* or *S. littoralis* were applied to the middle of the rosette on day 31. Enclosure of the larvae was guaranteed by assembly of the cylindrical upper part of the split-cage, preventing feeding on adult leaf blades simply by cage size. No difference in larval weight was detected between the wild type and *cpk28* after 12 d, while larval weight gain was significantly

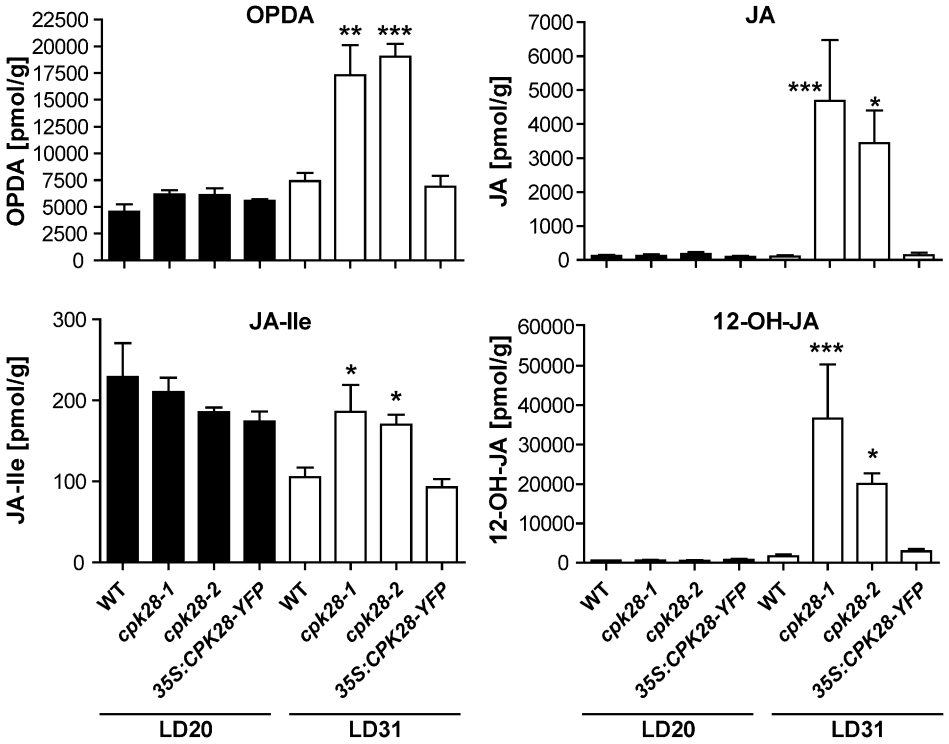

Figure 5. CPK28 Represses JA Accumulation Specifically in the Inner Rosette Area.

Levels of free OPDA, JA, JA-Ile, and 12-OH-JA in the wild type, *cpk28*, and transgenic line *35S:CPK28-YFP*. Central rosette tissue of plants was harvested at two different time points and jasmonates were determined via LC-MS/MS analysis. Data show mean ± SE of four biological replicates, each containing a pool of three central rosettes. Results were seen in two independent experiments (two-way ANOVA, Bonferroni post-test, *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

enhanced when larvae fed on inner rosette material of *aos* and *cpk28-1* × *aos* (Figures 7A and 7B). Likewise, no difference in fungal growth could be observed between *cpk28* and wild-type plants upon inoculation of *A. brassicicola* to the innermost whorl of rosette leaves on whole plants (Supplemental Figure 8), while at the same time, infection symptoms of *aos* were clearly enhanced. Importantly, quantification of fungal growth by fungal DNA quantification after *A. brassicicola* infection specifically on detached inner rosette tissue confirmed these results (Figures 7C and 7D). Plant material was prepared as described for JA metabolite measurements, inoculated with spores of *A. brassicicola* (Figure 7C), and kept in Petri dishes for 5 d. Quantitative PCR of fungal DNA, normalized to Arabidopsis DNA, revealed no differences in fungal multiplication within the inner rosette tissue of *cpk28* and the wild type, whereas fungal growth on *aos* as well as *cpk28-1* × *aos* was highly elevated (Figure 7D).

Our results demonstrate that spatially and temporally defined JA accumulation in *cpk28* does not contribute to resistance toward JA-affected pathogens in Arabidopsis, in contrast to VIGS-induced silencing of the homologous gene pair CDPK4 and CDPK5 in *N. attenuata* (Yang et al., 2012a). We therefore consider CPK28 to play an important role in the regulation of fine-balanced, tissue-specific, developmentally active JA metabolites, restricting the functionality of CPK28 in adult Arabidopsis plants strictly to developmental processes.

CPK28-Mediated Regulation of Plant Development Is Independent of Environmental Stress-Induced ROS and Calcium Perturbations

Recently, CPK28 has been identified as a negative regulator of PAMP-triggered immunity in a forward-genetics screen, which reduces levels of PAMP-induced ROS in the early seedling stage (Monaghan et al., 2014). To investigate whether altered basal JA levels have an impact on early PAMP-mediated events, we measured PAMP-triggered ROS elevation in the wild type, *cpk28*, *aos*, and *coi1-16*, as well as in the respective crosses *cpk28* × *aos* and *cpk28* × *coi1-16*, in a luminol-based ROS burst assay in adult plants at 31 dag (Supplemental Figure 9A). Upon treatment with the bacterial peptide flg22, a significant increase in ROS production was observed in *cpk28* compared with wild-type levels, independent of the presence of JA biosynthesis or JA signaling, while in single *aos* or *coi1-16* mutants, wild type-like flg22-induced ROS levels were observed.

Next, we generated *cpk28* × *rbohd* plants by crossing; in *rbohd* plants, the major enzyme responsible for ROS production during early PAMP-induced immune responses, NADPH oxidase RBOHD (Torres et al., 2002), is not produced. As expected, no flg22-triggered ROS elevation could be observed in these plants (Supplemental Figure 9B). Importantly, the growth phase- and JA-dependent developmental phenotype in adult *cpk28*

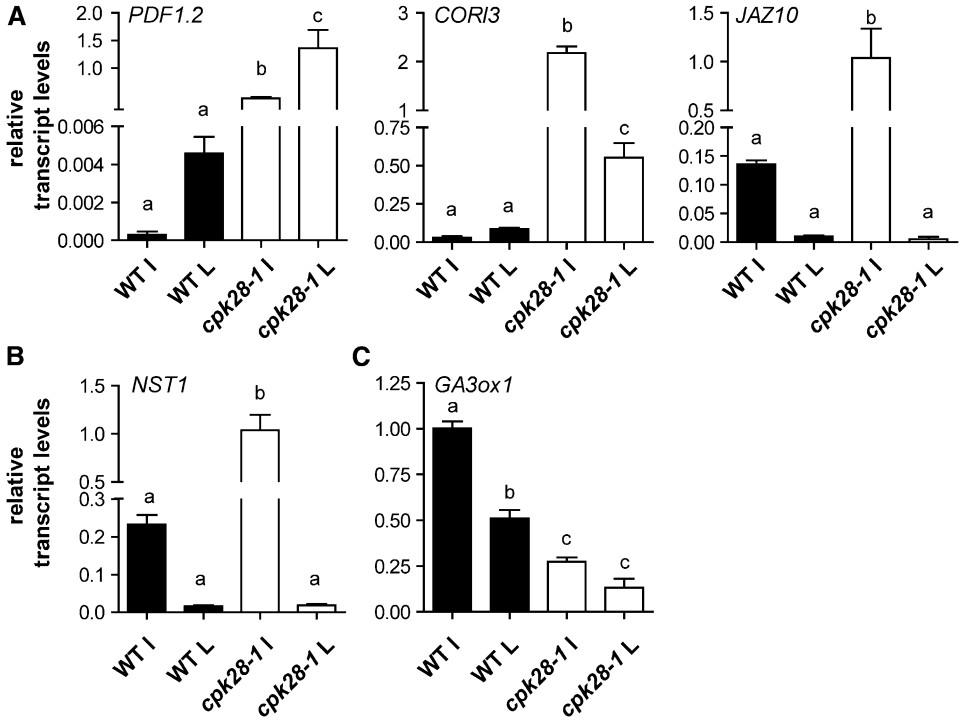

Figure 6. Tissue-Specific Gene Expression in the Wild Type and *cpk28-1*.

Leaf tissue (L) and inner rosette (I) (Supplemental Figure 7B) of the wild type and *cpk28-1* were harvested 31 dag and subjected to qRT-PCR analysis. Shown is expression of JA marker genes *PDF1.2*, CORI3, and *JAZ10* **(A)**, secondary growth marker gene *NST1* **(B)**, and GA marker gene *GA3ox1* **(C)**. Values represent means of three biological replicates (with pools of three plants per replicate) ± SE with two technical replicates per each biological replicate. Repetition of the experiment showed similar results (one-way ANOVA, Tukey's post-test, $P < 0.05$).

plants, characterized by stunted growth, was not abolished in these double mutants (Supplemental Figures 9C to 9E), although a slight increase in growth parameters could be detected for *cpk28* × *rbohd* compared with the *cpk28* parent. Consistently, the vascular phenotype of the *cpk28* stem, with ectopic lignification of phloem fibers, could also be detected in *cpk28* × *rbohd* (Supplemental Figures 9F to 9I). This suggests that RBOHD, although being a prerequisite of CPK28-dependent regulation of environmental signaling in the seedling stage, contributes only to a subtle extent to the JA-dependent, CPK28-mediated developmental changes in the adult plant.

cpk28 phenotypic features of growth phase-dependent altered development are reminiscent of those of a previously described mutant, *fatty acid oxygenation upregulated2* (*fou2*) (Bonaventure et al., 2007a). A detailed side-to-side comparison of phenotypes revealed that growth reduction as well as deformation of rosette leaves in *fou2* started earlier in development, and *fou2* plants displayed a significantly smaller rosette diameter than *cpk28* (Supplemental Figures 10A and 10B). In contrast, shoot elongation and therefore overall plant height was not as severely compromised in *fou2* compared with *cpk28*. Furthermore, although *fou2* showed phloem fiber lignification similar to that previously reported for *cpk28*, no severe differences in xylem development were detected in *fou2* and the wild type compared with *cpk28* (Supplemental Figure 10C), which shows a decrease in tracheary xylem elements (Matschi et al., 2013). *fou2* represents a gain-of-function allele of the vacuolar two-pore channel TPC1. Interestingly, *tpc1-2* plants carrying a loss-of-function mutation in this gene have recently been reported to be compromised in environmental stress-induced calcium signal propagation and distal stress-induced marker gene expression (Choi et al., 2014). We therefore conducted a genetic analysis in which *cpk28* was crossed to the null mutant of *TPC1*, *tpc1-2* (Peiter et al., 2005) (Supplemental Figure 10D). The parental line *tpc1-2* did not differ from wild-type growth; growth parameters of *cpk28-1* × *tpc1-2*, however, revealed that loss of function of TPC1 did not result in any reversion of the *cpk28* growth phenotype.

DISCUSSION

Developmental processes in plants are influenced by various endogenous and exogenous factors and are closely linked to a complex interplay of phytohormones leading to adaptation (Depuydt and Hardtke, 2011). We have previously described the calcium-dependent protein kinase CPK28 as a regulator of GA-mediated plant development in the generative growth phase (Matschi et al., 2013). Here, we present data supporting a dual function of a calcium-regulated protein kinase, with CPK28 negatively regulating two presumably independent processes during different developmental stages: While CPK28 modulates calcium-dependent activation of PAMP-triggered immunity in

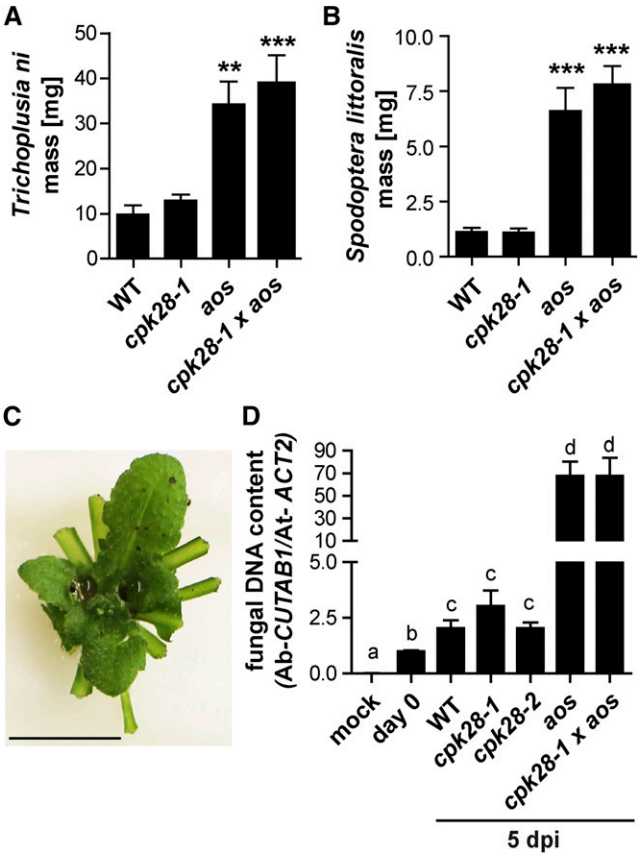

Figure 7. JA Accumulation in the Central Rosette Tissue of *cpk28* Does Not Lead to Tissue-Specific Increased JA-Dependent Resistance.

(A) and **(B)** Weight gain assays of different larvae caged on inner rosette tissue of 31-d-old Arabidopsis plants.

(A) Larval weight of *T. ni* on day 12 after restricted growth on central rosette tissue of the wild type, *cpk28*, *aos*, and *cpk28-1* × *aos* is depicted. Data show mean ± SE of $n = 12$ larvae.

(B) Repetition of the experiment with larvae of *S. littoralis* showed similar results (Student's *t* test; significance levels **$P < 0.01$ and ***$P < 0.001$).

(C) and **(D)** Quantification of in planta growth of *A. brassicicola* in the inner rosette area. Central rosette material of 31-d-old wild-type, *cpk28*, *aos*, and *cpk28-1* × *aos* plants was drop-inoculated with spores of *A. brassicicola* or water as mock treatment.

(C) Example of isolated central rosette tissue used for analysis in **(D)**. Bar = 1 cm.

(D) Quantitative PCR was used to analyze fungal growth on tissue shown in **(C)** by comparison of relative genomic DNA level of *A. brassicicola* cutinase gene *CUTAB1* after normalization to *ACTIN2* of Arabidopsis (Ab-*CUTAB1*/At-*ACT2*) 5 d postinfection (5 dpi). Controls of mock treatment and inoculation amount (day 0) are equaled over all genotypes. Data represent means ± SE of three biological replicates. Experiment was repeated three times with similar results (one-way ANOVA, Tukey's post-test, $P < 0.05$).

vegetative plants (Monaghan et al., 2014), coordinated growth of adult plants is, independent of environmental stresses, achieved by CPK28-dependent modification of tissue-specific JA content, hormonal balance of JA and GA, and resulting developmental processes, remarkably without influencing JA-mediated resistance.

JA Is a Prerequisite for *cpk28* Growth Inhibition in the Generative Phase

The phytohormone JA has been shown to govern defense responses as well as development via the establishment of defense mechanisms, including changes in gene expression and secondary metabolite composition, at the expense of growth promotion (Turner et al., 2002; Wasternack, 2007; Zhang and Turner, 2008). Growth reduction phenotypes as described for *cpk28* (Matschi et al., 2013) are reminiscent of plants showing elevated JA levels, achieved by repeated wounding of plants (Zhang and Turner, 2008) or in mutants exhibiting constitutively enhanced JA levels, such as *constitutive expression of VSP1* (*cev1*) (Ellis and Turner, 2001; Ellis et al., 2002). JA accumulation in these plants leads to a growth phenotype partially resembling that of *cpk28*, with reduced rosette growth, leaf deformation, and anthocyanin accumulation as well as elevated transcript levels of JA-regulated genes (Ellis and Turner, 2001; Ellis et al., 2002; Bonaventure et al., 2007a; Hyun et al., 2008). Loss of function of CPK28 indeed resulted in elevated JA marker gene expression and tissue-specific elevation of the levels of JA and related metabolites, such as OPDA, JA-Ile, and 12-JA-OH, interestingly, only in later stages of plant development (Figures 1 and 5). The phenotype was abolished by elimination of either JA biosynthesis or perception (Figure 2), demonstrating the essential role of JA in the establishment of the *cpk28* phenotype. CPK28 does not act in early JA-influenced processes such as root growth, shown by the wild-type behavior of *cpk28* in response to JA-mediated root growth inhibition (Staswick et al., 1992; Ellis and Turner, 2001) (Supplemental Figure 1). Unaltered JA content in early stages of *cpk28* development (Figure 5) confirmed the independence of CPK28 and early JA-dependent growth processes.

Also, the JA accumulating mutant *cev1*, carrying a defect in the cellulose synthase gene *CESA3*, displays growth reduction already in the seedling stage (Ellis et al., 2002), suggesting that it is unlikely that CPK28 plays a role in the direct regulation of cellulose biosynthesis. A link of CPK28 to (secondary) cell wall-related processes is nonetheless evident due to the deregulation of marker genes of secondary growth initiation and defects in shoot anatomy in *cpk28* (Matschi et al., 2013). *cev1* and its allelic mutant *ectopic lignin1* (*eli1*) exhibit ectopic lignification of the vasculature (Ellis et al., 2002; Caño-Delgado et al., 2003) and defects in xylem development (Caño-Delgado et al., 2000), similar to the anatomical phenotype of *cpk28* (Matschi et al., 2013), which is dependent on functional JA synthesis or signaling (Figure 2). Interestingly, cellulose synthesis inhibitor-treated wild-type plants mirror some of the *cev1* phenotypes, suggesting that cell wall integrity is involved in regulating JA responses (Ellis et al., 2002). Marker genes of secondary growth in the shoot, *NST1* and *NST3*, are regulated by CPK28 (Matschi et al., 2013), but their deregulation is dependent on JA biosynthesis or signaling (Figure 2; Supplemental Figure 3). JA was demonstrated to play a role in secondary growth (Sehr et al., 2010), with signal transduction components such as JAZ1 or JAZ10 as well as JA itself identified as regulating factors of cambium initiation and activity, which we show to be deregulated in *cpk28*. Therefore, CPK28 regulates JA-dependent

developmental processes, specifically during the generative growth phase, including secondary growth establishment.

CPK28-Mediated Balance of JA and GA

Simultaneous corepression of two CDPKs in the wild tobacco *N. attenuata*, *CDPK4* and *CDPK5*, by RNAi (*IRcdpk4/5*) and VIGS was reported to result in accumulation of JA and JA-induced secondary metabolites and subsequent enhanced resistance toward herbivores (Yang et al., 2012a). As both *N. attenuata* homologs as well as At-CPK28 are clustered into subgroup IV of the CDPK phylogenetic tree (Boudsocq and Sheen, 2013), functional homology of the wild tobacco and Arabidopsis genes is likely. Indeed, loss of function of both Na-CDPK4 and Na-CDPK5 or of At-CPK28 leads to an accumulation of different jasmonate metabolites (Figure 5; Yang et al., 2012a), disturbances in GA homeostasis (Heinrich et al., 2013; Matschi et al., 2013), and plants showing reduced growth, which are dependent on functional JA biosynthesis or signaling components (Figure 2; Yang et al., 2012a; Heinrich et al., 2013). These observations argue for a similar molecular function of these CDPK proteins, with functional redundancy in *N. attenuata*, presumably caused by gene duplication (Yang et al., 2012a), while only one protein, CPK28, adopts this function in Arabidopsis (Matschi et al., 2013).

JA and GA represent antagonistic components of the hormonal balance between growth induction and defense activation, and the hormone content and subsequent adaptations are regulated in response to changing external conditions (Kazan and Manners, 2012). This interplay of hormones is among others conferred by direct interaction of GA-regulating DELLA repressors with JA-dependent JAZ repressors (Hou et al., 2010; Wild et al., 2012; Yang et al., 2012b). The initial cause of the hormonal deregulation in *cpk28* is currently unclear: Do unknown mechanisms evoke CPK28-dependent JA accumulation leading to repression of GA-mediated growth processes (Yang et al., 2012b)? Or is CPK28, by direct or indirect means, responsible for GA biosynthesis, in which case, in *cpk28*, GA deficiency and accumulation of DELLA proteins cause enhanced DELLA-JAZ interaction and, without the repressing JAZ function, lead to increased JA responses (Hou et al., 2010)? On one hand, elevated JA marker gene expression in *cpk28* could not be reverted by GA treatment (Figure 3), similar to what is true for *NST* marker genes of *cpk28* and the connected secondary growth phenotype (Matschi et al., 2013). In the untreated GA biosynthesis mutant *ga1-3*, no enhanced JA marker gene expression can be observed (Hou et al., 2010), so genetically encoded GA deficiency does not automatically trigger enhanced JA gene transcription. On the other hand, constitutively JA accumulating plants were shown to exhibit reduced GA biosynthesis without treatment (Heinrich et al., 2013), and JA treatment of wild-type plants led to accumulation of DELLA proteins, which consecutively repressed GA signal transduction (Yang et al., 2012b). In the absence of a functional *CPK28* gene, the hormonal balance seems to tip to JA-mediated processes, possibly conferred by a deregulation of the JAZ-DELLA interaction or by an alteration of their stability, resulting in a constitutive repression of GA signal transduction. Plants with constitutively active DELLA repressors showed enhanced JA-dependent gene expression without any stimulus (Navarro et al., 2008). Enhanced JA signaling in *cpk28* could be caused by increased stability of DELLA proteins, potentially even regulated by a direct phosphorylation via CPK28. In rice (*Oryza sativa*), GA-dependent phosphorylation of the DELLA protein SLENDER RICE1 led to subsequent DELLA degradation and activation of GA signaling (Itoh et al., 2005). In Arabidopsis, the main DELLA repressors responsible for shoot elongation are RGA and GAI (Dill and Sun, 2001; Achard and Genschik, 2009). Importantly, abolishment of the functions of RGA or GAI in *cpk28* × *gai* or *cpk28-1* × *rga* did not lead to a reversion of the *cpk28* growth phenotype (Supplemental Figure 4). Additionally, RGA protein amounts of *cpk28* plants exhibiting the phenotype did not differ from wild-type levels. In conclusion, enhancing GA content by GA treatment or removal of GA repressors did not lead to a full reversion of the *cpk28* phenotype (Supplemental Figure 4; Matschi et al., 2013), whereas abolishment of JA biosynthesis or signal transduction completely reverted the growth reduction with all phenotypic properties (Figure 2; Supplemental Figures 2 and 3). Thus, the more likely scenario explaining the hormonal imbalance in *cpk28* is a primary JA accumulation consequently leading to GA deficiency.

CPK28 Does Not Influence JA-Dependent Defense Responses

Many observations made for *cpk28* are congruent with the description of *IRcdpk4/5* plants in *N. attenuata*. However, the major benefit achieved by cosuppression of Na-*CDPK4* and Na-*CDPK5*, enhanced resistance toward herbivores (Yang et al., 2012a), has not been observed in Arabidopsis as consequence of the loss of function of CPK28 (Figures 4 and 7). In *IRcdpk4/5* plants, JA accumulation was seen over a range of developmental stages and importantly in both leaf and stem tissue (Yang et al., 2012a; Heinrich et al., 2013). Conversely, CPK28 does not influence JA accumulation in the whole rosette but specifically in the central rosette tissue and only in the generative growth phase (Figure 5). No differences in JA content of the wild type and *cpk28* were detected when whole rosettes were analyzed (Supplemental Figure 6). Remarkably, even after wounding, JA levels in the whole *cpk28* rosette were similar to wild-type levels (Supplemental Figure 6), which most likely accounts for an unchanged resistance against herbivores or fungal infection on whole *cpk28* plants. This is in contrast to the description of *IRcdpk4/5* plants, showing elevated JA levels in rosette-staged plants before and after wounding or feeding (Yang et al., 2012a).

JA was demonstrated to be responsible for local as well as systemic defense responses, functioning as a signal required to transmit information about wounding or feeding both locally and to distant, nonwounded tissues (Schilmiller and Howe, 2005; Glauser et al., 2008). Plasma membrane-based wound-activated changes in surface potential were shown to be involved in distal JA-dependent defense-gene activation (Mousavi et al., 2013). In *cpk28*, elevated gene expression of JA-responsive genes was detectable in leaf tissue even without enhanced JA content (Figure 6A), suggesting that the spatially restricted elevated JA levels, at least to some extent, influence distal JA signaling and

likely result in changes in metabolite and protein profiles in the whole *cpk28* plant. This possibly accounts for the observed growth reduction on the whole-plant level, although so far, the cellular mechanisms underlying JA-mediated growth repression are not fully elucidated (Wasternack and Hause, 2013).

However, in *cpk28*, local elevation of JA metabolite levels does not lead to an enhanced wounding- or feeding-induced systemic response in leaf tissue. More importantly, even in tissue displaying elevated endogenous JA levels, no enhanced resistance toward herbivores or necrotrophic fungi could be detected (Figure 7). This suggests a different mode of action for the observed spatially restricted, developmentally active JA, repressed by CPK28 activity, which is independent of environmental stress stimuli. The existence of such "developmental JA" is supported by our observation of JA marker gene transcription gradually increasing in wild-type plants during plant development (Figure 1A). The unequal distribution of accumulating JA in *cpk28* plants fits with the observation of unelicited JA being concentrated in young apical sinks of untreated plants (Creelman and Mullet, 1995), and CPK28 seems to specifically contribute to this tissue-specific localization.

Therefore, our description of the role of CPK28 contributes to the ongoing discussion about the biological activities of JA metabolites and their putative functions in different biological contexts of development and plant defense (Zhang and Turner, 2008; Wasternack and Hause, 2013).

CPK28 Exhibits a Dual Function in Environmental Stress Response and Growth Regulation

CPK28 was shown to function as a negative modulator of PAMP-induced ROS generation and downstream immune signaling as early as the seedling stage, and loss of function of CPK28 resulted in increased resistance against bacterial infection (Monaghan et al., 2014). In this context, CPK28 adds a layer of regulation to the well-studied calcium-dependent defense response after this environmental challenge: Next to a calcium responsiveness of the ROS-generating enzyme RBOHD itself (Ogasawara et al., 2008), biochemical activation of the NADPH oxidase by phosphorylation via calcium-dependent protein kinases, including CPK5, and calcium-independent kinases such as BIK1 has been demonstrated (Dubiella et al., 2013; Gao et al., 2013; Kadota et al., 2014; Li et al., 2014). In contrast, with CPK28, a negative regulator was identified that diminishes PAMP-elicited ROS generation via the modification of BIK1 protein levels (Monaghan et al., 2014). Our genetic analyses of *cpk28* double mutants abolishing on one hand JA biosynthesis or signaling (*cpk28* × *aos* or *cpk28* × *coi1-16*) and on the other hand ROS production upon PAMP treatment (*cpk28* × *rbohd*) exclude a causal connection between ROS elevation and the observed JA accumulation during development in *cpk28* (Supplemental Figure 9). Both phenomena, occurring upon loss of function of the negative regulator CPK28, seem to act independently in different developmental stages and do not interdepend on each other: JA (signaling)-deficient plants still showed enhanced ROS levels, and ROS-deficient plants still displayed, although slightly less severe, the JA-dependent growth phenotype.

Calcium is thought to be an early key player of JA signaling because both wounding and herbivory result in a transient elevation of cytosolic calcium levels (Maffei et al., 2004). Calcium signal transduction components were characterized in herbivory-induced signal cascades (Kanchiswamy et al., 2010; Vadassery et al., 2012; Yang et al., 2012a), and elevation of gene expression as well as biochemical activation of a maize CDPK was seen in local and systemic tissue after touch and wounding stimuli (Szczegielniak et al., 2012). However, the nature of a calcium signal in a developmental context, activating CPK28 and, hence, subsequent specific modifications of the JA metabolism, is still unknown.

Interestingly, the *fou2* mutant in Arabidopsis, a gain-of-function variant of the vacuolar cation channel TPC1, was described to exhibit a stage-specific JA accumulation phenotype. In *fou2*, altered cytosolic calcium sensitivity has been observed, which had a direct effect on JA content and defense responses (Bonaventure et al., 2007a; Beyhl et al., 2009). However, unlike *cpk28*, only partial reversion of the phenotype could be achieved in *fou2* by abolishing JA biosynthesis or signaling, and this mutant showed increased resistance toward fungal infection (Bonaventure et al., 2007a, 2007b). Additionally, phenotypic comparison of *cpk28* and *fou2* revealed striking differences in growth parameters as well as shoot anatomy (Supplemental Figure 10), suggesting the presence of different calcium-related processes resulting in similar but not identical phenotypes. Remarkably, loss of function of TPC1 was recently shown to result in impaired systemic calcium signal propagation upon environmental stress stimuli (Choi et al., 2014), while local calcium responses to a variety of stimuli were unchanged (Ranf et al., 2008). The double mutant *cpk28-1* × *tpc1-2* did not revert the JA-dependent *cpk28* phenotype (Supplemental Figure 10), excluding the possibility of an overly active, *fou2*-like TPC1 channel in *cpk28*. More importantly, this result demonstrates that CPK28 presumably is activated by a unique developmental calcium signal, whose generation or propagation is independent of the TPC1-modulated calcium wave reported upon environmental stress perception (Choi et al., 2014).

Taken together, we demonstrate a dual function of CPK28, negatively regulating pathogen-elicited environmental stress signaling in plants as young as the seedling stage on one hand and regulating developmental JA-dependent growth processes, independent of any JA-mediated defense responses, in the adult plant on the other hand. Thus, CPK28 functions are allocated to two distinct biological contexts in separate growth phases during plant development. Future research should focus on dissecting the role of CPK28 in the calcium-dependent regulation of developmentally active JA-GA homeostasis, specifically by identifying in vivo substrates and elucidating the mechanism employed by a developmentally regulated calcium stimulus.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana accession Columbia-0 was used for all experiments. *cpk28* mutants, *35S:CPK28-YFP*, and *pCPK28:CPK28-YFP* lines were

described before (Matschi et al., 2013). Seeds of *aos* (SALK_017756), *rga28* (SALK_089146), *gai* (SAIL_587_C02), *rbohd* (SALK_070610), and *tpc1-2* (SALK_145413) were obtained from the Nottingham Arabidopsis Stock Center; *coi1-16* (*coi1-16B*; Noir et al., 2013) seeds were obtained from Alessandra Devoto (Royal Holloway, University of London); and *fou2* seeds were obtained from Edward E. Farmer (University of Lausanne). Homozygous double mutants were generated by crossing single mutants and confirmed by PCR in the F2 generation (for primers, see Supplemental Table 1). Plants were grown on soil under long-day conditions (16 h light, 8 h dark) at 22°C unless otherwise stated. GA treatments were performed by spraying plants with either 0.95% ethanol (mock) or 100 μM GA_3 every other day. Randomized grown plants were analyzed for morphological characteristics.

Determination of Anthocyanin Content and Stem Anatomy

Anthocyanin content was determined photometrically as described previously (Matschi et al., 2013). Hand-cut cross sections of the basal internode of the wild type and mutants were stained for lignin with 2% phloroglucinol and acidified with 37% HCl. Micrographs were taken using a Zeiss Axioplan microscope equipped with an AxioCam (Zeiss) and were processed through Photoshop.

RNA Isolation and RT-PCR Quantification

Material from two plants was pooled for each biological sample. RNA was extracted using the Trizol method (Chomczynski and Sacchi, 2006). Two micrograms of RNA was treated with RNase-free DNase (Fermentas) and reverse transcribed with Superscript III SuperMix (LifeTech) according to the manufacturer's protocols. qRT-PCR analysis was performed in a final volume of 10 μL using Power SYBR Green PCR Master Mix (LifeTech) according to the manufacturer's instructions using the CFX96 system (Bio-Rad). Specificity of the amplification reactions was assessed using postamplification dissociation curves. *ACTIN2* was used as an internal control for quantification of gene expression. Primer sequences are listed in Supplemental Table 2.

Larval Weight Gain and *Alternaria brassicicola* Infection Assays

Larval weight gain assays were performed according to Herde et al. (2013). Briefly, one (*Trichoplusia ni*) or three (*Spodoptera littoralis*) newly hatched larvae were transferred to 31-d-old plants grown in soil. Individual plants were confined in a cage and returned to the growth chamber. Fresh weights of larvae were determined individually after the respective days of feeding.

For weight gain assays on the central rosette tissue, laboratory-built split cages (2.5 cm diameter) were used. Plants were sown on soil to grow through a hole in the lower disc part of the cage from germination onward and growing leaves were elevated over the cage edges, if necessary. One larva of *T. ni* or *S. littoralis* and the cylindrical upper part of the cage were applied to the middle of the rosette on day 31 and fixed with a rubber band, preventing feeding on adult leaf blades due to cage size. Damage to the leaves and escape of caterpillars were prevented by attaching cotton wool to both edges of the cage parts.

For *A. brassicicola* inoculations, spores were prepared as described (Thomma et al., 1999) and diluted with water to a final density of 5×10^5 or 1×10^6 spores/mL. Different plant tissues were inoculated by placing one to four droplets of 5 μL spore suspension onto the leaf surface and kept at 100% relative humidity at 22°C under long-day conditions. Fungal growth was scored after 7 to 10 d by the classification of symptom severity: 1 = no symptoms, 2 = light necrotic lesions, 3 = severe necrotic lesions, 4 = spreading of lesions beyond infection site, 5 = whole leaf affected, and 6 = sporulation of the fungus.

JA Metabolite Measurements

OPDA, JA, JA-Ile, and 12-OH-JA contents were determined by LC-MS/MS according to Balcke et al. (2012) using 50 mg of plant material. Six biological replicates (single plants) per genotype, time point, and treatment were measured for whole-rosette analysis, while four replicates consisting of three pooled plants each per genotype and time point were measured in experiments in which inner rosette material was analyzed.

Fungal Growth Quantification by Quantitative PCR

A. brassicicola growth in infected plants was determined by relative quantification of fungal and plant DNA by means of quantitative PCR analysis. Total fungal and plant DNA was extracted as described (Gachon and Saindrenan, 2004) from one inoculated central rosette per sample after mock or pathogen inoculation 0 and 5 d postinfection. The relative quantity of *A. brassicicola* was calculated according to the abundance of the respective fungal *CUTINASE* gene (loci ABU03393), relative to the Arabidopsis-specific *ACTIN2* DNA measured by quantitative PCR. Analyses were performed in triplicate. Primer sequences (Berr et al., 2010) are detailed in Supplemental Table 2.

Root Growth Assays

Surface-sterilized seeds were plated on 0.5× Murashige and Skoog medium (pH 5.8) (Duchefa) supplemented with 0.8% agar. Plates were generally first incubated for 2 d at 4°C and then grown vertically at 22°C under LD conditions. Primary root growth was monitored every 3 to 4 d over 20 d. Evaluation of primary root growth inhibition by MeJA was performed 6 d after sowing seeds on 0.5× Murashige and Skoog medium containing 1% methanol (−) or 20 μM MeJA (+).

Protein Extraction and Immunoblot

Inner rosette material of three plants per genotype and replicate (31 dag) was pooled and frozen in liquid nitrogen. After homogenization, 45 mg of plant material per sample was extracted with extraction buffer (50 mM Tris-HCl, 150 NaCl, 0.5% Triton X-100, 10 μM MG132, 0.1 μM PMSF, and Sigma-Aldrich protease inhibitor cocktail, pH 7.5; Willige et al., 2007). Proteins were separated on a 12% SDS-PAGE gel and analyzed by protein gel blot. RGA protein detection was performed using anti-RGA antibody (1:500) (donated by AG Schwechheimer, TU München) as primary and anti-rabbit IgG antibody conjugated with horseradish peroxidase (Sigma-Aldrich) as secondary antibody (1:1000).

PAMP-Triggered ROS Measurements

Flg22-dependent ROS production was monitored in 31-d-old plants grown in LD conditions using a luminol-based assay described by Dubiella et al. (2013). Leaf discs (0.3 or 0.5 cm diameter) were cut out and floated on 100 μL water in a 96-well plate overnight. On the following day, 100 μL assay solution (final concentration: 0.034 mg/mL luminol and 0.02 mg/mL horseradish peroxidase) with or without 200 nM flg22 was added to the leaf discs, and total photon counts were immediately measured over one hour in a TriStar LB941 microplate reader (Berthold Technologies).

Statistical Analyses

Statistically significant groups were determined by Student's *t* test, one-way ANOVA followed by Tukey's multiple comparison post-test or two-way ANOVA followed by Bonferroni multiple comparisons post-test using GraphPad Prism 4.01 software.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: At5g66210 (*CPK28*), AT3G18780 (*ACT2*), At5g44420 (*PDF1.2*), At4g23600 (*CORI3*), At5g24770 (*VSP2*), At5g42650 (*AOS*), At2g06050 (*OPR3*), At1g19180 (*JAZ1*), At5g13220 (*JAZ10*), AT3G12500 (*PR3*), AT3G04720 (*PR4*), At2g39940 (*COI1*), At2g46770 (*NST1*), At1g15550 (*GA3ox1*), At2g01570 (*RGA*), At1g14920 (*GAI*), At5g47910 (*RBOHD*), and At4g03560 (*TPC1*).

Supplemental Data

Supplemental Figure 1. CPK28 Has No Influence on MeJA-Induced Root Growth Inhibition.

Supplemental Figure 2. JA Biosynthesis or JA Perception Is Required for the *cpk28* Growth Phenotype.

Supplemental Figure 3. JA Biosynthesis or JA Perception Is Required for Altered Gene Expression in *cpk28* Mutants.

Supplemental Figure 4. DELLA Abundance Is Not Influencing the *cpk28* Phenotype.

Supplemental Figure 5. CPK28 Does Not Influence Suppressed Resistance to Fungal Infection in JA Biosynthesis or Signaling Mutants.

Supplemental Figure 6. JA Metabolite Content in Whole *cpk28* Plants Is Similar to Wild Type Regardless of Age or Wounding Treatment.

Supplemental Figure 7. Harvesting Schemes of JA Metabolite Measurements and Tissue-Specific qRT-PCR.

Supplemental Figure 8. Tissue-Specific *Alternaria brassicicola* Infection Assay.

Supplemental Figure 9. Elevated ROS Levels Are Not the Cause of the JA-Dependent Growth Phenotype in *cpk28*.

Supplemental Figure 10. Environmental Stress-Induced Calcium Perturbations Are Not the Cause of the JA-Dependent Growth Phenotype in *cpk28*.

Supplemental Table 1. Genotyping Primers Used in This Study.

Supplemental Table 2. qRT-PCR Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

S.M., M.H., B.H., and T.R. designed the research. S.M. and K.H. performed most of the research. M.H. contributed knowledge and handling of larval feeding assays. B.H. carried out JA metabolite measurements. S.M. and T.R. wrote the article.

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