

## Probing the Unknowns in Cytokinin-Mediated Immune Defense in *Arabidopsis* with Systems Biology Approaches

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**ABSTRACT:** Plant hormones involving salicylic acid (SA), jasmonic acid (JA), ethylene (Et), and auxin, gibberellins, and abscisic acid (ABA) are known to regulate host immune responses. However, plant hormone cytokinin has the potential to modulate defense signaling including SA and JA. It promotes plant pathogen and herbivore resistance; underlying mechanisms are still unknown. Using systems biology approaches, we unravel hub points of immune interaction mediated by cytokinin signaling in *Arabidopsis*. High-confidence *Arabidopsis* protein–protein interactions (PPI) are coupled to changes in cytokinin-mediated gene expression. Nodes of the cellular interactome that are enriched in immune functions also reconstitute sub-networks. Topological analyses and their specific immunological relevance lead to the identification of functional hubs in cellular interactome. We discuss our identified immune hubs in light of an emerging model of cytokinin-mediated immune defense against pathogen infection in plants.

**KEYWORDS:** systems biology, plant hormones, interaction networks, gene expression, cytokinin

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### Introduction

The small molecule hormone cytokinin occurs in different  $N^6$  derivatives of adenines. It is indispensable for almost every aspect of plant growth and development. In *Arabidopsis*, the signal of cytokinin is conveyed by two-component signaling systems (TCS; all abbreviations in Table 1).<sup>1</sup> The first component of this system is composed of membrane-spanning *Arabidopsis* histidine kinases (AHKs), which sense the binding of cytokinin to its CHASE domain. AHKs are not only limited to plasma membrane but also located on endoplasmic reticulum (ER).<sup>2</sup> The second component of TCS is provided by nuclear response regulators (*Arabidopsis* response regulators type-B (type-B ARR)), which are linked to AHKs by *Arabidopsis* histidine phosphotransfer proteins (AHPs). The type-B ARRs are positive regulators of cytokinin signaling and are transcriptional activators of type-A ARRs. The latter

inhibit cytokinin signaling.<sup>1,2</sup> Cytokinin plays an important role in controlling various functions such as cell proliferation and differentiation, nodulation, nutrient acquisition, circadian rhythm, abiotic stress responses, light responses, senescence, and immunity as signaling outputs.<sup>1–3</sup> The interplay between cytokinin and auxin as well as ethylene (Et) has already been established.<sup>4,5</sup> However, efforts elucidating the crosstalk between cytokinin and immune pathways in plants are still lagging behind. Generally, plants defend themselves against biotrophic infections through salicylic acid (SA)-mediated resistance and deter necrotrophic pathogens through the jasmonic acid (JA) pathway.<sup>6</sup> Enhanced plant cytokinin responses have been shown to enhance plant immunity against diverse pathogens such as biotrophs,<sup>7</sup> hemi-biotrophs,<sup>8,9</sup> as well as necrotrophs.<sup>9,10</sup> We, therefore, hypothesize that cytokinin cellular circuitry has broader crosstalk possibilities to immune



defense pathways in plants. By adopting systems biology approaches, we spotlight immune regulatory hub proteins in *Arabidopsis* and discuss their importance in the interplay between cytokinins and defense pathways.

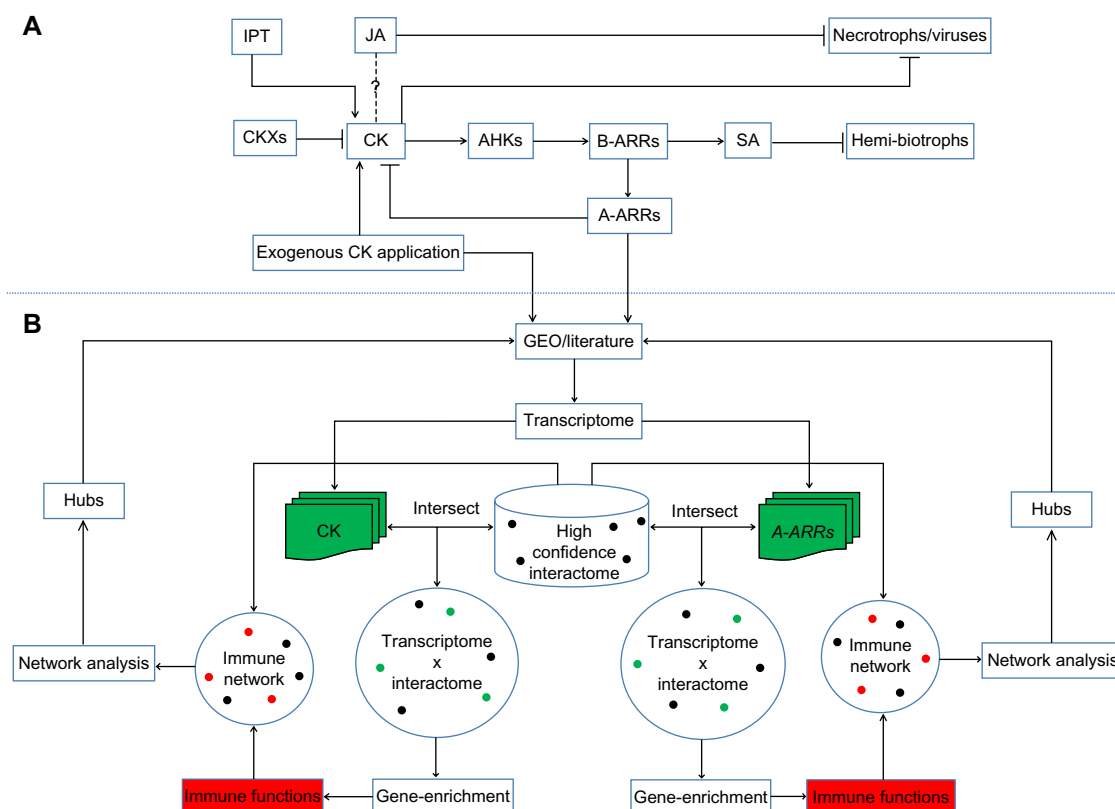
Although profoundly analyzed with respect to fungal biotrophy<sup>11</sup> and gall-causing pathogens,<sup>12</sup> the potential connection of cytokinins to plant immune pathways such as the SA–JA/Et backbone of immunity<sup>6</sup> was still not known until recently. Transgenic *Arabidopsis* plants overexpressing the gene encoding cytokinin pathway enzyme isopentenyltransferase (IPT) have been shown to increase the expression of SA defense marker gene pathogenesis-related protein 1 (PR1) and enhance protection against infection with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000).<sup>9</sup> To achieve this protection, type-B cytokinin response regulator ARR2 interacts with transcription factor 3 (TGA3) both in an SA-dependent as well as in nonexpresser of PR genes 1 (NPR1)-dependent manner and thus regulates defense gene expression. Exploring the impact of cytokinin transcriptional networks on plant immunity, Argueso et al.<sup>7</sup> found that negative regulators of cytokinin signaling (type-A ARR) negatively regulate plant immunity. *Arabidopsis* type-A ARR quadruplet and sextuplet mutants in comparison to wild-type plants are shown to have a high basal expression of SA marker genes and increased immunity against infection with *Hyaloperonospora arabidopsidis* isolate Noco2 (*Hpa* Noco2).<sup>7</sup> Two-weeks-old wild-type or type-A ARR mutant (*arr3,4,5,6,8,9*) plants were used in these experiments. The corresponding transcriptome analysis data are acquired from the study of Argueso et al.<sup>7</sup> *Hpa* is an absolute obligate biotrophic (Fig. 1A) pathogen whose population coevolves with its host, *Arabidopsis thaliana*.<sup>13</sup> More recently, multiple aspects of cytokinin-mediated immune defense such as a positive effect on PTI (PAMP-triggered immunity), synergism between pathways of cytokinins and SA, and an antagonism between cytokinins and auxin have been highlighted.<sup>8,14</sup> Unlike the impact of cytokinins on the SA pathway of resistance, the interaction between cytokinin and the JA sector of immunity has not been congruently analyzed. However, increased cytokinin-mediated protection against infection with necrotrophic pathogens<sup>9,10</sup> and certain viruses,<sup>15</sup> and attack by herbivores<sup>16</sup> point to a potential cross-talk between cytokinins and the JA pathway of resistance.

Network biology tools are valuable and highly instrumental in exploring previously hidden biological inferences particularly by resolving complex hormonal interplays in plants. Quantitative transcriptome repository and interactome (protein–protein interactions (PPI)) databases can be exploited for the investigation of the hormonal interplay occurring in plant immunity. To achieve this, we (i) mapped high-confidence PPI from STRING database.<sup>17</sup> (ii) An efficient overlay of gene expression data on the cellular interactome not only couples dynamics of change to the network but also can lead to the identification of regulatory switches in the system. (iii) To evolve this further, we adopted and reanalyzed

**Table 1.** Key terms in plant immune defense.

NAME	ABBREVIATION
<i>Arabidopsis</i> histidine phosphotransfer proteins	AHPs
<i>Arabidopsis</i> histidine kinases	AHKs
<i>Arabidopsis</i> response regulator 2	ARR2
<i>Arabidopsis</i> response regulators type-A	type-A ARRs
<i>Arabidopsis</i> response regulators type-B	type-B ARRs
<i>Arabidopsis thaliana</i> basic pathogenesis-related protein 1	ATPRB1
<i>Arabidopsis thaliana</i> mitogen-activated protein kinase	AtMPK
Biological Networks Gene Ontology tool	BiNGO
Coronatine insensitive 1	COI1
Coronatine induced 3	COR13
COI1 suppresser 1	COS1
Delta sub-unit of ATP synthase	cpATPase
Cytochrome P450, family 81, subfamily F, polypeptide 2	CYP81F2
Cytokinin oxidases	CKXs
Differentially expressed genes	DEGs
Effectors-triggered immunity	ETI
Ethylene	Et
Gene Expression Omnibus	GEO
Gibberellin 3-oxidase 1	GA4
<i>Hyaloperonospora arabidopsidis</i>	<i>Hpa</i>
<i>Hyaloperonospora arabidopsidis</i> isolate Noco2	<i>Hpa</i> Noco2
Isopentenyltransferase	IPT
Jasmonic acid	JA
Lipoxygenase 3	LOX3
Nonexpresser of PR genes 1	NPR1
Pathogenesis-related protein 1	PR1
PAMPs triggered immunity	PTI
Penetration 2	PEN2
Phytoalexin deficient 3	PAD3
Phytochrome B	phyB
Phytohormone interacting Factor 3	PIF3
Protein–protein interactions	PPI
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	<i>Pst</i> DC3000
Salicylic acid	SA
Superroot 1	SUR1
Transcription factor 3	TGA3
Tryptophan synthase alpha chain 1	TSA1
Two-component signaling systems	TCS
WRKY DNA-binding protein	WRKY

previously deposited gene expression data to GEO (Gene Expression Omnibus) on the application of cytokinin to *Arabidopsis* as well as gene expression changes due to the deletion of type-A ARRs. (iv) The integration of data on transcriptome



**Figure 1.** A model of cytokinin responses in plant immunity and systems biology tools for the identification of cytokinin-linked immune regulators. **(A)** Model and hypothesis. Cytokinin promotes plant immunity against infection with some of the biotrophic (*Hpa Noco2*), necrotrophic (*Alternaria*), as well as hemi-biotrophic pathogens (boxes, arrows). The presence of cytokinin is perceived by membrane-spanning receptors AHKs (plasma membrane or ER membrane may house AHKs) and transmit the phosphorylated signal to type-B ARR via AHPs. Type-B ARR, ARR2 interacts with TGA transcription factors and promotes SA signaling for resistance against infection with *Pst* DC3000 (hemi-biotroph). Type-A ARRs, which are negative regulators of cytokinin signaling, are transcriptionally activated by type-B ARRs. Deletion of type-A ARRs enhances cytokinin signaling and derepresses SA responses in promoting *Arabidopsis* immunity against infection with *Hpa Noco2* (biotroph). Enhanced cytokinin levels and signaling also promotes *Arabidopsis* resistance to infection with *Alternaria* (necrotroph). The potential link between cytokinin and JA pathway is still unknown. The signal of cytokinin is fortified through enhanced endogenous production by overexpressing IPT enzyme gene or by external application of cytokinin. Also, deletion of type-A ARRs relieves its inhibition to cytokinin signaling. The impact of exogenous application of cytokinin and deletion of type-A ARRs on plant immune defense is further analyzed by adopting systems biology approaches. **(B)** Methodology to address hypothesis. Transcriptome data on the exogenous application of *trans*-Zeatin and type-A ARRs sextuplet mutants are collected from GEO and studies of Argueso et al.,<sup>7</sup> respectively (green files). High-confidence (edge score > 0.9) PPI from the STRING database are mapped and visualized with Cytoscape. DEGs from transcriptomes and their corresponding proteins in interactome are identified as intersecting coverage between the two levels of omics data (transcriptome × interactome). Intersected nodes (green dots) are subjected to functional enrichment analysis by using Cytoscape plugin BiNGO. Proteins of the network relevant to plant immune defense processes (red dots) are considered as immune nodes of the network. Immune networks are composed of immune-enriched nodes (red dots) as well as their interactors (black dots) from the basal interactome. Taking the degree of connectivity and neighborhood (network analysis) as well as biological relevance to immune defense as parameters, we then looked for regulatory hubs mediated by cytokinin signaling in *Arabidopsis*.

and interactome in the light of biological functions and network analysis leads us to link cytokinin signaling and immune regulatory hubs in *Arabidopsis*. These analyses enabled us to compare different sectors of plant immunity either by direct cytokinin application or by deleting type-A response regulators.

## Material and Methods

According to our hypothesis of a “missing link between cytokinin signaling and plant immune regulators,” we integrated two large-scale omics data sets: interactome (cellular PPI) and transcriptome (genome-wide changes in gene expression) as

shown in Figure 1B. We collected the *Arabidopsis* interactome from STRING database.<sup>17</sup> A stringent criterion for the selection of interactions as combined edge score greater than 0.9 (*P*-values for Bayesian confidence score) resulted in a basal network, composed of 3056 nodes and 18233 interactions. For visualization of nodes and interactions, we used Cytoscape version 2.8.3. We acquired microarray data from GEO (GSE 6832.I). In this experiment, *trans*-Zeatin was exogenously applied for 1 h to 2-week-old wild-type *Arabidopsis* plants in comparison to mock treatment. GEO-2R-based analysis resulted in 5489 differentially expressed genes (DEGs). Furthermore, we collected significantly regulated *Arabidopsis*



genes of wild-type (Col-0) vs. *arr3,4,5,6,8,9* (sextuplet mutant) plants treated with *Hpa* Noco2. Next, we searched for intersecting genes differentially regulated in transcriptome data sets and their corresponding protein nodes present in our high-confidence basal interactome. Nodes representing these genes are differentially colored in the visualized interactome (see Figures 1B, 2A and 3A; also see Supplementary Material Tables 1 and 2). We then used the Cytoscape plugin Biological Networks Gene Ontology (BiNGO) tool to find out biological functions in which the transcriptome–interactome-intersected genes are involved. These analyses identified diverse functional processes. Out of them, we only selected immune relevant functions and their corresponding participatory nodes (see Figure 1B, see also Supplementary Material Tables 1 and 2). We then reconstructed immune networks based on immune-enriched nodes and which interactor (neighbors) nodes exist in the basal interactome (see Figures 1B, 2B, and 3B). Next, we performed topological analysis of immune networks using the Cytoscape plugin NetworkAnalyzer and plotted the number of degree and average number of neighbors (see Figures 1B, 2C, and 3C). Based on criteria such as network connectivity and topology, expression, and immunological relevance, we identified functional hubs (see Figures 1B, 2D, and 3D). Databases such as TAIR, ARAMEMNON, and AHD (see Supplementary Material Table 3) were used to mine functional implications of these identified regulatory hub nodes from the previously established experimental data.

## Results and Discussion

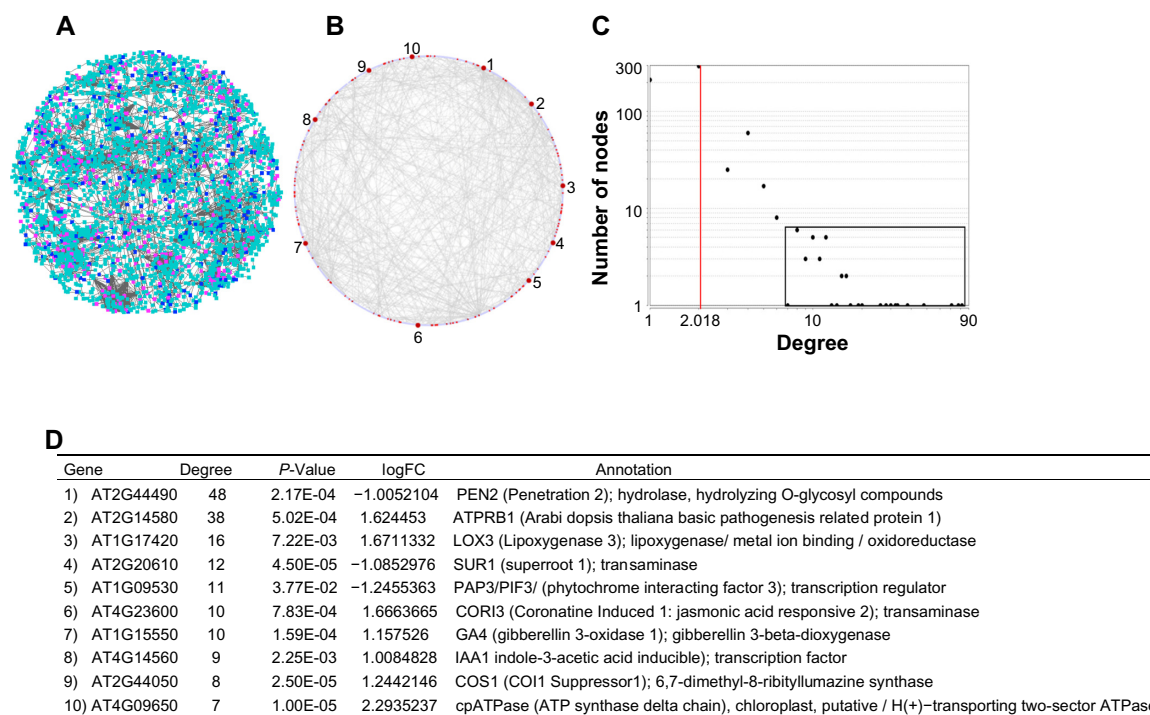
Cytokinin promotes defense against various plant pathogens and therefore entails a broader role in modulating immune dynamics of the host plant. To generalize understanding regarding the implications of cytokinins in plant immunity, we integrated aforementioned findings into a logical model (Fig. 1A). According to the model, enhanced cytokinin levels<sup>8</sup> or increased sensitivity<sup>9</sup> is perceived as a defense signal by the plant systems. The former can be achieved by exogenous application of various types of cytokinins such as *trans*-Zeatin, benzyladenine, and kinetin as well as various derivatives of phenylurea such as thidiazuron.<sup>9,18</sup> The overexpression of genes encoding various isoforms of the cytokinin pathway rate-limiting enzyme IPT can enhance the levels of plant cytokinins.<sup>9,16</sup> Contrarily, overexpression of genes encoding catabolic enzymes such as cytokinin oxidases (CKXs) reduces *Arabidopsis* defense against *Pst* DC3000.<sup>8</sup> Overexpression of cytokinin receptor histidine kinases such as *AHK2* in *Arabidopsis* enhances cytokinin sensitivity with concomitant increase in pathogen defense (Fig. 1A).<sup>7,19</sup> Moreover, type-B ARR are positive regulators of cytokinin responses. Overexpression of one such ARRs, the *ARR2* also enhances pathogen defense and their loss of function mutants failed to manifest optimal protection.<sup>9</sup> As shown in the model (Fig. 1A), derepression of cytokinin signaling can improve cytokinin responses including plant defense. Deletions of various type-A ARRs have

been shown to promote plant immunity against biotrophic pathogen *Hpa* Noco2.<sup>7</sup> Our model describes modulating inputs to the cytokinin cellular circuitry and immune responses as output of the system. It is anticipated that cytokinin-mediated immune modulation is the consequence of complex interactions between cytokinins and immune pathways operating in plants. Here, we identify and highlight the role of potential regulatory proteins in these interactions.

The adoption of systems biology approaches has opened up new avenues in plant immunity.<sup>20</sup> Systems biology has high potential in resolving the complex hormonal interactions in plant immunity.<sup>8</sup> To this end, public PPI databases such as STRING, AtPID, and Plant Interactome Database can be exploited for mapping the basal *Arabidopsis* interactome (Fig. 1B). Moreover, quantitative transcriptome repositories such as GEO, Genevestigator, and Array Express (see Figure 1B and supplementary material for methodology, links, and references to these databases) provide genome-wide changes in gene expression for multiple stimuli including phytohormones. An efficient overlay of gene expression data on the cellular interactome not only couples dynamics of change to the network but also can lead to the identification of hub regulators important for immune defense in plants (Fig. 1B). To establish a link between cytokinin signaling and plant immunity, we applied above-mentioned approaches and thereby probed into immune protein nodes in cellular PPI-networks. We compared two different case studies: (i) exogenous application of cytokinin and (ii) deletion of type-A ARRs. Each augments the signal of cytokinin and leads to changes in gene expression with altered immune outputs but the means how this is achieved differ strongly in both experiments. Does this affect different pathways? Where are the hub proteins for the response? These questions are also tackled by our methodology (Fig. 1).

Exogenous application of cytokinins has been shown to protect *Arabidopsis* against infection with several types of plant pathogens such as *Pst* DC3000,<sup>8</sup> *Hpa* Noco2,<sup>7</sup> as well as *Verticillium longisporum*.<sup>21</sup> To investigate the mechanism of this broader resistance and involvement of underlying immune regulators, we acquired and analyzed GEO (GSE6832.I)-based microarray data on the treatment of *Arabidopsis* with *trans*-Zeatin. Furthermore, we mapped high-confidence *Arabidopsis* PPI from STRING and visualized these with Cytoscape (Fig. 2). From 5489 cytokinin-modulated genes, 575 corresponding proteins are present in our high-confidence interactome (10.48% of DEGs, 18.82% of interactome). We differentially visualized genes encoding these 327 up- and 248 down-regulated proteins in the interactome in comparison to non-overlapping proteins of the same network (Fig. 2A). Our results from functional analysis show that for the high-confidence interacting proteins, their corresponding 575 genes are involved in 363 biological functions. Among these processes, only 35 (9.46%) are relevant to immune defense in *Arabidopsis* (supplementary material). Mining the connectivity of these





**Figure 2.** Systems biology analysis for the assessment of the impact of exogenous cytokinin application on plant immune defense in *Arabidopsis*. **(A)** Transcriptome  $\times$  interactome mapping and visualization. Proteins are indicated by little squares. The color for non-intersecting nodes between transcriptome and interactome is cyan, for intersecting up-regulated nodes between transcriptome and interactome is violet, and for intersecting down-regulated nodes between transcriptome and interactome is dark blue; interactions are drawn as gray lines. Transcriptome changes due to the exogenous application of cytokinin in comparison to mock application in *Arabidopsis* are collected from GEO (GSE6832.1). GEO2R-based normalization of selected samples resulted in 5489 DEGs ( $\log_{2}FC > \pm 1$ ). High-confidence interactome ( $>0.9$  as combined edge score) of *Arabidopsis* was acquired from STRING (3056 protein nodes, 18233 interactions) and visualized with Cytoscape, version 2.8.3 ([www.cytoscape.org](http://www.cytoscape.org)). 575 nodes are shared between interactome and transcriptome (327 up regulated: violet, 248 down regulated: dark blue). **(B)** Biological function analysis and reconstruction of the immune network. 10 functional hubs were identified and are shown as bold red dots with numbers from 1 to 10 on the rim of the immune network. Overall, there are 130 nodes with immune enrichment (red little circles on the rim) and 525 immune interactors (light blue circles). The latter are overlapping and covering the rim. 1039 interactions between all proteins are given in gray and cross the interior of the figure. The 575 shared nodes were analyzed with Cytoscape plugin tool BiNGO to detect over-represented biological functions in the interactome adapted to the cytokinin responses. We identified 363 biological functions for 501 genes; 74 genes had no BiNGO-based annotation (see supplementary material). We only focused on 35 immune and defense-related terms (see Supplementary Table 1). 130 protein nodes of the intersecting interactome participating in these 35 immune functions form a base for the reconstruction of the immune network. **(C)** Network analysis and identification of functional hubs. Topological analysis of immune network **(B)**: Network properties such as number of degree and average number of neighbors were next computed applying the Cytoscape plugin NetworkAnalyzer. The double logarithmic plot shows (black dots) for each degree (number of connection to other nodes,  $x$ -axis), the number of nodes found ( $y$ -axis). The average degree is shown as red vertical line. Well-connected proteins (“hubs”) with degree equal to 7 or higher are framed in bold. Based on network connectivity and topology, expression  $P$ -values, and immunological relevance, 10 functional hubs are identified and shown as bold red dots with numbers from 1 to 10 in the immune network **(B)**. They are tabulated with quantitative details in **(D)**.

immune-enriched nodes in the basal interactome (Fig. 2A) enabled us to extract a sub-network (an immune network). This immune network is composed of 130 immune-enriched nodes (22.61% of transcriptome–interactome mapping) and their interactors, which consists of 525 nodes and 1039 edges (Fig. 2B). We then conducted network analyses such as degree of connectivity and average number of neighbors for our immune network (Fig. 2C). According to these analyses, average connectivity in our immune network is 2.018 (Fig. 2C). Thus on average, each protein has above two neighbors. To identify functional hubs, we further computed the degree of connectivity and specified nodes as potential hubs having a degree more than the average number of neighbors (Fig. 2C,

enclosed in the rectangle). Besides connectivity and topological parameters, we also considered  $P$ -values (expression of genes encoding network nodes) and immune relevance as criteria for hubs in our immune network. We analyzed the top 10 such connected nodes (Fig. 2D) and discussed their implications in plant immunity with special reference to cytokinins.

The node of immune network ATPRB1 (*Arabidopsis thaliana* basic pathogenesis-related protein 1) is among the densely connected nodes. Being responsive to hormonal cues, the gene encoding ATPRB1 is also significantly up regulated by the treatment of cytokinin (Fig. 2D). Genes of pathogenesis-related protein 1 belong to thaumatin protein family; the well-known example of this family is PR1, which is a marker

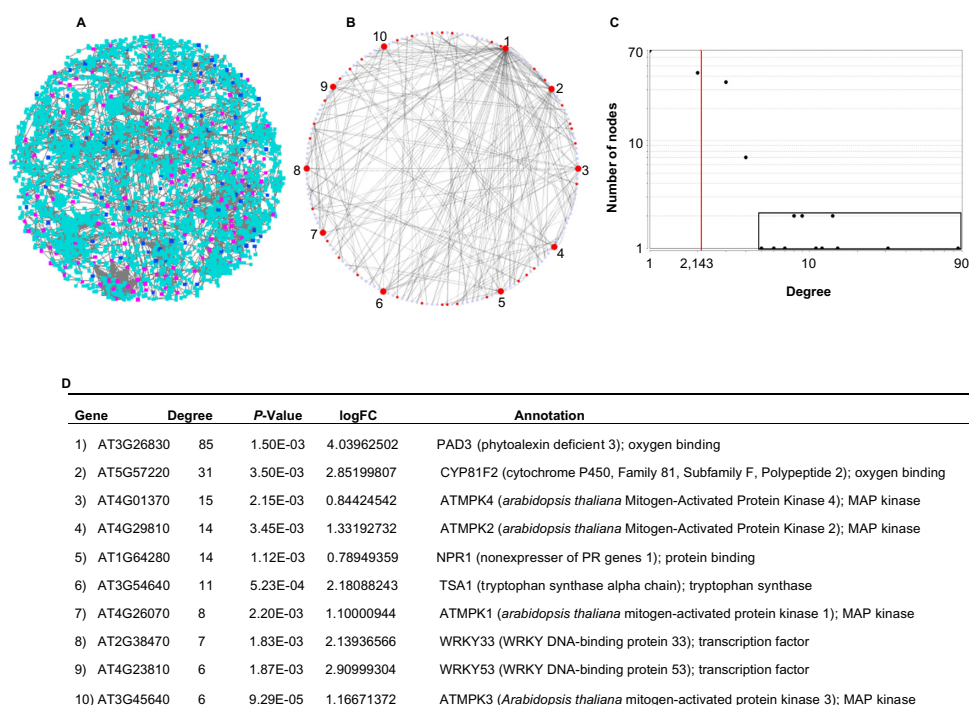


of the SA pathway of resistance that shows higher induction with exogenous cytokinin application.<sup>7,8</sup> The promoter of the *ATPRB1* gene has been shown to have organ-specific expression patterns and is specifically responsive to the treatment of Et and Me-JA while treatment of SA represses its expression.<sup>22</sup> Similarly, LOX3 (lipoxygenase 3) is another highly connected node of the immune network with higher induction upon cytokinin treatment (Fig. 2D). Besides being an important oxylipin (precursors of jasmonates) biosynthetic pathway enzyme, LOX3 is responsive to a multiplicity of internal and external cues.<sup>23</sup> The enhanced cytokinin-mediated resistance against infection with necrotrophic pathogens such as *Alternaria*<sup>9</sup> and *Botrytis cinerea*<sup>10</sup> may partially be due to a link between cytokinin and JA pathways through LOX3. Likewise, the COR13 (coronatine induced 3) or JR2 (JA responsive 2) is an important node of jasmonate signaling pathway in plants and regulates both growth and defense response to pathogens and herbivores.<sup>24</sup> This could also be another point of connection between JA and cytokinin signaling with an important role in plant immune defense against necrotrophs, viruses, and herbivores (Fig. 2D).

Moreover, GA4 (gibberellin 3-oxidase 1) is involved in the later steps of the GA biosynthetic pathway in *Arabidopsis*. Densely connected in our immune network (Fig. 2B), it is significantly up regulated by cytokinin. Enhanced levels of GA have been shown to degrade DELLA proteins and promote resistance against infection with biotrophic pathogens.<sup>25</sup> Similarly, IAA1 (indole-3-acetic acid inducible) is an Aux/IAA transcription factor and is an active repressor of auxin responsive gene expression. Enhanced auxin responses lead to susceptibility of plants against infection with biotrophic pathogens and resistance against necrotrophic pathogens.<sup>6,26</sup> Cytokinin-mediated stability of Aux/IAA1 may enhance immune responses against biotrophic pathogens.<sup>14</sup> According to our analysis, the *Arabidopsis* COS1 (coronatine insensitive 1 (COI1) suppressor 1) is another immune regulator with above average degree of connectivity in our extracted immune network (Fig. 2B) and is significantly induced by the treatment of cytokinin (Fig. 2D). The gene of *COS1* encodes lumazine synthase, a key component of riboflavin pathway, and suppresses the JA signaling node COI1.<sup>27</sup> Cytokinin-mediated increase in the expression of *COS1* and its suppressive behavior to JA pathway may lead to better protection in *Arabidopsis* against infection with biotrophic pathogens. Moreover, the *Arabidopsis* cpATPase (delta subunit of ATP synthase) plays a pivotal role: it is stabilizing the cpATPase complex to maintain optimal thylakoid composition as well as photosynthesis.<sup>28</sup> The gene encoding cpATPase is highly induced by the application of cytokinin but moderately connected in our analysis (Fig. 2D). Cytokinin in general maintains organelle integrity and increases photosynthetic activities;<sup>29</sup> hence, it protects plants against stresses leading to the derangement of cellular organelles or their functions.

Our analysis of immune functional hubs highlights nodes of the immune network (Fig. 2B): Their genes are significantly down regulated by cytokinin application but their proteins are densely connected in the network (Fig. 2D). Among them is the *Arabidopsis* protein PEN2 (penetration 2), which is a type of myrosinase enzyme that triggers broad-spectrum anti-fungal defense particularly at the host–pathogen interface.<sup>30</sup> Many fungal biotrophs are capable of producing cytokinin at the host–pathogen interface.<sup>11</sup> By accumulating cytokinins at the host–pathogen interface, biotrophic pathogens might turn down the PEN2 gene expression for their survival in the invading plant tissue. Our analysis shows that SUR1 (superroot 1) is connected with 12 different protein nodes and is significantly down regulated by cytokinin treatment (Fig. 2D). SUR1 is strongly induced by auxin and also plays an important role in auxin homeostasis.<sup>31</sup> Down-regulation of SUR1 by cytokinin will improve plant resistance to biotrophic pathogens, which is otherwise compromised in higher auxin status of the plant. Similarly, PIF3 (phytohormone interacting factor 3) is down regulated by cytokinin and is connected to 11 different nodes in our immune network (Fig. 2D). PIF3 is negative regulator of phyB (phytochrome B). Negative regulation of phyB leads to decreased plant resistance against infection with necrotrophic pathogen, *B. cinerea*.<sup>32</sup> Increased cytokinin levels have been shown to promote resistance against *Botrytis*.<sup>10</sup> Thus, cytokinin-mediated down-regulation of PIF3 might have positive impact on phyB activation and protection against infection with *Botrytis*. Taken together, these analyses emphasize a module where the cytokinin signaling pathway interacts with the jasmonate pathway of resistance and PIF3 and DELLA proteins exert its regulatory impact on the interaction.

**Response regulators mutant data set.** Type-A ARRs are negative regulators of cytokinin signaling<sup>1</sup> and repress immune responses in *Arabidopsis*.<sup>7</sup> Here, the sextuplet type-A ARR mutant (*arr3,4,5,6,8,9*) provides a unique opportunity to investigate the impact of the deletion of six individual ARRs in a single mutant background on plant immunity.<sup>7</sup> We mapped and visualized the high-confidence STRING interactome and collected significantly *Hpa* Noco2-regulated genes in mutant *arr3,4,5,6,8,9* vs. Col-0 (Fig. 3A). We found an overlay of 227 nodes, which is 7.43% of interactome and 14.39% of DEGs, and marked these 145 up- and 82 down-regulated nodes as distinct points on the visualized PPI network (Fig. 3A). All such intersecting nodes were next subjected to biological function analysis. We found 15 immune relevant functions (3.62%) looking at a total of 414 biological processes. According to our analyses, 43 (18.94% of transcriptome–interactome-mapping) nodes participate in these immune functions. We sorted out the connectivity of these nodes from the basal interactome and visualized them as sub-network consisting of 168 nodes and 261 edges, where immune-enriched nodes are connected to their interactors (Fig. 3B). Based on network analysis, the average number of neighbors for this immune network is 2.143 (Fig. 3C). To identify functional hubs, we further computed



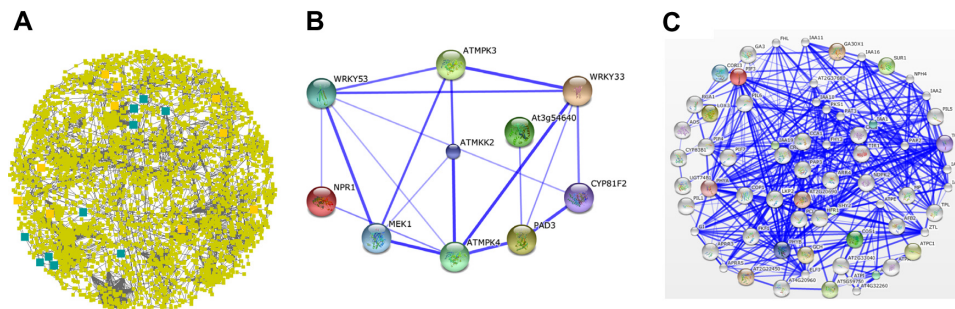
**Figure 3.** Systems biology analysis to determine the effect of derepression of cytokinin signaling on plant immunity. Type-A ARR attenuate cytokinin signaling. Hence, plant mutants deficient in type-A ARRs enhance cytokinin responses including pathogen defense (color code as in Fig. 2). **(A)** Transcriptome  $\times$  interactome mapping and visualization. Proteins are indicated by little squares. The color for non-intersecting nodes between transcriptome and interactome is cyan, for intersecting up-regulated nodes between transcriptome and interactome is violet, and for intersecting down-regulated nodes between transcriptome and interactome is dark blue; interactions are drawn as gray lines. Transcriptome data of sextuplet type-A ARRs mutant (*arr3,4,5,6,8,9*) *Arabidopsis* plants vs. wild-type Col-0 and *Hpa Noco2* treated vs. mock treated are adopted from the study of Argueso et al.<sup>7</sup> 1577 genes are differentially regulated. Interactome of *Arabidopsis* (3056 protein nodes, 18233 interactions), acquired from STRING, is visualized with Cytoscape. 227 nodes are shared between transcriptome and interactome data sets (145 up regulated: violet, 82 down regulated: dark blue). **(B)** Biological function analysis and reconstruction of immune network. Functional hubs are shown as bold red dots on the rim, immune enrichment as red little circles on the rim, interacting proteins as overlapping light blue circles on the rim, and protein interactions as gray lines traversing the circle to find the protein partner. Overrepresented biological functions in the interactome adaptation to the pathogen are detected by the Cytoscape plugin BiNGO tool. 414 biological functions for 214 genes are identified, 13 genes had no BiNGO-based annotation (see supplementary material), and 43 immune process-related protein nodes participate in 15 immune functions (red ones, see supplementary Table 1). The reconstructed immune network of these proteins and their interactors (light blue, maintained from step A) consists of 168 nodes and 261 edges (gray). **(C)** The double logarithmic plot shows (black dots) for each degree (number of connection to other nodes, x-axis), the number of nodes found (y-axis). The average degree is shown as red vertical line. Well-connected proteins (“hubs”) with degree equal to 7 or higher is framed in bold. Based on network connectivity and topology, expression *P*-values, and immunological relevance, 10 functional hubs are identified and shown as bold red dots with numbers from 1 to 10 in the immune network (B). They are tabulated with quantitative details in **(D)**.

the degree of connectivity and specified nodes as potential hubs having a degree more than the average number of neighbors (Fig. 3C, enclosed in the rectangle). Besides connectivity and topological parameters, we also considered *P*-values (expression of genes encoding network nodes) and immune relevance as criteria for hubs in our immune network.

According to our analysis, PAD3 (phytoalexin deficient 3) is a paralogue of PAD4 implicated in plant pathogen defense and instrumental in the biosynthesis of a class of phytoalexins in *Arabidopsis*, the camalexin (Fig. 3D).<sup>32</sup> PAD3 is connected with 85 nodes in our immune network (Fig. 3B) and is significantly up regulated by *Hpa NoCo2* infection in sextuplet *arr3,4,5,6,8,9* mutants. The PAD3 mutant plants are deficient in the accumulation of camalexin and are susceptible to infection by necrotrophic pathogen *B. cinerea*.<sup>33</sup>

Although enhanced plant camalexin levels do not protect *Arabidopsis* against infection with biotrophic pathogens, derepression of cytokinin responses with concomitant increase in camalexin would give better protection to plants against infection with necrotrophic pathogens. Recent studies on *Arabidopsis* and tobacco show that increased plant cytokinin levels also lead to higher accumulation of camalexin<sup>8</sup> and capsidiol<sup>18</sup> and inhibit bacterial growth. Moreover, our analysis shows that the *Arabidopsis* CYP81F2 (cytochrome P450, family 81, subfamily F, polypeptide 2) protein is a type of P450 monooxygenase that plays a crucial role in the synthesis of glucosinolate<sup>30</sup> and is implicated in broad-spectrum antifungal and antibacterial defense (Fig. 3D). Our analysis shows that CYF81F2 is densely connected in the immune network and is positively induced due to deletion of type-A ARRs. It





**Figure 4.** An integrative view on modulations in cytokinin pathway at two different levels and their impact on immune regulatory hubs in *Arabidopsis*. **(A)** Localization of hubs due to the deletion of type-A ARR (big dark green squares) compared to hubs due to the exogenous application of cytokinins (big yellow squares). The hub nodes are projected on the background of all nodes from the high-confidence interactome (proteins: small light green squares; interactions: gray lines). **(B)** Compact interactome and short, direct interaction paths (blue lines; strength: confidence level) between hub protein nodes (colored, drop-like circles) are observed after the deletion of type-A ARR. This was drawn according to information from STRING database. **(C)** Complex, non-compact connectivity paths (blue lines; strength: confidence level) among hub proteins (colored, drop-like circles) affected after the exogenous application of cytokinins. This was drawn according to information from STRING database.

remains to be established whether derepression of cytokinin signaling accumulates higher glucosinolate and thereby protects plants against biotrophic infections. The gene of *TSA1* (tryptophan synthase alpha chain 1) is significantly up regulated and encodes the small subunit of tryptophan synthase. *TSA1* mutants are deficient in callose deposition at the site of pathogen infection.<sup>34</sup> Elevated cytokinin levels and signaling have been shown to increase callose deposition in transgenic *Arabidopsis* plants.<sup>35</sup> Conversely, enhanced cytokinin catabolism in *CKX4* transgenic plants deposits significantly less callose in comparison to wild-type plants after flagellin22 treatment.<sup>35</sup>

Furthermore, we found that derepression of cytokinin signaling also lead to the induction of MAPK-cascade such as *AtMPK1*, *AtMPK2*, *AtMPK3*, and *AtMPK4* (*Arabidopsis thaliana* mitogen-activated protein kinases 1, 2, 3, 4). These various stress-integrating and signaling kinase hubs are also densely connected to other nodes in our analysis (Fig. 3D). The MAPK pathway plays a central role in both PTI and ETI (effectors-triggered immunity).<sup>1,6,19</sup> The signal of cytokinin is conveyed through *TCS*,<sup>1,2</sup> and a link between cytokinin signaling and MAPK signaling in immunological perspective is still not known. However, application of *trans-Zeatin* to *Brassica napus* has been shown to induce *MAPK4* and manifests better protection against infection with necrotrophic pathogen *Alternaria*.<sup>34</sup> According to our analysis, *NPR1*, which is SA signal perception receptor,<sup>35</sup> is connected to 14 other nodes in our immune network and is also significantly induced. *Arabidopsis NPR1* mutants are highly susceptible to biotrophic infections.<sup>36</sup> Type-B ARR, the *ARR2* interacts with TGA transcription factors and activates defense response only in the presence of *NPR1* in SA-dependent manner.<sup>9</sup> Similarly, acting downstream of MAPK pathway, WRKY transcription factors (contain a 60 amino acid protein domain with N-terminal sequence WRKYGQK and a zinc finger motif)

play an important role in regulating immune dynamics in plants. Two of them, *WRKY33*<sup>37</sup> and *WRKY53*<sup>38</sup> (*WRKY* DNA-binding proteins 33, 53), are induced by the deletion of ARR and are densely connected in our immune networks (Fig. 3D).

How does now the integrated picture for both sets of experimental data look like? Recent studies show that plant immune signaling networks are highly interconnected and bear a sector switching property.<sup>39</sup> This modular behavior of plant immune networks suggests that barely a small sector of the whole immune signaling interactome is highly active for each experimental setting. To visualize this picture of cytokinin-based modularity within plant immune networks, we projected in Figure 4 A all identified hubs (from Figs. 2D and 3D) on our high-confidence interactome. The hubs for both stimuli are in partly overlapping network areas as they both contribute to plant immune function (including shared target genes), but they operate in clearly different sectors of immunity (no shared hubs). These results support that modulation in cytokinin pathways at various levels leads to differential sub-network responses. Also the compactness in the two immune networks differs (Figs. 4B and 4C). Hubs due to the deletion of type-A ARR are directly connected and thus show the shortest path connectivity (Fig. 4B). For comparison, hubs in the immune networks due to the application of cytokinins require more intermediate nodes for the shortest paths and hence represent a large module (Fig. 4C). A plausible explanation for more direct connectivity among the hubs due to the deletion of type-A ARR might be due to subtle change in the deletion of ARR vs. global activation of the whole pathway with exogenous cytokinin application starting from perception of the hormone via AHKs and transduction to type-B ARR via AHPs.

Taken together, these analyses show that our basic hypothesis has good support: we can identify hub proteins



as cross-linking agents between cytokinins and defense pathways. We show that the two different approaches for the modulation of cytokinin responses activate different sectors in the cellular proteome. Thus, deletion of type-A ARR leads to de-repression of cytokinins responses, which promotes the SA pathway of resistance. Furthermore, our systems biology analysis points to a link between cytokinin signaling and WRKY transcription factors in controlling immune dynamic in *Arabidopsis*. External application of cytokinin also modulates immune responses by activating jasmonate pathway nodes in our analysis. We, therefore, conclude that our system biology approaches culminate in vital target proteins important for cytokinin-mediated immune defense in plants. Future experiments concerning the detailed functional role of these proteins would be intriguing and will further elucidate the important role of cytokinins in plant immunity.

### Author Contributions

Conceived and designed the experiments: MN. Analyzed the data: MN, MK, TD. Wrote the first draft of the manuscript: MN, MK, TD. Contributed to the writing of the manuscript: MN, MK, TD. Agree with manuscript results and conclusions: MN, MK, TD. Jointly developed the structure and arguments for the paper: MN, TD. Made critical revisions and approved final version: MN, MK, TD. All authors reviewed and approved of the final manuscript.

### DISCLOSURES AND ETHICS

As a requirement of publication the authors have provided signed confirmation of their compliance with ethical and legal obligations including but not limited to compliance with ICMJE authorship and competing interests guidelines, that the article is neither under consideration for publication nor published elsewhere, of their compliance with legal and ethical guidelines concerning human and animal research participants (if applicable), and that permission has been obtained for reproduction of any copyrighted material. This article was subject to blind, independent, expert peer review. The reviewers reported no competing interests.

### Supplementary Files

- Supplementary Table 1.** arr3, 4, 5, 6, 8, 9; Bingo-Analysis.
- Supplementary Table 2.** Genes participating in the tabulated biological processes and also belonging to the immune network (Hubs and Interactors) in Figure 2C.
- Supplementary Table 3.** Expression values for intersecting genes.

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