Extracellular ATP Shapes a Defense-Related Transcriptome Both Independently and along with Other Defense Signaling Pathways[1][OPEN]

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ATP is not only an essential metabolite of cellular biochemistry but also acts as a signal in the extracellular milieu. In plants, extracellular ATP is monitored by the purinergic receptor P2K1. Recent studies have revealed that extracellular ATP acts as a damage-associated molecular pattern in plants, and its signaling through P2K1 is important for mounting an effective defense response against various pathogenic microorganisms. Biotrophic and necrotrophic pathogens attack plants using different strategies, to which plants respond accordingly with salicylate-based or jasmonate/ethylene-based defensive signaling, respectively. Interestingly, defense mediated by P2K1 is effective against pathogens of both lifestyles, raising the question of the level of interplay between extracellular ATP signaling and that of jasmonate, ethylene, and salicylate. To address this issue, we analyzed ATP-induced transcriptomes in wild-type Arabidopsis (*Arabidopsis thaliana*) seedlings and mutant seedlings defective in essential components in the signaling pathways of jasmonate, ethylene, and salicylate (classic defense hormones) as well as a mutant and an overexpression line of the P2K1 receptor. We found that P2K1 function is crucial for faithful ATP-induced transcriptional changes and that a subset of genes is more responsive in the P2K1 overexpression line. We also found that more than half of the ATP-responsive genes required signaling by one or more of the pathways for the classical defense hormones, with the jasmonate-based signaling being more critical than others. By contrast, the other ATP-responsive genes were unaffected by deficiencies in signaling for any of the classical defense hormones. These ATP-responsive genes were highly enriched for defense-related Gene Ontology terms. We further tested the ATP-induced genes in knockout mutants of transcription factors, demonstrating that MYCs acting downstream of the jasmonate receptor complex and calmodulin-binding transcription activators are nuclear transducers of P2K1-mediated extracellular ATP signaling.

ATP, a universal biological energy currency and genetic building block, is maintained at a high concentration (approximately millimolar) intracellularly. By contrast, the resting extracellular ATP concentration is roughly 6 orders of magnitude lower (Watt et al., 1998; Weerasinghe et al., 2009). This steep chemical gradient provides an elegant basis for cellular signaling (Verkhratsky and Burnstock, 2014). Transmembrane receptors monitor changes in extracellular ATP concentration to detect either uncontrolled ATP release caused by necrosis of nearby cells (Davalos et al., 2005; Song et al., 2006) or active ATP release induced after pathogen detection or treatment with, for example, salicylic acid (Chivasa et al., 2009; Chen et al., 2017). In this manner, extracellular ATP can be perceived as a sign of damaged self, or a damage-associated molecular pattern (DAMP; Tanaka et al., 2010a, 2014; Cho et al., 2017). For general discussions of the DAMP concept, see reviews by Heil (2009), Boller and Felix (2009), and Lotze et al. (2007). In animals, extracellular ATP has been well studied due to its significant medical implications, and its important roles in physiological processes and signal transduction mechanisms are documented in detail (Burnstock, 2017). In contrast, extracellular ATP signaling in plants is only beginning to be understood.

A plant extracellular ATP receptor, P2K1, originally identified by genetic screen as an Arabidopsis (*Arabidopsis thaliana*) mutant, *does not respond to nucleotides1* (*dorn1*), is a legume-type lectin receptor kinase, LecRK-I.9 (Choi et al., 2014). ATP binding to the extracellular domain and kinase activity of the intracellular domain

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of P2K1 are essential for the plant response to extracellular ATP (Choi et al., 2014). P2K1 is expressed during the major stages of plant growth and development (Cho et al., 2017), suggesting that extracellular ATP is involved in various physiological processes, including seedling growth, stomata movement, pollen tube development, root hair growth, gravitropism, and abiotic and biotic stress responses.

Mechanisms of extracellular ATP signal transduction through P2K1 are largely unknown, although a growing body of circumstantial evidence indicates a role in plant defense against pathogens. For example, overexpression of P2K1 protects plants against the oomycete pathogen *Phytophthora brassicae* and the bacterial pathogen *Pseudomonas syringae* (Bouwmeester et al., 2011; Balagué et al., 2017). A recent study demonstrated that extracellular ATP-induced plant protection against the fungal pathogen *Botrytis cinerea* depended on a functional jasmonate signaling pathway but not jasmonate biosynthesis (Tripathi and Tanaka, 2018; Tripathi et al., 2018). Moreover, ATP-induced stomatal immunity against the bacterial pathogen *P. syringae* was caused by P2K1-mediated activation of the plasma membrane-localized NADPH oxidase RBOHD that produces reactive oxygen species (Chen et al., 2017). Lastly, application of extracellular ATP to lima bean (*Phaseolus lunatus*) induced an indirect defense, the secretion of extrafloral nectar (Heil et al., 2012). Therefore, extracellular ATP is likely involved in different resistance mechanisms.

Plant pathogens can be divided into biotrophs and necrotrophs based on their lifestyles. Plant defense against hemibiotrophs/biotrophs (e.g. *P. syringae* and *P. brassicae*) and necrotrophs (e.g. *B. cinerea*) generally relies on salicylate-based and jasmonate/ethylene-based defensive signaling pathways, respectively (Glazebrook, 2005). Interestingly, the above-mentioned ATP-induced resistances were effective against both types of pathogens (i.e. necrotrophs and biotrophs). This led us to hypothesize that the extracellular ATP signaling pathway interacts with other signaling pathways of classical defense hormones (i.e. jasmonate, ethylene, and salicylate).

To characterize the interaction between extracellular ATP signaling and that of these classical defense hormones, we investigated the ATP-induced transcriptomes in several mutants defective in signaling of jasmonate, ethylene, and salicylate (*coi1*, *ein2*, and *npr1*) as well as the *dorn1* mutant and a P2K1 overexpression line. Our results reveal that (1) the ATP-responsive transcriptome depends on a functional P2K1 receptor, (2) many ATP-responsive genes depend on one or more of the signaling pathways of the classical defense hormones, (3) nearly 30% of ATP-responsive genes are solely dependent on jasmonate-mediated signaling, and (4) nearly half of ATP-responsive genes are unaffected by deficiencies in the classical defense signaling pathways of jasmonate, ethylene, and salicylate. These results suggest that extracellular ATP induces a number of defense-related genes, half of them independent of, but the other half acting cooperatively with, the classical defense hormones. Finally, we find that MYC transcription factors and a calmodulin-binding transcription activator (CAMTA3) are important signaling components for ATP-responsive gene expression.

RESULTS

Experimental Design and Validation of Transcriptome Profiling for Extracellular ATP-Induced Responses

In a preliminary dose-response experiment, we found that treating seedlings with increasing ATP concentrations increased the induction of selected ATP-responsive genes, with 500 μM ATP treatment maximally stimulating expression, although significant gene induction could also be detected with treatments of ATP as low as 50 μM (Fig. 1A). We also tested whether addition of the polyvalent anion ATP to the test solution would alter the pH. As expected, even though its stock solution was prepared in MES buffer, ATP reduced the pH level in a dose-dependent manner, with 1 mM ATP lowering the pH to 5.3 (Fig. 1B). This result led us to further examine the effect of the pH drop on ATP-responsive gene expression. RT-qPCR demonstrated no changes in the gene expression tested even in the lowest pH condition (i.e. pH 5.3) in the absence of ATP (Fig. 1A). Therefore, we concluded that ATP has bona fide effects on plant physiology (gene expression in our case) regardless of its anionic effect in the treatment solution.

To define the interaction of ATP-responsive transcription with classical defense signaling pathways, we treated 8-d-old seedlings of wild-type and mutant lines with 500 μM ATP or vehicle solution for 30 min. The mutants used were *coi1-30*, defective in all known jasmonate responses (Xie et al., 1998; Yang et al., 2012); *ein2-1*, defective in most known ethylene responses (Alonso et al., 1999); *npr1-3*, deficient in all known salicylate responses (Cao et al., 1997); and *dorn1-3*, defective in ATP-induced cytosolic calcium transients and ATP-induced pathogen resistance (Choi et al., 2014; Tripathi et al., 2018). In addition, we examined *OxP2K1*, an overexpression line of the extracellular ATP receptor (Choi et al., 2014). The experiment was replicated six times in order to have the statistical resolving power needed to determine quantitative differences between the treatments. Seventy-two RNA samples were used in mRNA sequencing (mRNA-seq) analysis, using 1 × 100-bp Illumina sequencing. The mRNA-seq libraries were prepared from total RNA using poly(A) enrichment of the mRNA. Hierarchical clustering and principal component analysis indicated a high level of data quality, with different samples of treatment/genotype combinations clustering together (Supplemental Figs. S1 and S2). Moreover, RT-qPCR analysis of a subset of these genes indicated a high concordance with the sequencing by synthesis-based gene expression estimates (R^2 = 0.98; Supplemental Fig. S3), which confirmed the validity of the mRNA-seq data in this study.

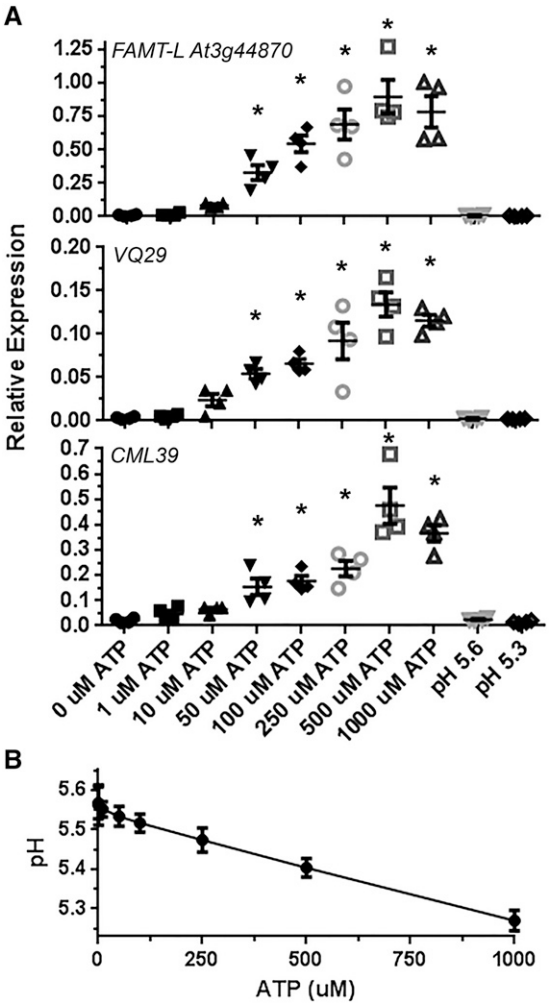

Figure 1. Dose-dependent effect of ATP on gene expression in wild-type plants and pH in the treatment solution. A, Eight-day-old seedlings were treated with varying concentrations of ATP or with different pH levels for 30 min, and gene expression of the indicated genes was estimated by reverse transcription quantitative PCR (RT-qPCR; mean, individual replicate values and SE are shown, $n = 4$). The expression of each gene was normalized by comparison with the internal control *PP2A* (Czechowski et al., 2005). Gene expression different from the non-ATP treatment is indicated by asterisks ($P < 0.05$). B, Addition of ATP to the test solution decreases the pH. Note that ATP treatment induces ATP-responsive gene expression but that pH decrease does not.

Extracellular ATP-Responsive Gene Expression in the Wild Type, the *P2K1* Overexpression Line, and the *dorn1* Mutant

The response to extracellular ATP in wild-type seedlings led to the induction of 500 genes and the repression of 43 genes, with a cutoff at false discovery rate (FDR) < 0.05 and fold change (FC) > 1.5. All gene expression changes after ATP treatment are shown in Supplemental Data Set S1. The ATP-responsive genes were significantly enriched in Gene Ontology (GO) biological processes (Fig. 2A) related to response to wounding (FDR = 2.89e-12) and response to chitin (FDR = 2.96e-12), similar to what was described for ATP response in root tissue (Cao et al., 2014; Choi et al., 2014). In addition, protein phosphorylation (FDR = 8.52e-7), response to jasmonic acid (FDR = 1.35e-7), regulation of innate immune response (FDR = 1.55e-4), salicylic acid biosynthesis (FDR = 1.22e-3), and abscisic acid-activated signaling pathway (FDR = 8.21e-3) were also highly enriched biological process GO terms (Fig. 2A). We further performed MapMan mapping in the metabolism overview pathway, demonstrating that the ATP-induced transcriptome was highly enriched in the category biotic stress (Supplemental Fig. S4). With regard to molecular function (Fig. 2B), the ATP-responsive genes were enriched for calcium ion binding (FDR = 1.13e-5), calmodulin binding (FDR = 3.12e-5), and protein Ser/Thr kinase activity (FDR = 1.08e-4). Genes responsive to ATP were highly enriched for plasma membrane cellular components (FDR = 9.10e-12; Fig. 2C). In summary, ATP-responsive genes in wild-type seedlings tend to encode plasma membrane-localized defense-related proteins with kinase or calcium-binding activity.

We further compared the ATP-induced transcriptome in the wild type with that in *OxP2K1*. The data indicated that there was substantial overlap between ATP-responsive gene expression in the wild type and *OxP2K1*, with 96% of genes identified in the wild type also being identified in the P2K1-overexpressing line (523 of 543; Fig. 3A). A total of 329 of the 523 ATP-responsive genes in common between the wild type and *OxP2K1* were greater than 2-fold more responsive in the *OxP2K1* line compared with the wild type (red dots in Fig. 3B; $P = 7.54e\text{-}79$, two-tailed heteroscedastic Student's t test), indicating that most of the ATP-responsive genes are regulated in correlation with the amount of the receptor, P2K1. Those 329 genes were composed of the same ontological components based on GO analysis (Supplemental Fig. S5). In parallel, 3,576 genes were identified as ATP responsive in *OxP2K1* that were not differentially expressed in the wild type. The genes identified in *OxP2K1* but not in the wild type were enriched for biological processes also noted in the wild type (Supplemental Fig. S6), such as response to chitin (FDR = 1.42e-20), response to wounding (FDR = 3.47e-4), response to jasmonic acid (FDR = 1.08e-3), regulation of innate immune response (FDR = 5.97e-3), and plasma membrane localization (FDR = .72e-17). In addition, DNA-binding transcription factor activity (FDR = 3.83e-20), ethylene-activated signaling pathway (FDR = 1.50e-4), response to gibberellin (FDR = 2.29e-3), and response to karrikin (FDR = 5.10e-3) were included.

In contrast, only a single gene (AT1G80240; DUF642) was identified as ATP responsive in the *dorn1-3* mutant ($\log_2$FC = 1.45, FDR = 0.038). The expression of this gene of unknown function was also induced by ATP in a similar manner in the wild type ($\log_2$FC = 1.28, FDR = 6.36e-4) and *OxP2K1* seedlings ($\log_2$FC = 1.33, FDR = 1.85e-5), perhaps indicating the existence of another signaling pathway that mediates extracellular ATP-responsive gene expression irrespective of P2K1

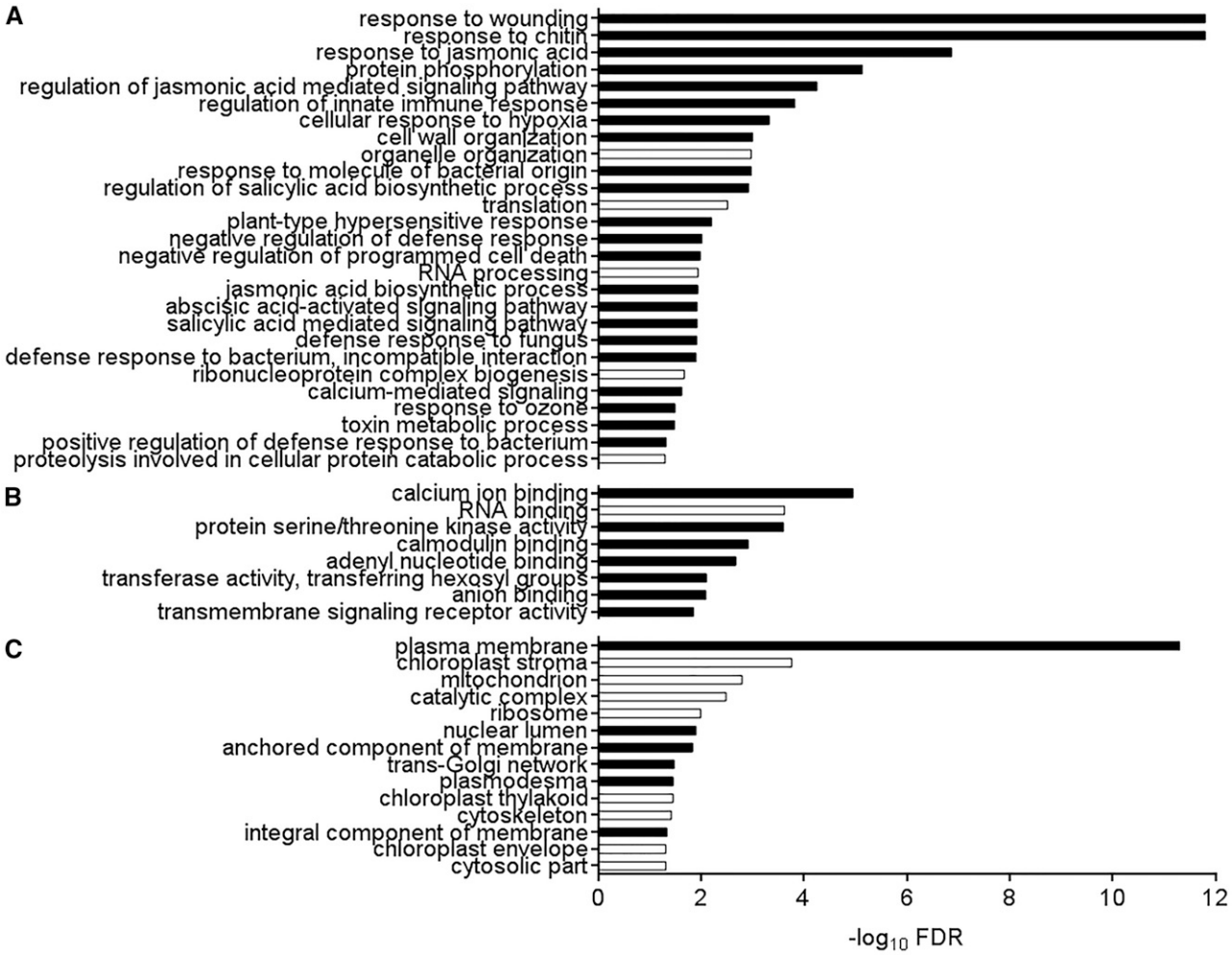

Figure 2. GO term enrichment for ATP-responsive gene expression in the wild type. Biological processes (A), molecular functions (B), and cellular components (C) significantly enriched (FDR < 0.05) in 543 ATP-responsive genes in wild-type seedlings are shown. Black and white bars indicate enrichment and depletion, respectively. Note that, perhaps because of the low number of ATP-repressed genes, no functional categories were detected to be enriched in ATP-repressed genes, so these GO terms represent all 543 ATP-responsive genes whether repressed or induced.

function. However, the vast majority (99.8%) of ATP-responsive genes identified here depended on P2K1.

To identify conserved promoter elements that may be responsible for ATP-responsive transcription, we submitted the 1,000 bp upstream of the transcription start site of the 543 ATP-responsive genes to the regulatory sequence analysis tools (RSAT) server to search de novo for nucleotide sequences enriched relative to the promoters of the Arabidopsis genome (Defrance et al., 2008; Nguyen et al., 2018). Among the 33 significantly enriched 8-mer sequences ($P < 3e\text{-}5$; Supplemental Table S1), 23 contained a CGCG motif, the core motif of an element referred to as a CAM-box and known to be bound by so-called CAMTAs (Benn et al., 2014). Eight of the 33 sequences contained a TGAC element, the core motif of the WRKY transcription factor-bound W-box (Eulgem et al., 1999); one was composed of GAAAATTC, a portion of the minimal heat shock element (HSE) bound by heat shock transcription factors (Kumar et al., 2009); and one was a pyrimidine-rich sequence (CCTCCTCC), which may represent enrichment of the Y patch, which has previously been proposed to be a general component of core plant promoters and for which a binding factor is unknown (Yamamoto et al., 2007). To directly evaluate the enrichment of the CAM-box, the W-box, and the HSE in these promoters, we scored the presence of these elements in ATP-responsive gene promoters and in the promoters of the Arabidopsis genome, evaluating significance by hypergeometric distribution. Given the apparent role for extracellular ATP signaling in pathogen defense, we also tested for the enrichment of elements previously implicated in defense-related gene expression. These additional elements are the G-box (CACGTG), implicated in jasmonate-responsive expression (Fernández-Calvo et al., 2011; Figueroa and Browse, 2012); the abscisic acid response element (ABRE; Anderson et al., 2004; Adie et al., 2007); and the

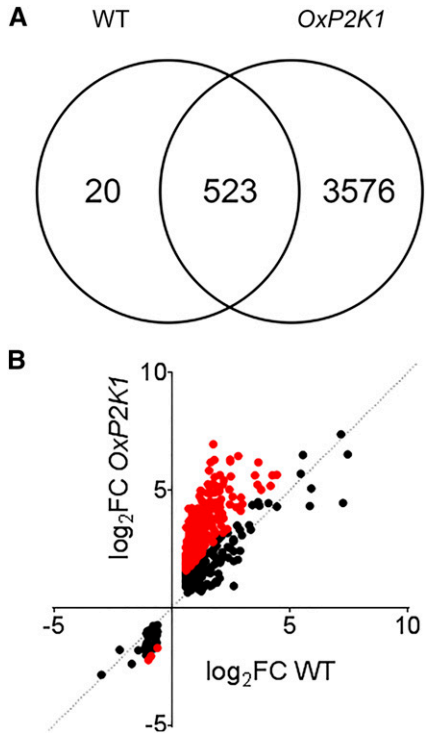

Figure 3. Comparison of gene expression between *OxP2K1* and the wild type. A, Venn diagram showing overlap between genes responsive to ATP in the wild type (WT) and *OxP2K1*. B, The $\log_2$FCs (ATP/mock) were compared for the 523 genes differentially expressed in both the wild type and *OxP2K1*. Red dots represent the 329 genes whose expression was greater than 2-fold more highly responsive in *OxP2K1* than in the wild type.

GCC-box (GCCGCC), implicated in ethylene-responsive gene expression (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998). These analyses are summarized in Table 1. Notably, the CAM-box was highly enriched (2-fold, P = 5.1e-26) in ATP-responsive promoters in the wild type. Significant enrichment was also detected for the W-box (P = 1.1e-9), the HSE (P = 3.7e-4), the G-box (P = 4.1e-3), and the ABRE (P = 0.017). The GCC-box was marginally significantly (P = 0.058, fold enrichment = 0.94) underrepresented in these promoters.

We similarly analyzed the enrichment of these promoter elements in the 4,099 genes responsive to ATP in *OxP2K1* seedlings, where broadly comparable results were seen (Supplemental Table S2), although here the GCC-box was significantly, although subtly, underrepresented (P = 0.019, fold enrichment = 0.977).

ATP-Responsive Gene Expression in Mutant Seedlings Defective in Jasmonate, Ethylene, and Salicylate Signaling

Next, we compared ATP-responsive gene expression in wild-type seedlings with that of the *coi1-30*, *ein2-1*, and *npr1-3* mutants. Similar numbers of genes were responsive to ATP in these mutants and wild-type plants (Supplemental Table S3). However, hierarchical clustering of gene expression in the different genotype/treatment combinations revealed a clear disparity between ATP-responsive gene expression in *coi1-30* and the three other genotypes; both ATP- and mock-treated samples of *coi1-30* were more similar to mock-treated than ATP-treated samples of the other genotypes (Fig. 4B). On average, genes responding to ATP in the wild type were 67% less responsive to ATP in *coi1-30* (P = 4.8e-20, two-tailed heteroscedastic Student's t test), where no such differences were observed in comparisons between wild-type seedlings and *ein2-1* or *npr1-3* (P = 0.58 and 0.14, respectively).

The unique transcriptomic response to ATP in the *coi1-30* mutant was also evident in heat maps comparing the responses of these four genotypes for classes of GO terms enriched in the wild type. Hierarchical clustering of these data revealed that wild-type, *npr1-3*, and *ein2-1* responses were more similar to each other in comparison with *coi1-30* in GO classes including response to wounding, response to chitin, regulation of salicylic acid biosynthesis, calcium ion binding (Fig. 5), and protein phosphorylation and compared with genes encoding plasma membrane-localized proteins (Supplemental Fig. S6).

There were 154 genes responsive to ATP in the wild type that were solely dependent on intact COI1 but at the same time unaffected by the NPR1 and EIN2 functions. In contrast, there were only six and 21 genes dependent solely on EIN2 (not COI1 and NPR1) and NPR1 (not COI1 and EIN2), respectively (FDR $<$ 0.05 and absolute fold change $>$ 1.5; Fig. 4A). Interestingly, 129 genes depended on two or more of these pathways. With regard to GO enrichment, the 154 COI1-dependent ATP-responsive genes were significantly enriched for eight biological processes (Fig. 6), in contrast to NPR1-dependent and EIN2-dependent genes in which no enrichment for any GO term was detected. COI1-dependent ATP-responsive genes were enriched in expected GO biological processes such as response to wounding (FDR = 3.45e-9), response to chitin (FDR = 6.33e-9), and response to jasmonic acid (FDR = 3.05e-6).

Finally, we examined the promoters of these genes dependent on COI1, NPR1, or EIN2 to look for elements enriched in putative promoters of genes responsive to ATP in wild-type seedlings (Table 2). As observed for ATP-responsive gene promoters in wild-type plants, the CAM-box was significantly enriched in promoters of COI1-dependent genes (P = 1.59e-4). Although G-box-binding basic helix-loop-helix transcription factors such as MYC2, MYC3, and MYC4 are important primary signal transducers in COI1-dependent signaling (Chini et al., 2007; Fernández-Calvo et al., 2011; Niu et al., 2011), we observed that G-box elements were not significantly enriched in COI1-dependent ATP response genes (P = 0.09) but rather were found 2-fold enriched in NPR1-dependent genes (P = 0.04). Interestingly, the W-box, long known as a salicylate-responsive element, was enriched in COI1-dependent ATP-responsive promoters (P = 1.20e-5) but not found enriched in NPR1-dependent promoters (P = 0.137).

Table 1. *Enrichment of defense-related cis-elements in promoters of ATP-responsive genes in wild-type seedlings*

The presence or absence of the indicated promoter elements in the presumptive promoters (−1,000 bp upstream of the transcriptional start site) was scored in 543 ATP-responsive genes and 33,693 genes of the Arabidopsis nuclear genome, and significant enrichment was tested by hypergeometric test. Italicized values indicate that the element was not significantly enriched ($P > 0.05$). Fold enrichment is calculated as (percentage presence in ATP-responsive genes)/(percentage presence in all Arabidopsis genes).

Element	Sequence	Presence	Fold Enrichment	*P*
CAM-box	VCGCGB	206	2.01	5.1e-26
W-box	TTGACY	428	1.17	1.1e-9
HSE	GAANNTTC	215	1.18	3.7e-4
G-box	CACGTG	93	1.24	4.1e-3
ABRE	ACGTGKC	82	1.16	0.017
GCC-box	GCCGCC	*43*	*0.94*	*0.058*

Furthermore, to our knowledge, the HSE has not been identified previously as enriched in COI1-dependent promoters, yet we found it enriched in COI1-dependent ATP-responsive genes (P = 8.93e-4).

ATP-Responsive Gene Expression Independent of COI1, NPR1, and EIN2

In total, we found 233 genes induced by extracellular ATP in the wild type that were also responsive in *coi1-30*, *ein2-1*, and *npr1-3*, indicating that these genes are regulated independently of the functioning of any signaling pathways of classical defense hormones (i.e. jasmonate, ethylene, and salicylate; Fig. 4A). These 233 genes were termed as putative ATP-specific genes for convenience. As shown in Figure 7, GO term enrichment in the putative ATP-specific genes presented defense response to bacterium (FDR = 3.43e-7), response to chitin (FDR = 3.33e-6), salicylic-acid-mediated signaling pathway (FDR = 8.79e-6), calcium and calmodulin binding (FDR = 7.96e-10 and 1.16e-6, respectively), and genes encoding plasma membrane-localized proteins (FDR = 1.73e-13).

As for genes responsive to ATP in wild-type seedlings, we submitted the presumptive promoters for these genes to the RSAT server to search de novo for enriched sequence elements. No novel elements were identified. Rather, in 23 identified 8-mer sequences, 21 contained a CGCG CAM-box and two contained a W-box (Supplemental Table S4). Indeed, the VCGCGB CAM-box is highly enriched in these promoters relative to Arabidopsis nuclear genome promoters (2.6-fold, P = 3.6e-26) and is more enriched in these promoters than in the 543 ATP-responsive genes in the wild type. The W-box, G-box, ABRE, and HSE are also significantly enriched in these ATP-specific promoters (Table 2).

Contribution of MYC Transcription Factors to ATP-Specific Gene Expression

Given the larger number of genes dependent on COI1 for ATP-responsive expression, and given the importance of MYC transcription factors for jasmonate signaling (Fernández-Calvo et al., 2011; Niu et al., 2011), we investigated the contribution of MYC transcription factors to ATP-responsive transcription. We treated 8-d-old wild type, the *myc2-1* mutant (Boter et al., 2004), and the *myc2-1 myc3-1 myc4-1* triple mutant (Major et al., 2017) for 30 min with 500 μM ATP or vehicle solution and performed RT-qPCR analysis on COI1-dependent or ATP-specific transcripts. We chose 14 ATP-responsive genes for analysis: five dependent on COI1 and nine ATP specific. As shown in Figure 8A, genes dependent on COI1 for induction by ATP were, to differing degrees, dependent on MYC function for ATP response. *TAT3*, well known as a COI1- and MYC2-dependent jasmonate response marker gene (Titarenko et al., 1997; Jung et al., 2015), and *FAMT-L* (AT3G44870), an uncharacterized gene encoding a protein 93% identical to a farnesoic acid methyl transferase (Yang et al., 2006), were induced by ATP treatment in the wild type but not in *myc2* or the *myc2 myc3 myc4* triple mutant. *VQ29*, a gene encoding a VQ motif protein dependent on JAZ3 degradation for jasmonate-responsive induction (Chini et al., 2007), was more subtly perturbed in ATP response, with significantly reduced induction only in the triple mutant. Among tested ATP-specific genes, *ERF13*, encoding an ethylene-responsive element-binding factor, was induced similarly, irrespective of MYC mutation (Fig. 8B). *WRKY38* and the uncharacterized gene AT4G23515, encoding a Toll/IL-1 receptor homology domain-containing protein, were similarly responsive to ATP treatment in *myc2* and the wild type, and substantially reduced induction was observed in the triple mutant.

Contribution of CAMTA3 to ATP-Responsive Gene Expression

Since the CAM-box is highly enriched in ATP-responsive genes and Arabidopsis CAM-box-binding factors are known, we compared ATP-responsive gene expression in 8-d-old seedlings of wild-type Arabidopsis and the *atsr1-1* mutant, a null mutation of the CAM-box-binding transcription factor CAMTA3 (Du et al., 2009). Seven of the tested ATP-responsive genes had significantly lower expression after ATP treatment in the *atsr1* mutant than in wild-type seedlings

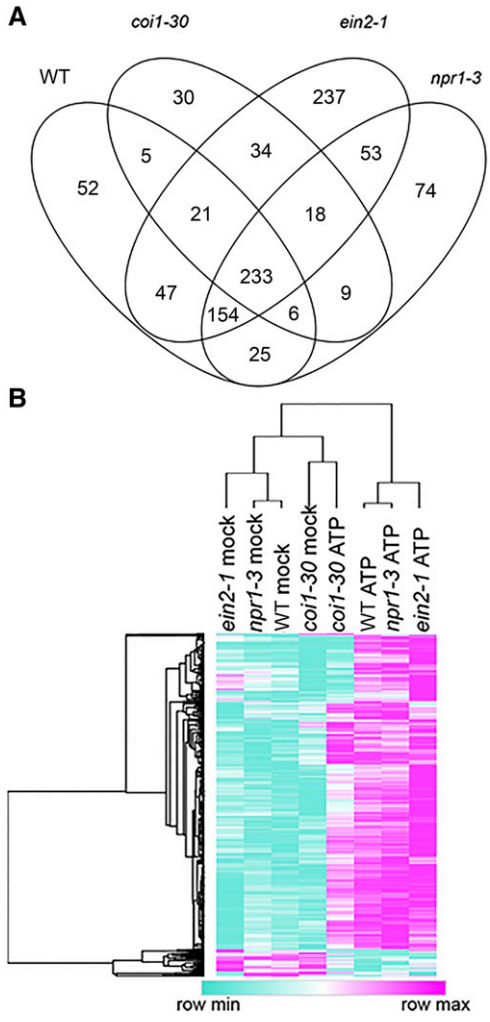

Figure 4. Overview of ATP-responsive gene expression in the wild type and *coi1-30*, *npr1-3*, and *ein2-1* mutants. A, Overlap between ATP-responsive gene expression in the indicated genotypes 30 min after treatment with 500 μM ATP. B, Hierarchical clustering of average expression [$\log_2$(cpm+1)] for 543 genes identified as ATP responsive in the wild type (WT). Rows and columns were clustered by 1 − Pearson correlation using the Morpheus online tool (https://software.broadinstitute.org/morpheus), and the tree was drawn using average linkage.

($P < 0.05$, Fisher's LSD; Fig. 9A). Because a recent study indicated that this mutant exhibits an autoimmunity phenotype due to constitutive activation of nucleotide-binding, Leu-rich repeat guard proteins (Lolle et al., 2017), and this immune activation could potentially alter the transcriptional response to extracellular ATP, we also tested the expression of two immune markers reported to be highly expressed in mature *atsr1-1* plants, *PATHOGENESIS-RELATED1* (*PR1*) and *ISOCHORISMATE SYNTHASE1* (*ICS1*; Du et al., 2009; Kim et al., 2017; Lolle et al., 2017). As shown in Figure 9B, *PR1* and *ICS1* were not significantly accumulated under mock conditions in *atsr1*; in fact, *ICS1* was slightly less abundant ($P = 0.012$, Fisher's LSD). Based on this result, we conclude that CAMTA3 function is required for proper gene induction in response to extracellular ATP. Furthermore, lack of immunity marker gene expression suggests that autoimmunity due to loss of CAMTA3 function is not likely to be responsible for the perturbed ATP-responsive expression.

DISCUSSION

ATP, a fundamental building block of life on Earth, is an extracellular signaling molecule in animals (Verkhratsky and Burnstock, 2014) and is recently coming to be considered an extracellular signaling molecule in plants as well (Tanaka et al., 2010a). Although the physiological function and signal transduction downstream of plant extracellular ATP recognition remain poorly understood, our study provides several insights from mRNA-seq analysis, as discussed below, and demonstrates that extracellular ATP treatment causes transcriptional reprogramming of defense-related genes that fully depends on the functional P2K1 receptor.

Extracellular ATP Orchestrates Plant Defense Responses Independent of, But in Parallel with, the Classical Defense Hormones

Several studies have indicated that extracellular ATP plays an important role as a DAMP that contributes to successful plant defense against hemibiotrophic and necrotrophic pathogens (Bouwmeester et al., 2011; Balagué et al., 2017; Chen et al., 2017; Tripathi et al., 2018). Plant defense responses depend to varying degrees on signaling of the classical defense hormones salicylate, ethylene, and jasmonate (Glazebrook, 2005; Spoel et al., 2007; Hillmer et al., 2017).

Our transcriptomics experiments demonstrated that more than half (57%, 310 of 543) of ATP-responsive genes required signaling by one or more of the pathways of the classical defense hormones. This result is in line with a recent report in which 65% (5,189 of 7,918) of flg22-responsive transcriptional changes depended on one or more of the pathways of the classical defense hormones (Hillmer et al., 2017). Moreover, our data indicated that GO biological processes for response to jasmonic acid and for salicylic acid-mediated signaling are both enriched in the set of genes responsive to extracellular ATP treatment in the wild type. Indeed, the genes induced include primary signal transduction components of both salicylic acid and jasmonate signaling pathways, such as several *JAZ* genes, *MYC2*, *JAM1*, and *NPR3* (Sasaki-Sekimoto et al., 2013; Ding et al., 2018). In addition, there were genes involved in the regulation of biosynthesis of salicylic acid, such as *SARD1*, *PAD4*, *CBP60G*, and *CBP60A* (Zhou et al., 1998; Zhang et al., 2010), and nearly all of the genes encoding jasmonate biosynthetic enzymes. Although ethylene signaling and biosynthesis were not identified as enriched processes in ATP-responsive genes in the

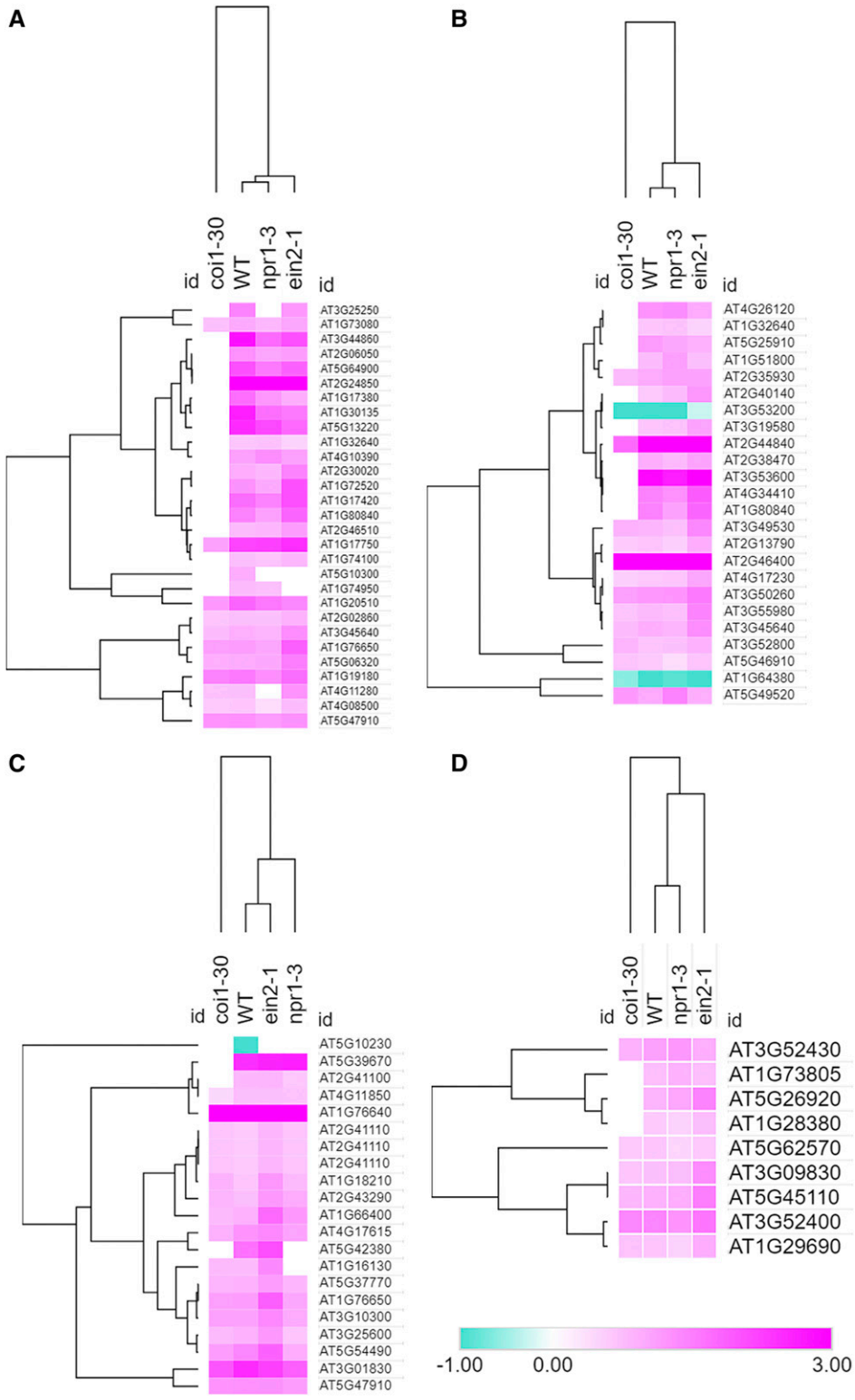

Figure 5. Regardless of the biological process, molecular function, or cellular component, *coi1-30* shows a markedly different transcriptional response to ATP stimulus. Log_2FC for genes responsive to ATP in the wild type (WT) in selected GO categories, response to wounding (A), response to chitin (B), regulation of salicylate biosynthesis (C), and calcium ion binding (D), is shown. If a given gene had FDR > 0.1 in a given genotype, the raw FC value was set to 1, and log_2FC and genotype were clustered using Morpheus.

wild type, the ethylene-activated signaling pathway was found to be enriched in *OxP2K1*. Moreover, several ERFs were induced in the wild type, as was AMINOCYCLOPROPANE CARBOXYLIC ACID SYNTHASE6, which is required for ethylene biosynthesis induced by MAPK6 activation (Liu and Zhang, 2004). Our results suggest one way that extracellular ATP confers resistance against different types of pathogens is by direct enhancement of the downstream signaling pathways activated by these classic

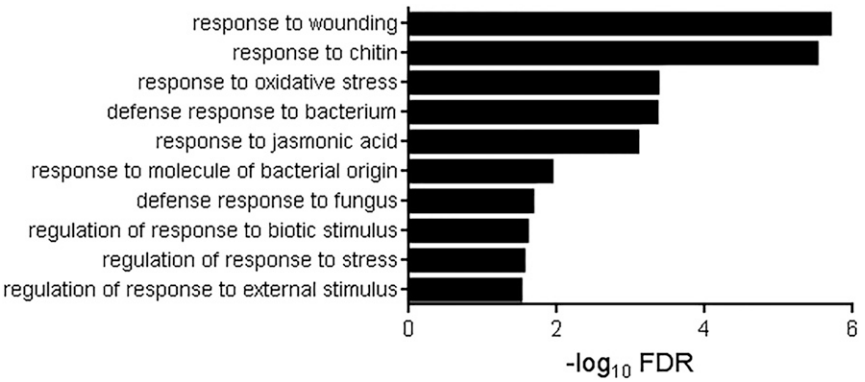

Figure 6. GO term enrichment for ATP-responsive gene expression dependent on COI1. Biological processes significantly enriched in 154 genes solely dependent on COI1 for ATP responsiveness are shown. Note that no functional categories were detected in 21 NPR1-dependent and six EIN2-dependent genes (see text for details).

defense hormones, with later pathogen challenge providing specificity.

Other evidence supporting the idea that extracellular ATP enhances defensive adaptation nonspecifically is found in the induction of many genes involved in the detection or response to pathogens. These include the induction of 29 genes encoding proteins containing a Toll/IL-1 receptor homology domain or a nucleotide-binding, Apaf1, Resistance, CED4 domain. Proteins containing these domains are associated with the recognition of microbe-derived molecules (i.e. effectors) or with microbe-modified host proteins (Jones et al., 2016). This result suggests that extracellular ATP induces the capacity for recognition of a broad range of danger-associated molecules, regardless of their source.

In addition, a number of ATP-responsive genes (233 of 543) were not disrupted in the *coi1*, *ein2*, and *npr1* mutant backgrounds; we term these genes putative ATP-specific genes. These genes are highly enriched in defense-related processes, including response to bacteria, response to chitin, and salicylic acid-mediated signaling, and include *NPR3*, *JAZ1*, several defense-related *ERF* and *WRKY* transcription factors, jasmonate biosynthetic genes, the two PEP receptors, *AtPEPR1* and *AtPEPR2*, and the PEP precursors *PROPEP3* and *PROPEP4* (Yamaguchi et al., 2010). The enrichment of defense-related genes among ATP-specific genes again highlights the importance of extracellular ATP signaling in fortification against pathogen attack, as has been discussed elsewhere (Tanaka et al., 2014). Our results highlight the role for extracellular ATP as a cue for the adoption of a general defensive posture.

Defense-Related Transcription Factors Play an Important Role in the ATP-Induced Transcriptome

The enrichment in ATP-responsive promoters also highlights the indiscriminate induction of defense platforms. For example, the G-box and W-box are both enriched in the ATP-responsive promoters. The W-box is a salicylic acid- and pathogen elicitor-responsive promoter element bound by WRKY and TGA transcription factors (Katagiri et al., 1989; Rushton et al., 1996; Yu et al., 2001), while the G-box, depending on the promoter context, can act as a jasmonate-inducible element bound by basic helix-loop-helix transcription factors (e.g. MYCs; Fernández-Calvo et al., 2011; Figueroa and Browse, 2012). Another enriched element in the ATP-responsive promoters was ABRE, which has long been known as an abiotic stress and abscisic acid-responsive element bound by bZIP transcription factors such as ABI5 (Guiltinan et al., 1990; Skriver et al., 1991; Uno et al., 2000). Abscisic acid signaling is more recently becoming recognized as important for pathogen defense (Anderson et al., 2004; Adie et al., 2007). The

Table 2. *Enrichment of defense-related cis-elements in COI1-, NPR1-, and EIN2-dependent ATP-responsive promoters and ATP-specific responsive promoters*

The presence or absence of the indicated promoter elements in the presumptive promoters (−1,000 bp upstream of transcriptional start site) was scored in 154 COI1-dependent, 21 NPR1-dependent, and six EIN2-dependent ATP-responsive genes as well as in 233 ATP-specific genes (Fig. 3) and 33,693 genes of the Arabidopsis nuclear genome, and significant enrichment was tested by hypergeometric test. Italicized values indicate that the element was not significantly enriched ($P > 0.05$). Fold enrichment is calculated as (percentage presence in ATP-responsive genes)/(presence in all Arabidopsis genes).

Element	Sequence	*P* (Fold Enrichment)			
		COI1 Dependent	NPR1 Dependent	EIN2 Dependent	ATP Specific
CAM-box	VCGCGB	1.59e-4 (1.62)	*0.171 (1.26)*	*0.286 (0)*	3.60e-26 (2.62)
W-box	TTGACY	1.20e-5 (1.22)	*0.137 (1.13)*	*0.273 (1.24)*	1.60e-3 (1.12)
G-box	CACGTG	*0.090 (1.04)*	0.040 (2.07)	*0.394 (1.21)*	1.80-e3 (1.46)
ABRE	ACGTGKC	0.049 (0.748)	0.033 (2.20)	*0.433 (0)*	4.20e-3 (1.42)
HSE	GAANNTTC	8.93e-4 (1.34)	*0.169 (0.854)*	*0.262 (0.498)*	0.021 (1.13)
GCC-box	GCCGCC	*0.105 (0.848)*	*0.280 (1.13)*	*0.590 (0)*	*0.091 (0.94)*

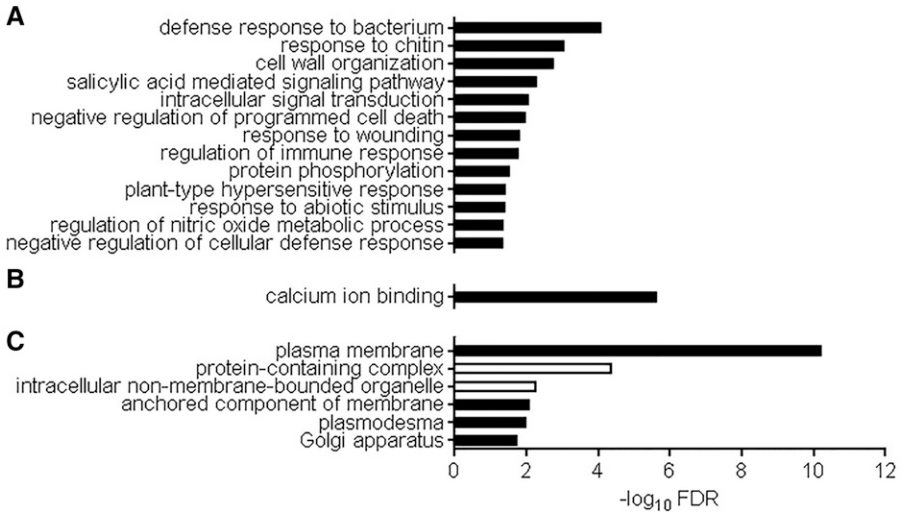

Figure 7. GO term enrichment for ATP-specific genes that require neither COI1, NPR1, nor EIN2 for expression. Biological processes (A), molecular function (B), and cellular components (C) for proteins significantly enriched in ATP-specific genes are shown (FDR < 0.05). Black and white bars indicate enrichment and depletion, respectively.

most enriched promoter element in ATP-responsive promoters is the CAM-box (Whalley et al., 2011), bound by CAMTAs that have been implicated in defense against pathogens and insects as well as response to abiotic stress (Bouché et al., 2002; Yang and Poovaiah, 2002; Du et al., 2009; Qiu et al., 2012; Benn et al., 2014). The analysis of promoter element enrichment suggests the ATP-responsive activation of multiple defense-responsive pathways.

Having delineated the importance of COI1 for ATP-responsive gene expression, we examined a subset of ATP-responsive genes in *myc2* and the *myc2 myc3 myc4* triple mutant. Among five COI1-dependent genes tested by RT-qPCR, four depended on MYC transcription

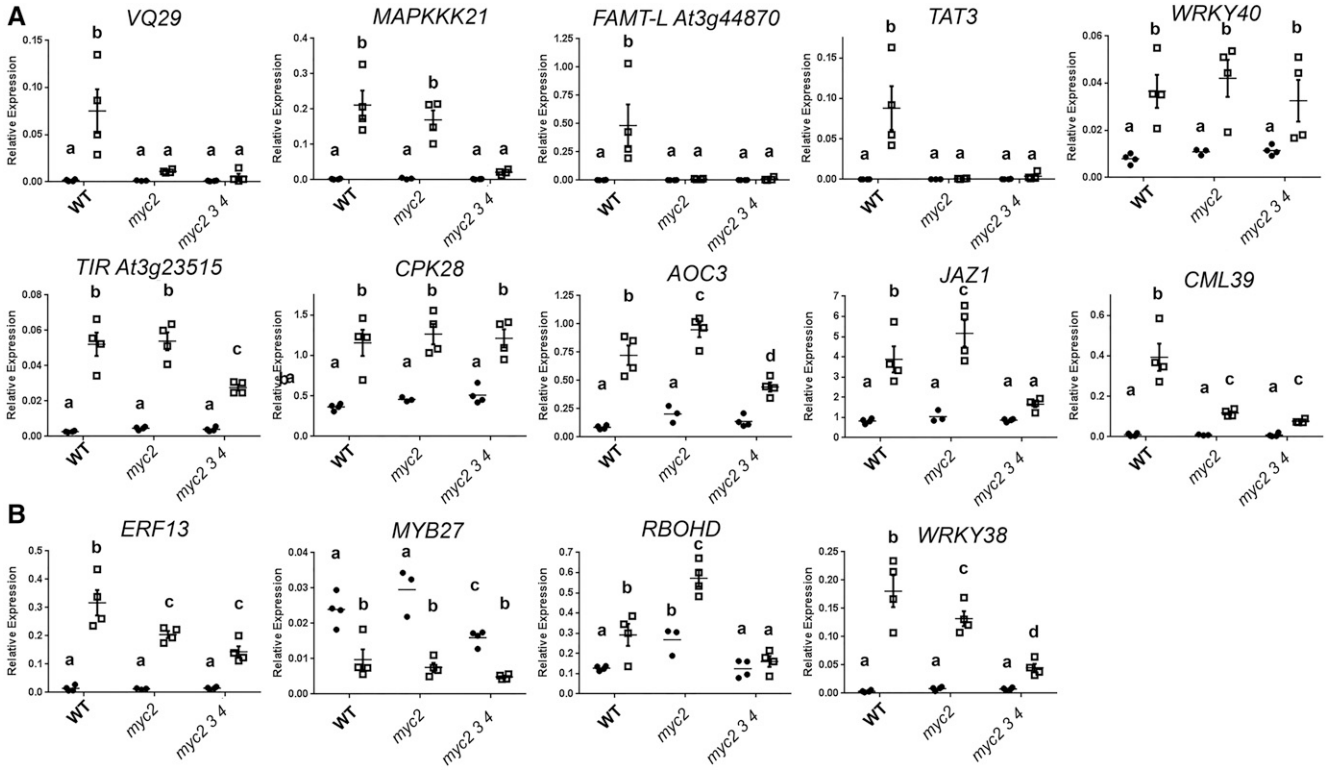

Figure 8. Involvement of MYC transcription factors in ATP-responsive transcription. Wild-type (WT) seedlings, the *myc2-1* single mutant, and the *myc2-1 myc3-1 myc4-1* triple mutant were treated with 500 μM ATP or vehicle solution for 30 min. Expression of COI1-dependent genes (A) and ATP-specific genes (B) in the indicated genotypes is shown. The expression of each gene was normalized by comparison with the expression of *PP2A*. Data indicate means, individual replicate values and SE (n = 3–4). In a subpanel, columns with different letters are significantly different ($P < 0.05$, Fisher's LSD).

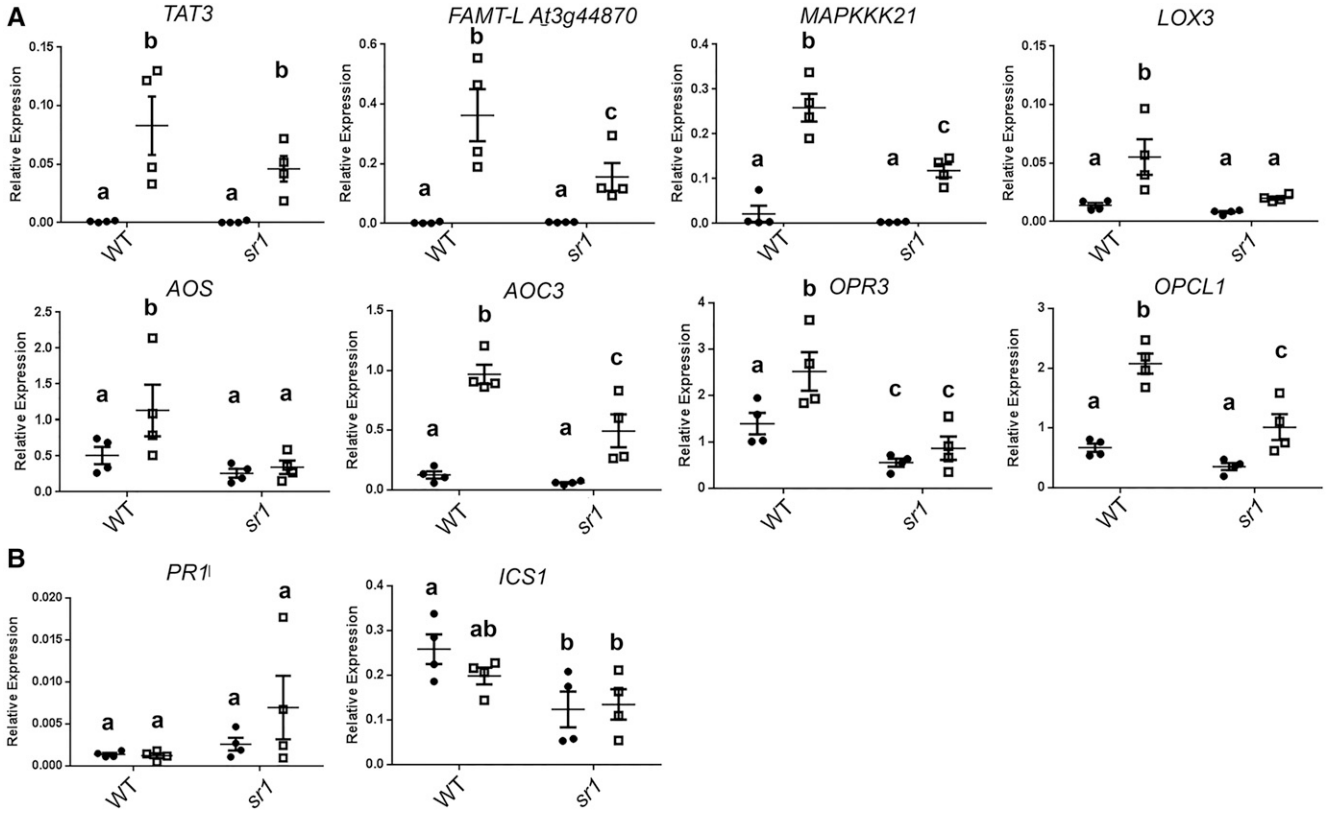

Figure 9. Involvement of CAMTA3 in ATP-responsive transcription. Wild-type (WT) seedlings and the *atsr1-1* (*camta3*) mutants were treated with 500 μM ATP or vehicle solution for 30 min. Expression of ATP-responsive genes (A) and autoimmunity marker genes, *PR1* and *ICS1* (B), in the wild type and *atsr1-1* is shown. The expression of each gene was normalized by comparison with the expression of *PP2A*. Data indicate means, individual replicate values and SE (n = 3–4). In a subpanel, columns with different letters are significantly different ($P < 0.05$, Fisher's LSD).

factors. We also examined ATP-specific genes (i.e. expression was independent of COI1, EIN2, and NPR1 functions) and found that most of the tested genes showed a clearly reduced induction by ATP in the *myc2* or *myc2 myc3 myc4* background. As COI1 controls the activity of these MYC transcription factors by regulating the abundance of the JAZ corepressors (Chini et al., 2007), it is somewhat unexpected that COI1-independent genes (although those are induced by ATP) were dependent on the MYCs for activation, although we can speculate that MYC factors control the activity of transcriptional repressors or that known protein-protein interactions of MYC could explain this phenomenon (Jaspers et al., 2009; Hong et al., 2012; Shin et al., 2012). Indeed, several of the MYC-dependent genes lack the MYC-regulated G-box in their presumptive promoters, so ATP-responsive MYC regulation of these genes is likely indirect.

Since extracellular ATP is known to induce cytosolic calcium elevation in a P2K1-dependent manner, it is exciting to find significant enrichment for the CAM-box-containing sequences. The CAM-box is bound by calcium-regulated CAMTA transcription factors (da Costa e Silva, 1994; Yang and Poovaiah, 2002; Choi et al., 2005; Doherty et al., 2009; Nie et al., 2012; O'Malley et al., 2016), and this element is transcriptionally activated by CAMTA3 in a tobacco (*Nicotiana tabacum*) transient expression system (Benn et al., 2014). Given that extracellular ATP treatment induces an increase in cytosolic calcium content (Tanaka et al., 2010b), it is attractive to hypothesize that the cytosolic calcium increases upon ATP perception promote CAMTA-mediated transcriptional activation of ATP-responsive genes. Our RT-qPCR analysis in a CAMTA3 loss-of-function mutant, *atsr1*, demonstrated that most tested genes showed reduced ATP-induced response compared with the wild type. Importantly, the expression of two autoimmune markers, *PR1* and *ICS1*, was not induced in the mutant, suggesting that the autoimmune phenotype of the CAMTA3 mutant is not manifested in the plants under our growth conditions and is unlikely to disturb the ATP response, although the possibility cannot be discounted entirely. Other studies found high expression of the autoimmune marker genes in soil-grown *atsr1* mutant plants aged from 3 to 6 weeks (Du et al., 2009; Kim et al., 2017; Lolle et al., 2017), while our axenically grown seedlings were 8 d old, which could explain the difference in immune marker expression. Our study suggests an important role for extracellular ATP in modulating the

calcium-dependent cellular response, where the CAMTA-mediated ATP signaling can be responsible for regulating the ATP-specific genes, which contribute to GO for calcium- and calmodulin-binding molecular functions such as calmodulin and calmodulin-like proteins, calcium-dependent protein kinases, EF-hand domain-containing proteins, and putative calcium channels.

CONCLUSION

In conclusion, we present a model (Fig. 10) in which ATP binding to P2K1 results in defense gene activation, where more than half of ATP-responsive genes required signaling by one or more of the pathways through EIN2, NPR1, and COI1, but with a more substantial reliance on COI1. MYC transcription factors participate in some level of COI1-independent activation of ATP response. Cytosolic calcium transients induce some proportion of COI1/EIN2/NPR1-independent genes, likely with the participation of CAMTA transcription factors.

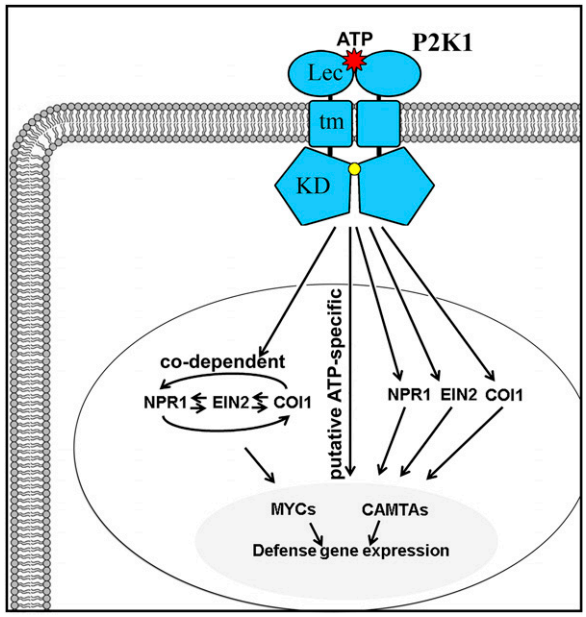

Figure 10. Proposed model of extracellular ATP signaling pathway to the defense-related transcriptome. Once extracellular ATP binds to the P2K1 receptor on the plasma membrane, which induces the autophosphorylation of the receptor and transphosphorylation of downstream targets (Chen et al., 2017), the activated P2K1 receptor results in transcriptional reprogramming of 542 genes. More than half of the ATP-responsive genes required signaling by one or more of the pathways through EIN2, NPR1, and COI1, for example, 154 genes in the COI1-dependent pathway, 21 genes in the NPR1-dependent pathway, six genes in the EIN2-dependent pathway, and 129 genes in the co-dependent signaling pathway. The other half of the ATP-responsive genes (233 genes) are independent of COI1/NPR1/EIN2-mediated pathways, the putative ATP-specific pathway. Many of the ATP-responsive genes are regulated under the participation of MYC and CAMTA transcription factors. KD, Kinase domain; Lec, lectin domain; tm, transmembrane domain.

MATERIALS AND METHODS

Plant Materials and Treatments

Arabidopsis (*Arabidopsis thaliana*) *OxP2K1*, *dorn1-3*, *ein2-1*, *npr1-3*, and *coi1-30* were described previously (Cao et al., 1997; Alonso et al., 1999; Yang et al., 2012; Choi et al., 2014). The wild type (Columbia-0; CS70000), *coi1-30* (Salk_035548), *ein2-1* (CS3071), and *npr1-3* (CS3802) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. Seeds of *myc2-1* (Niu et al., 2011) and *myc2-1 myc3-1 myc4-1* (Major et al., 2017) were provided by John Browse. Seeds of *atsr1-1* were from Joe Poovaiah (Du et al., 2009). The genotype of mutant parental lines was verified as follows. For *coi1-30*, genomic DNA was used as a template in a three-primer PCR using primers shown in Supplemental Table S5, and hemizygous plants were selected. Genomic DNA of parental lines of *ein2-1* and *npr1-3* was amplified using primers shown in Supplemental Table S5, and the products were sequenced to verify that they carried the causative mutation. The following lines are T-DNA insertional mutants that lack expression of full-length transcripts of the respective mutant genes: *coi1-30*, *dorn1-3*, *myc2-1*, *myc2-1 myc3-1 myc4-1*, and *atsr1-1* (Du et al., 2009; Niu et al., 2011; Yang et al., 2012; Choi et al., 2014; Major et al., 2017). The *EIN2* gene in the *ein2-1* mutant contains an ethyl methanesulfonate-induced mutation that introduces a premature stop codon prior to the 3′ region encoding 839 amino acids of the C-terminal domain essential for ethylene-induced signal transduction (Alonso et al., 1999). Likewise, the *npr1-3* allele contains a premature stop codon in the NPR1 coding sequence that should result in a truncated protein lacking the C-terminal 194 amino acids (Cao et al., 1997).

Seeds were surface sterilized, sown on rectangular plates of one-half-strength Murashige and Skoog medium, 1% (w/v) Suc, 0.05% (w/v) MES, pH 5.7, and 1% (w/v) agar, and stratified in darkness at 4°C for 3 d. Homozygous mutants of *coi1-30* were grown with inclusion of 20 μM methyl jasmonate (Bedoukian Research) in the medium and selected based on their obvious long-root phenotype. After seed sterilization and sowing, plates were transferred to a 22°C growth chamber (Conviron) with 12 h of light of 100 to 120 μmol photons m^{-2} s^{-1} and grown vertically for 7 d. On day 7, 20 seedlings of each genotype were transferred to six-well plates, each well containing 2 mL of one-half-strength Murashige and Skoog medium as above but lacking agar (liquid medium). All media were sterilized by autoclaving. On day 8, 2 mL of 1 mM ATP in liquid medium (ATP treatment) or 2 mL of liquid medium (mock treatment) was added to each well, and the plates were returned to the growth chamber. After 30 min, the seedlings were removed from the wells, gently blotted dry, and snap frozen in liquid nitrogen. Each genotype and treatment was a true biological replicate sampled on different days.

Preparation of ATP Solution

ATP was purchased from Sigma-Aldrich. Stock solution of ATP was prepared in 2 mM MES buffer at 0.1 M, buffered to pH 5.7, and filter sterilized. The stock solution was stored at −20°C until use.

RNA-Seq Library Construction, Sequencing, and Analysis

After tissue disruption using a bead beater, total RNA was isolated using a quick-RNA miniprep kit with on-column DNase treatment per the manufacturer's instructions (Zymo Research). Using the TruSeq RNA Sample Preparation Kit v2 (Illumina; catalog no. RS-122-2002), the poly(A) RNA was isolated from 2 μg of the total RNA from each treatment sample using magnetic oligo (dT) beads. Following purification, the mRNA was fragmented by zinc treatment at 94°C for 5 min and reverse transcribed to synthesize first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. Second-strand cDNA synthesis was performed, and the products were then subjected to end repair and phosphorylation, and an A base was added to the 3′ end of the blunt phosphorylated DNA fragments. Illumina multiple indexing adapters were ligated to the fragments, as described by Illumina's TruSeq RNA Sample Preparation V2 Guide. The cDNA fragments flanked by Illumina PE adapters were selected and purified by AMPure XP beads for downstream enrichment. The cDNA fragments were amplified by PCR Primers PE 1.0 and PE 2.0 (Illumina) that anneal to the ends of the adapters, using the PCR program of 30 s at 98°C followed by 15 cycles of 10 s at 98°C, 30 s at 60°C, 30 s at 72°C, and

a final elongation step of 5 min at 72°C. The products were purified using AMPure XP beads to create an Illumina paired-end library. Library quality control was performed with a Bioanalyzer DNA 1000 Chip Series II (Agilent). A qPCR method was employed for quantifying libraries in advance of generating clusters. The libraries were diluted to a final concentration of 10 nM. The paired-end libraries were applied for cluster generation at a concentration of 10 pM on a flowcell in a cBOT (Illumina). Sequencing was performed on an Illumina HiSeq 2500 platform by Macrogen with one lane of 12 pooled libraries to generate 100-bp single-end reads. The base-calling and quality-value calculations were performed by the Illumina data-processing pipelines CASAVA v1.8.2 and v1.7.0, respectively. Various quality controls, including removal of reads containing primer/adaptor sequences, trimming of read length, and filtering of high-quality reads based on the score value, were performed using Illumina CASAVA v1.7.0. After trimming and filtering, reads were aligned to the Araport11 annotation of the Arabidopsis genome (Cheng et al., 2017) using TopHat version 2.1.1 (Kim et al., 2013) on the Kamiak High Performance Computing Cluster at Washington State University. Transcript reads were counted on Kamiak using HTSeq-0.7.2 (Anders et al., 2015). Significant differences in gene expression between mock and ATP-treated samples were calculated using edgeR release 3.7 (Robinson et al., 2010) with a cutoff for FDR < 0.05 and absolute FC > 1.5. Enrichment of GO terms in a given set of transcripts was determined by accessing the PANTHER classification system through TAIR (https://www.arabidopsis.org/; Lamesch et al., 2012). The PANTHER overrepresentation test version 13.1 was used (Fisher's exact test with FDR multiple test correction) by comparison with GO database release 2018-08-09 (Thomas et al., 2003). All gene expression changes after ATP treatment are shown in Supplemental Data Set S1.

RT-qPCR

For RT-qPCR, seedlings were grown and treated, and RNA was prepared as above for the RNA-seq experiments. After isolation of RNA, 1 μg of RNA was used in a 20-μL reverse transcription reaction according to the manufacturer's instructions (iScript; Bio-Rad). After reverse transcription, the products were diluted 5-fold with water, and 2 μL was used in a 20-μL reaction with a SYBR Green dye/polymerase mix (SsoAdvanced; Bio-Rad) in a CFX96 thermocycler (Bio-Rad). Genes of interest were normalized to the *PP2A* (AT1G13320) reference gene as described (Czechowski et al., 2005; Rieu and Powers, 2009). Primers used are listed in Supplemental Table S5.

Promoter Analysis

For de novo identification of enriched promoter elements in a given list of genes, promoter sequences 1,000 bases upstream of the transcriptional start site as annotated in Araport11 were accessed via Araport (www.araport.org) and submitted to the oligoanalysis tool (van Helden et al., 1998) using the default settings as implemented in RSAT (Defrance et al., 2008; http://rsat.eead.csic.es/plants). To determine significant enrichment of a particular element in promoters of a list of genes, presence or absence was scored in promoters of the genes of interest and in the Araport11 Arabidopsis nuclear genome using the RSAT dna-pattern tool. Significance was determined using the hypergeometric distribution function in Microsoft Excel 2016.

Accession Numbers

Quality-filtered mRNA sequencing data are available at National Center for Biotechnology Information BioProject PRJNA494862 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA494862).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. High between-replicate concordance of ATP-responsive gene expression in this study: hierarchical clustering.

Supplemental Figure S2. High between-replicate concordance of ATP-responsive gene expression in this study: principal component analysis.

Supplemental Figure S3. Reliability of RNA-seq-based estimates of gene expression in this data set.

Supplemental Figure S4. MapMan enrichment analysis of cellular function overview and biotic stress.

Supplemental Figure S5. GO term enrichment for ATP-responsive genes common to *OxP2K1* and the wild type.

Supplemental Figure S6. GO term enrichment for ATP-responsive gene expression in *OxP2K1*.

Supplemental Table S1. Promoter element analysis for ATP-responsive gene expression in wild-type plants.

Supplemental Table S2. Enrichment of defense-related cis-elements in promoters of ATP-responsive genes in *OxP2K1* seedlings.

Supplemental Table S3. Number of ATP-responsive genes in each genotype.

Supplemental Table S4. Promoter element analysis for ATP-specific gene expression.

Supplemental Table S5. Primer sequences used in this study.

Supplemental Dataset S1. Table of gene expression changes after 30 min 500 μM ATP treatment.

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