Dual Regulation of Gene Expression Mediated by Extended MAPK Activation and Salicylic Acid Contributes to Robust Innate Immunity in Arabidopsis thaliana

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Abstract

Network robustness is a crucial property of the plant immune signaling network because pathogens are under a strong selection pressure to perturb plant network components to dampen plant immune responses. Nevertheless, modulation of network robustness is an area of network biology that has rarely been explored. While two modes of plant immunity, Effector-Triggered Immunity (ETI) and Pattern-Triggered Immunity (PTI), extensively share signaling machinery, the network output is much more robust against perturbations during ETI than PTI, suggesting modulation of network robustness. Here, we report a molecular mechanism underlying the modulation of the network robustness in Arabidopsis thaliana. The salicylic acid (SA) signaling sector regulates a major portion of the plant immune response and is important in immunity against biotrophic and hemibiotrophic pathogens. In Arabidopsis, SA signaling was required for the proper regulation of the vast majority of SA-responsive genes during PTI. However, during ETI, regulation of most SA-responsive genes, including the canonical SA marker gene PR1, could be controlled by SA-independent mechanisms as well as by SA. The activation of the two immune-related MAPKs, MPK3 and MPK6, persisted for several hours during ETI but less than one hour during PTI. Sustained MAPK activation was sufficient to confer SA-independent regulation of most SA-responsive genes. Furthermore, the MPK3 and SA signaling sectors were compensatory to each other for inhibition of bacterial growth as well as for PR1 expression during ETI. These results indicate that the duration of the MAPK activation is a critical determinant for modulation of robustness of the immune signaling network. Our findings with the plant immune signaling network imply that the robustness level of a biological network can be modulated by the activities of network components.

Introduction

How network properties, such as robustness against network perturbations, emerge from biological networks has been a central question in systems biology [1,2]. Possible modulation of network robustness in a biologically relevant context and mechanisms underlying the modulation are areas of study that have rarely been explored.

Innate immunity, in which defense responses are induced through signaling events initiated by recognition of pathogen attack, constitutes a major part of plant immunity [3]. PAMP/ Pattern-Triggered Immunity (PTI) and Effector-Triggered Immunity (ETI) are modes of plant innate immunity defined by the way pathogens are detected [4,5]. PTI is triggered by recognition of microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) by the cognate pattern-recognition receptors (PRRs), which are typically receptor-like kinases or receptor-like proteins [6]. For example, Arabidopsis thaliana FLS2 is the PRR for flg22, an elicitor-active epitope of flagellin from Gram-negative bacteria [7]. While most non-adapted pathogens cannot overcome PTI, adapted pathogens deliver effectors into the plant cell that manipulate plant cell functions to facilitate their infection by, for instance, interfering with PTI signaling [8,9]. ETI is triggered by specific recognition of effectors by resistance (R) proteins, which are often nucleotide-binding leucine-rich repeat (NB-LRR) proteins [10]. For example, the Arabidopsis intracellular NB-LRR R proteins RPS2 and RPM1 independently recognize perturbations of the PTI signaling component RIN4 by the effectors AvrRpt2 and AvrRpm1/AvrB, respectively, of a Gram-negative bacterial pathogen, Pseudomonas syringae [3]. In addition to proteinaceous effectors, some P. syringae strains deliver coronatine, which is a
Dual Regulation by Extended MAPK Activation and SA

Author Summary

Robustness of a network is defined by how consistently it performs upon removal of some of its components. It is a common strategy for plant pathogens to attack components of the plant immune signaling network in an attempt to dampen plant immunity. Therefore, it is crucial for the plant immune signaling network to have a high level of robustness. We previously reported that the robustness level of the plant immune signaling network is higher during Effector-Triggered Immunity (ETI) than Pattern-Triggered Immunity (PTI). Here we discovered a molecular switch that determines two robustness levels during ETI and PTI. Salicylic acid (SA) is a major plant immune signal molecule that regulates many immune-related genes. SA-independent alternative mechanisms also regulated the majority of SA-responsive genes during ETI but not PTI. One of the SA-independent mechanisms was mediated by prolonged activation of MAP kinases (MAPKs). MAPK activation was prolonged during ETI but transient during PTI. Thus, the duration of MAPK activation switches the robustness level of the plant immune signaling network. Our findings imply that the robustness level of a biological network can be modulated by activities of its components.

jasmonic isoleucine mimic, in order to suppress plant immunity [11]. Recently, it was shown that coronatine suppresses immune responses dependent on salicylic acid (SA) as well as independent of SA [12,13]. Thus, there are evolutionary arms races between hosts and pathogens. Pathogens evolve much faster than hosts, rapidly changing effector repertoires, thereby changing points of attack in host immune networks. As hosts cannot match the speed of pathogen evolution, it is important that hosts develop robust immune networks that remain functional in the face of effector attack. Mechanisms underlying network robustness are thus a critical aspect of immunity.

SA is a signal molecule controlling a major portion of immunity against biotrophic and hemibiotrophic pathogens, including P. syringae [14]. SID2 encodes a key enzyme for SA biosynthesis in response to pathogen infection [15]. In Arabidopsis sid2 mutants, pathogen-induced SA accumulation is almost undetectable [14]. Hundreds of genes are transcriptionally regulated by SA signaling, mediated mainly by a positive regulator of SA signaling, NPR1 [14]. PR1 is one SA-inducible gene used as a canonical SA marker [14].

Arabidopsis has 20 mitogen-activated protein kinases (MAPKs) [16], and four of them, MPK3, MPK4, MPK6 and MPK11, have been described as immune signaling components [17]. MPK3 and MPK6 are associated with immune responses, such as reactive oxygen species (ROS) production, ET production/signaling, phytoalexin production and cell death [17]. For instance, ethylene production is positively controlled by dual regulation of enzymes (ACS) synthesizing the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid. MPK6 stabilizes ACS2 and ACS6 by their phosphorylation, and MPK3 and MPK6 control gene expression through a transcription factor, WRKY33, which is activated by the MAPKs [18,19]. The same cascade is required for production of a phytoalexin, camalexin, by controlling expression of a biosynthetic gene, PAD3 [20]. A double mutant deficient in MPK3 and MPK6 is embryonic lethal but the single mutants are viable, suggesting functional redundancy between them in development [21]. MPK3 phosphorylates the bZIP type transcription factor VPI1 whose phosphorylation is required for its nuclear translocation [22]. Transient over-expression of VIP1 led to weak induction of PR1 in Arabidopsis protoplasts although involvement of SA in this PR1 induction is not known [23].

The overall spectra of induced defense responses are overlapping between PTI and ETI whereas the kinetics and intensity of the responses seem different [4,24]. In Arabidopsis, knocking out the hub genes of four major signaling sectors abolished 80% of flg22-triggered PTI (flg22-PTI) and AvrRpt2-triggered ETI (AvrRpt2-ETI), indicating extensively shared signaling network machinery between PTI and ETI [25]. Relationships among these signaling sectors are part compensatory and part synergistic in flg22-PTI but are predominantly compensatory in AvrRpt2-ETI, which explains a high level of robustness in the ETI level against network perturbations [25]. Single mutations (ide2, ein2, pad4 and sid2) weakly but significantly compromised flg22-PTI but not AvrRpt2-ETI while the quadruple mutation largely abolished both. These observations demonstrated differences in the robustness of the highly overlapping signaling networks during the two modes of plant immunity. However, the molecular mechanism controlling modulation of the network robustness is not known.

Here we report a molecular mechanism that affects the robustness of the plant immune signaling network. Although Arabidopsis MPK3 and MPK6 are activated during both PTI and ETI, the duration of the activation was much longer during ETI than PTI. Only sustained activation of the MAPKs supported expression of a majority of SA-responsive genes in the absence of SA. The roles of MPK3 and SA signaling during AvrRpt2-ETI were compensatory, contributing to network robustness against perturbations during ETI. Our findings demonstrate that a biologically important differential network property, robustness, can emerge from duration of the activity of a network component.

Results

Most SA-responsive genes were properly regulated in the absence of SA during ETI

We previously reported that ETI is more robust against network perturbations than PTI due to a higher level of network compensation [25]. We hypothesized that this compensation occurred at the level of gene regulation. To test this hypothesis, we examined expression of a canonical SA marker gene, PR1, during ETI. Transcriptional induction of PR1 was completely dependent on SID2, which is a key SA biosynthetic enzyme, and hence completely dependent on SA signaling during PTI [26]. We found that PR1 induction was only partially dependent on SID2 and NPR1 at a late time point of 24 hours post inoculation (hpi) with ETI-triggering P. syringae pv. tomato DC3000 (Pto) strains expressing the effectors AvrRpt2 (Pto AvrRpt2) or AvrRpm1 (Pto AvrRpm1) (Figure 1A and Figure S1). While AvrRpt2 and AvrRpm1 are recognized by the CC-type NB-LRR proteins RPM2 and RPM1, AvrRps4 is recognized by the TIR-type NB-LRR protein RPS4 [3]. We also observed SID2- and NPR1-dependent PR1 induction during AvrRps4-triggered ETI although induction levels were lower compared to AvrRpt2- and AvrRpm1-ETI (Figure S1). In contrast, PR1 induction was completely dependent on SID2 in the case of the non-ETI triggering Pto strain carrying an empty vector (Pto EV). Inoculation of the ETI-triggering strains at a high dose can trigger a form of programmed cell death called a hypersensitive response (HR) [3]. The inoculation dose used in this experiment was relatively low (OD600 = 0.001), and we did not observe a macroscopic HR within 24 hpi. To test the possibility that the SA level increased independently of SID2 during ETI, we measured the SA level in these tissues. The increased SA accumulation was completely dependent on SID2 in all conditions.
The SA level is shown on a log10 scale. Asterisks and standard errors of two biological replicates calculated using a mixed linear model. The vertical axis shows the log2 expression level relative to Actin2 expression level in leaves at 6 or 24 hpi with Pto AvrRpt2 inoculation (Figure S2 and Table S2). Thus, initiation of ETI appears to be the key for activation of this SA-independent mechanism(s).

Activation of MPK3 and MPK6 was sustained in ETI but transient in non-ETI

We hypothesized that a kinetic difference in activation of network components is responsible for activation of SA-independent mechanism(s). A prior study suggested that the duration of MPK3 and MPK6 activation is longer during ETI than non-ETI [27]. We compared the duration of MAPK activation in ETI and PTI. When wild-type seedlings in a liquid medium were treated with the PTI inducer flg22, activation of the MAPKs was observed after 10 min and returned to the basal level within one hour (Figure 3A), confirming previous observations [28]. The possibility that flg22 was rapidly degraded in the liquid culture was excluded since the MAPKs were activated similarly when fresh seedlings were placed in the liquid medium containing flg22 that had been incubated with other seedlings for 3 hours (Figure 3A). Thus, MAPK activation is truly transient after flg22 treatment. We employed transgenic seedlings carrying an estradiol-inducible AvrRpt2 transgene (XVE-AvrRpt2) to measure MAPK activation during ETI in the absence of PTI. The MAPKs were activated by three hours and remained active for at least 7 hours after estradiol treatment (Figure 3B). This sustained MAPK activation was ETI-specific as no such activation was observed in the rps2 mutant background, which lacks the corresponding receptor (Figure 3C). PR1 induction during AvrRpt2-ETI was independent of SA in XVE-AvrRpt2 transgenic seedlings (Figure S3), which is consistent with the results obtained using adult leaves inoculated with a Pto strain expressing AvrRpt2 (Figure 1). Similar trends in MAPK activation duration were observed when adult leaves were inoculated with Pto strains: sustained activation of the MAPKs was observed with Pto AvrRpt2 in a manner dependent on the R gene RPS2, but not with the strains that do not trigger ETI (Figure 4). While the amounts of activated MPK3 and MPK6 were similar during AvrRpt2-ETI triggered in XVE-AvrRpt2 transgenic plants (Figure 3), there was more activated MPK3 than activated MPK6 during AvrRpt2-ETI triggered by Pto AvrRpt2 (Figures 4, S4 and S5), suggesting that MPK3 plays a major role during AvrRpt2-ETI in bacterial infection. We also observed sustained MAPK activation during AvrRps4-ETI although levels of activation were weaker compared to AvrRpt2-ETI (Figure S4). Since there are 20 MAPKs in Arabidopsis [16], we determined the identities of the activated MAPKs. Indeed, the activated MAPKs during AvrRpt2- and AvrRps4-ETI were MPK3 and MPK6 (Figure S4). Previously, Beckers et al (2009)
Figure 2. Sustained MAPK activation supports transcriptional regulation of a majority of SA-responsive genes without SA. (A) A heatmap of the SA-responsive genes. Leaves were collected at 24 hpi with the indicated Pto strains (OD$_{600}$ = 0.001) or mock. Independently, leaves of DEX-MKK4DD plants were collected at 24 hpi with 2 μM DEX or mock and subjected to mRNA profiling analysis using a whole genome DNA microarray. SA-responsive genes were selected for reproducible SID2-dependent responsiveness to the Pto strains as described in Experimental Procedures. The log$_2$ ratios compared to mock for 187 SA-responsive genes were subjected to agglomerative hierarchical clustering analysis. The log$_2$ ratio of DEX/mock for the DEX-MKK4DD sid2 plant (MKK4DD/sid2) samples was weighted by a factor of 0.5 to reduce its effects on the clustering pattern. The log$_2$ ratios used were averaged from three independent experiments. Green indicates negative values, red indicates positive values and black indicates zero: see the color scale. The arrow indicates the position of PR1. Means and standard errors of (B) Cluster I, 85 genes; (C) Cluster II, 20 genes; (D) Cluster III, 25 genes are shown.
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reported that an SA analog, benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH), induced priming of MPK3 activation by inducing expression of MPK3 [29]. In contrast, sustained activation of MPK3 during AvrRpt2-ETI was independent of SA (Figure S5). The sustained activation was not due to an increased amount of MPK3 as we did not observe obvious changes in the MPK3 protein level during AvrRpt2-ETI (Figure 3). Taken together, our data show that sustained activation of the MAPKs is SA-independent and occurs during ETI but not during non-ETI responses.

Sustained activation of MPK3 and MPK6 is sufficient for PR1 induction in the absence of SA

To test if sustained activation of MPK3 and MPK6 can induce PR1 in an SA-independent manner, transgenic plants expressing constitutively active forms of MKK4 (MKK4DD) or MKK5 (MKK5DD) under the control of a dexamethasone (DEX)-inducible promoter were employed (DEX-MKK4DD and DEX-MKK5DD). MKK4 and MKK5 are MAP kinase kinases, whose activated forms phosphorylate and activate MPK3 and MPK6 [17]. DEX-induced expression of MKK4DD or MKK5DD leads to sustained activation of MPK3 and MPK6 (Figure S6) [30]. Induction of PR1 was observed 9 hours after DEX treatment (Figure 5A), suggesting that sustained activation of MPK3 and MPK6 is sufficient for induction of PR1. Induction of FRK1 is thought to be a good marker for activation of MPK3 and MPK6 [31] and was observed 3 hours after DEX treatment while PR1 was not (Figure 5B). FRK1 was strongly induced 30 minutes after flg22 treatment [32], and the induction did not require SA accumulation (Figure S7). Thus, although transient MAPK
activation of MPK3 and MPK6 is sufficient for FRK1 induction, sustained MAPK activation is necessary and sufficient for SA-independent PR1 induction. The sustained activation of MPK3 and MPK6 by DEX-induced MKK4DD or MKK5DD did not increase the level of SA (Figure 6A). Furthermore, a wild-type-like PR1 induction 24 hours after DEX treatment was observed in plants deficient in SID2 or NPR1 (Figure 6B). Since PR1 induction was not observed in a DEX-inducible β-glucuronidase (GUS, an arbitrary reporter gene) line after DEX treatment, PR1 induction was not caused by the DEX-inducible system or DEX but by induced expression of MKK4DD or MKK5DD. Although MPK4 was activated as well as MPK3 and MPK6 during PTI and ETI (Figure 4; [17]), expression of MKK4DD or MKK5DD does not lead to strong activation of MPK4 [30]. Therefore, it is unlikely that MPK4 plays a role. We conclude that sustained activation of MPK3 and/or MPK6 causes PR1 induction in an SA-independent manner.

We tested whether mpk3 and mpk6 single mutations had effects on PR1 induction by MKK4DD or MKK5DD expression. PR1 induction was unaffected in mpk6 but strongly reduced in mpk3 plants (Figure S8A). MKK4DD induction was also strongly reduced in mpk3 plants (Figure S8B), so the reduction of PR1 induction in DEX-MKK4DD/mpk3 may be due to reduction of MKK4DD expression. MKK5DD induction in DEX-MKK5DD/mpk3 was reduced compared to DEX-MKK5DD/Col yet 10 times higher than MKK4DD induction in DEX-MKK4DD/mpk3 while PR1 induction was similarly compromised in both plant lines. Thus, these results suggest that MPK3 is required for SA-independent PR1 induction conferred by forced MKK5 activation while MPK6 is dispensable.

Sustained activation of MPK3 and MPK6 supported transcriptional regulation of most SA-responsive genes

We tested whether sustained activation of MPK3 and/or MPK6 also regulates other SA-responsive genes. Leaves of the DEX-MKK4DD transgenic lines in wild type (Col) or sid2 backgrounds were treated with DEX or mock control and were collected for mRNA profiling at 24 hours after treatment. The transcriptomic changes caused by DEX treatment were very similar between Col and sid2 (Figure S9 and Table S3), indicating that gene regulation by sustained activation of the MAPKs is mostly independent of SA. Therefore, only the mRNA profile from the DEX-MKK4DD sid2 line was included in the following analysis. The heatmap in Figure 2A shows that a majority of the SA-responsive genes responded in the DEX-treated DEX-MKK4DD sid2 line similarly to sid2 plants during AvrRpt2-ETI: most up-regulated or down-regulated SA-responsive genes in sid2 during AvrRpt2-ETI were up-regulated or down-regulated, respectively, in the DEX-treated DEX-MKK4DD sid2 line. This suggests that sustained activation of the MAPKs regulates a majority of SA-responsive genes in an SA-independent manner during AvrRpt2-ETI.
Three gene clusters were selected for further analysis (Clusters I–III in Figure 2A). The expression level changes of genes in each cluster were averaged and shown in Figure 2B–D. Clusters I and III include genes up- or down-regulated, respectively, in a SID2-independent manner during AvrRpt2-ETI and by sustained activation of the MAPKs. Thus, these genes appear to be regulated by sustained activation of the MAPKs during ETI. Cluster II includes genes that were up-regulated in a largely SID2-independent manner during ETI but not up-regulated by sustained activation of the MAPKs. Thus, up-regulation of the Cluster II genes during ETI is supported by a mechanism(s) other than the mechanism mediated by the MAPKs. When the GO terms associated with the clusters were examined, Cluster I, but none of the other clusters, was enriched with genes related to biological stresses (response to biotic stimulus, $P=2.8\times10^{-5}$; response to other organism, $P=1.1\times10^{-3}$; multi-organism process, $P=5.9\times10^{-3}$). The results imply that genes induced by both SA and the MAPKs are important for biological stress responses.
Compensatory relationships between the MAPKs and SA signaling confer robustness to AvrRpt2-ETI

We investigated if compensation between MPK3/MKP6 and SA signaling could be detected in the PRI expression level during ETI. Leaves of wild type (Col), mpk3, mpk6, sid2, mpk3 sid2 and mpk6 sid2 plants were inoculated with Pto AvrRpt2 or Pto AvrRpm1, and PRI expression levels were determined 24 hpi (Figure 7A). While PRI expression was compromised in sid2 but not in mpk3 or mpk6 during AvrRpt2-ETI, it was compromised in mpk3 sid2 more than in sid2 (blue bar), suggesting compensation between MPK3 and SID2 on PRI expression during AvrRpt2-ETI. To quantify the level of compensation between MPK3 and SID2 on PRI expression, a signaling allocation analysis was applied [25]. In this analysis, the effects of the genes and their interactions were estimated for contribution to the PRI expression level after inoculation. We estimated the individual contribution of MPK3 on the PRI expression level as the difference in expression levels between sid2 and mpk3 sid2, that of SID2 as the difference in PRI expression levels between mpk3 and mpk3 sid2 and their combined contribution as the difference in PRI expression levels between the wild type and mpk3 sid2. The value of the genetic interaction between MPK3 and SID2 was calculated by subtracting the sum of the individual contributions of MPK3 and SID2 from their combined contribution. Their combined contribution in the wild type was less than the sum of the individual contributions of SA and MPK3, which is signifies by the negative interaction between them. We previously defined this less-than-additive combined contribution as compensation [25]. Such compensation was observed for AvrRpt2-ETI (Figure 7B, top). Thus, signaling mediated by MPK3 and SA is compensatory on PRI expression during AvrRpt2-ETI. No significant effects of MPK6 or the interaction [MPK6:SID2] on PRI expression were detected during AvrRpt2-ETI (Figure 7A and B). No significant effects of MPK3, MPK6 or their interactions [MPK3:SID2] and [MPK6:SID2] on PRI expression (Figure 7A and B, red bar) or resistance (Figure S11) were detected during AvrRpm1-ETI, suggesting a divergence in the mechanisms that modulate network robustness between different cases of ETI.

A similar trend was observed with the effects of MPK3 and MPK6 on bacterial resistance in AvrRpt2-ETI (Figure 7C). AvrRpt2-ETI is defined as the difference in in planta growth of Pto EV and Pto AvrRpt2 on a log10-scale [25]. The compensation between MPK3 and SID2 was clear from the signaling allocation analysis, as both had positive effects and their interaction was negative (Figure 7D, left). We did not detect significant effects of MPK6 or the interaction [MPK6:SID2], although we observed a similar pattern to the case of MPK3 (Figure 7D, right). Thus, compensation of SA signaling by a signaling mechanism involving MPK3 exists in inhibition of bacterial growth, as well as in PRI expression, during AvrRpt2-ETI.

Lethality of the double mutants mpk3 mpk6 [21] does not allow us to determine combined contributions of MPK3 and MPK6 to compensation of SA signaling during ETI. It is possible that MPK6 is not a major factor in SA signaling compensation during ETI and that a signaling mechanism(s) other than that involving MPK3 or MPK6 is important during AvrRpm1-ETI. Nonetheless, these results clearly demonstrate that at least during AvrRpt2-ETI, SA signaling can be compensated by MPK3-mediated signaling in regulation of SA-responsive gene expression and that this compensation increases the robustness of the network output. This allows immunity to be maintained even if the major network sector, SA signaling, is compromised.

Discussion

In this study, we identified a mechanism that can increase the robustness of the plant immune signaling network during AvrRpt2-ETI. Our results demonstrate that (1) MPK3 and MPK6 are activated in a sustained manner during ETI and in a transient manner during non-ETI; (2) Transient MAPK activation during non-ETI such as PTI does not contribute to SA-independent regulation of the SA-responsive genes; (3) Sustained MAPK activation activates an SA-independent alternative mechanism that regulates the SA-responsive genes; (4) SA-independent alternative mechanisms which regulate the SA-responsive genes were activated during AvrRpt2-ETI; (5) SA signaling compensation by the signaling sector involving MPK3 contributes to increased robustness against network perturbations during AvrRpt2-ETI (Figure 8).

Factor(s) controlling differential activation duration of the MAPKs

A prior study implied that the duration of MPK3 and MPK6 activation is longer during ETI compared to during non-ETI upon P. syringae infection [27]. However, it did not rule out the possibility that the effector AvrRpt2 caused sustained MAPK activation through a mechanism independent of recognition of AvrRpt2 via RPS2. We clearly demonstrated that sustained MAPK activation occurs when ETI is triggered (Figures 3 and 4). The duration of MPK3 and/or MPK6 activation is the determinant for activation of the SA-independent alternative mechanism to regulate the SA-responsive genes: only sustained MAPK activation results in activation of the alternative mechanism. One potential cause of the differential activation duration is rapid turnover of PTI receptors, PRRs. FLS2 is rapidly degraded and disappears within one hour upon exposure to flg22 [33,34]. Although turnover rates of other PRRs are not known, if many PRRs turn over rapidly upon activation, this could explain transient activation of the MAPKs by Pto hrcC (Figure 4), which presents multiple MAMPs [11]. The turnover rates of R proteins, the ETI receptors, upon their activation are largely unknown. Whether turnover rate is involved or not, this hypothesis that the duration of MAPK activation and, consequently, the robustness of the network can be tuned to each receptor is attractive because it would enable network robustness to be evolutionarily adapted according to what pathogen-derived signals are recognized by the receptors. Another potential but not mutually exclusive cause of the differential activation duration is involvement of protein phosphatases that dephosphorylate and inactivate the MAPKs: activation of the MAPKs may be negatively regulated by a phosphatase(s) during non-ETI responses while the phosphatase may be inactivated during ETI, resulting in the sustained activation of the MAPKs. Multiple types of such phosphatases including MAPK phosphatases are known in Arabidopsis [35]. Differential regulation of these phosphatases during ETI and non-ETI responses may explain the differential duration of MAPK activation.

Decoding of the activation duration information

Switching of downstream signaling by differential duration of MAPK activation is known in animals and yeast [36–38]. In one case, it is explained by nuclear translocation of a MAPK that occurs only after its sustained activation [36]. In this way, sets of substrates available to the MAPK are distinct between its transient and sustained activation, which could lead to distinct downstream
Figure 7. Compensation between MPK3 and SA contributes to the robust ETI levels. (A) The PR1 expression level in leaves of the indicated genotypes at 24 hpi with Pto AvrRpt2 (blue bars) or AvrRpm1 (red bars) (OD$_{600} = 0.001$) was determined by qRT-PCR. Bars represent means and standard errors of three biological replicates calculated using a mixed linear model. The vertical axis shows the log$_2$ expression level relative to Actin2 (At2g18780). Statistically significant differences are indicated by different letters ($P < 0.01$, two-tailed $t$-tests). (B) The signaling allocations for the PR1 expression level shown in (A) were estimated for MPK3 and SID2 (upper panel) or MPK6 and SID2 (lower panel). (C) The bacterial counts of Pto EV (left panel) or AvrRpt2 (right panel) (inoculation dose, OD$_{600} = 0.0001$) at 0 or 2 dpi in leaves of the indicated genotypes were measured. Bars represent means and standard errors of three independent experiments with at least 4 or 12 biological replicates for 0 dpi or 2 dpi, respectively. Statistically significant differences are indicated by different letters per strain per dpi ($P < 0.01$, two-tailed $t$-tests). (D) The signaling allocations for AvrRpt2-ETI
ETI may provide robustness under perturbation by coronatine

*Pto* produces the small molecule coronatine, which is a molecular mimic of the JA-Ile conjugate and promotes virulence by suppressing SA signaling [13]. *Pto* is highly virulent on Arabidopsis plants while ETI-triggering strains of *Pto*, such as *Pto AvrRpt2*, are much less virulent. Nevertheless, coronatine could suppress SA signaling. Therefore, SA-independent alternative mechanism(s) to regulate expression of the SA-responsive genes, such as that mediated by the MAPKs, may have a substantial role against perturbation of the immune signaling network by coronatine. This hypothesis is consistent with our observation that loss of both MPK3 and SA led to increased susceptibility to *Pto AvrRpt2* (Figure 7).

Can *AvrRpt2-ETI* overcome suppression effects by other effectors?

*Pto* DC3000 possesses type III effectors which directly or indirectly suppress MAPK activation [45–48]. However, we observed sustained activation of MPK3 and MPK6 during *AvrRpt2-ETI* when *AvrRpt2* was delivered from *Pto DC3000* (Figure 4). We speculate that the amounts of such MAPK-inhibiting type III effectors delivered and/or the kinetics of their delivery are not optimal to effectively suppress MAPK activation when the type III effectors are delivered from *Pto DC3000*, which represents a relatively natural context.

The effectors HopAI1 from *Pto DC3000* can physically interact with and inactivate MPK3 and MPK6 by removing the phosphate group from phosphothreonine via a phosphothreonine lyase activity [45]. HopAI1 also targets MKK4 and decreases MKK4 activity [48]. Decreased MKK4 activity appears to be monitored by the NB-LRR protein SUMM2, resulting in triggering ETI. Overexpression of HopAI1 in wild-type Col-0 plants but not *summ2* mutant plants leads to dwarfism and constitutive activation of immune responses [48]. However, *Pto DC3000* does not trigger SUMM2-mediated ETI. Consistently, *HopAI1* of *Pto DC3000* is disrupted by an insertion in its promoter region [49]. Thus, the amount of HopAI1 delivered from *Pto DC3000* appears insufficient for effective inhibition of MPK3 and MPK6 activation during *AvrRpt2-ETI*.

Another effector, HopF2, from *Pto DC3000* can also suppress activity of MPK3, MKK4 and MPK6 by targeting the upstream MKK5 and likely other MKKs as well [46,47]. When overexpressed in plants, HopF2 interferes with *AvrRpt2-ETI* by inhibiting MKK4-mediated RIN4 degradation [50]. Again, the reason that HopF2 cannot suppress sustained activation of MPK3 and MPK6 triggered by *AvrRpt2* when it is delivered from *Pto DC3000* (Figure 4) is likely insufficient HopF2 or inappropriate timing of its delivery. Delivery of *AvrRpt2* may precede that of HopF2 [50].

Is the low level of robustness required during PTI?

One enigma is why plants need to make the robustness of the immune signaling network lower during PTI when the network itself has the capacity to be highly robust. If the network output during PTI were as robust as during ETI, the chance that “true” pathogens will overcome PTI would be much lower. We speculate that the lower robustness during PTI is selected through evolution as trade-offs with other requirements. Many MAMPs are shared...
among pathogens and benign microbes and provide low quality information about pathogen attack. It is probably not adaptive for plants to respond to a MAMP with strong and sustained immune responses similar to those during ETI since in many cases, plants encounter benign microbes and ETI-type responses cost fitness. A strategy apparently selected is to respond weakly first and wait to intensify the response until further information increases the probability that a true pathogen is present [24]. In contrast, since effectors are a hallmark of true pathogens and provide high quality information, during ETI plants can induce rapid and strong immune responses with a very low chance of needless fitness costs.

Concluding remarks

The signaling sector activated by sustained activation of the MAPKs during ETI and the SA signaling sector can regulate the common set of genes. This is one of the mechanisms underlying robustness of the immunity level against network perturbations during ETI. This modulation of the network robustness is controlled by signaling kinetics of a network component. Our findings imply that properties of biological networks can be modulated through network component activities.

Materials and Methods

Free SA measurement, MAP kinase assays, bacterial growth assays and the signaling allocation analysis were performed as described previously [25,26].

Plant materials and growth conditions

Arabidopsis plants were grown in a controlled environment at 22 ºC with a 12 h photoperiod and 75% relative humidity. Arabidopsis thaliana accession Col-0 was the background of all mutants used in this study. Arabidopsis mpk3-1 (SALK_151394) [21], mpk6-2 (SALK_073907) [18], npr1-1 [51], npr2 101C [52] and sid2-2 [15] were previously described. We generated the double mutants mpk3 sid2 and mpk6 sid2 by standard genetic crosses. Estradiol-inducible AvrRpt2 transgenic lines [53] and the DEX-MKK4DD and -MEKK3DD transgenic lines [30] were previously described. We crossed DEX-MKK4DD and -MEKK3DD into the mutant backgrounds mpk3, mpk6, npr1, sid2 and npr1. Primers and restriction enzymes used for screening of the mutants are listed in Table S4.

Chemicals

Flg22 peptide was purchased from EZBiolab Inc (Westfield, IN, USA). Estradiol (E8875) and DEX (D1756) were purchased from Sigma (St Louis, MO, USA).

Quantitative RT-PCR analysis

Pto DC3000 strains (or water for mock) or 2 μM DEX (or 0.1% ethanol for mock) were infiltrated into leaves of 4-week-old plants. Leaves were collected at the indicated time points. Total RNA isolation and qRT-PCR analysis were carried out as described previously [54,55]. The following models were fit to the relative Ct isolation and qRT-PCR analysis were carried out as described previously. Leaves were collected at the indicated time points. Total RNA was extracted as described previously [26] and profiled using the NimbleGen DNA microarray (A. thaliana Gene Expression 12×135K array TAIR9.0) following the manufacturer’s protocol (Roche Applied Science, Indianapolis, IN, USA). Three independent experiments (biological replicates) were performed. The microarray data were submitted to Gene Expression Omnibus (Accession, GSE40555). Probe signal values were subjected to the robust multi-array average (RMA) summarization algorithm [36] using the standard NimbleGen software to obtain the expression level values of the transcripts. Among transcripts of a single gene, those with higher expression values were selected as the representative transcripts of the genes. The following models were fit to log2 expression values using the lmFit function in the limma package in the R environment: $S_{\text{gy}} = G_{\text{gy}} R_{\text{gy}} + e_{\text{gy}}$, where $S$, log2 expression value, $G$, genotype:time:interaction, and random factors; $R$, biological replicate; $e$, residual. The eBayes function in the limma package was used for variance shrinkage in calculation of the p-values and the Storey’s q-values were calculated from the p-values using the qvalue function in the qvalue package. First, genes whose expression was up-regulated or down-regulated (q values<0.01 and more than 2 fold change) in both Pto EV and Pto AvrRpt2-infected Col compared to mock were selected (2828 genes). Second, SID2-dependent genes in Pto EV infection (inductions/suppression in sid2 are less than 20% compared to Col) were selected (187 “SA-responsive” genes) for the clustering analysis. Heatmaps were generated by CLUSTER [57] using uncentered Pearson correlation and complete linkage, and visualized by TREEVIEW [57].

Accession numbers

The accession numbers for the Arabidopsis genes discussed in this article are as follows: Actin2 (At2g18780), Chitinase (At1g02360), CHS (At5g13930), FRK1 (At2g19190), MKK4 (At1g51660), MKK3 (At5g21220), MPK3 (At3g45640), MPK4 (At4g03170), MPK6 (At2g43790), NPR1 (At1g64280), RPM1 (At3g07040), RPS2 (At4g26090) and SID2 (At1g74170).

Supporting Information

Figure S1 PR1 induction during ETI is largely NPR1-independent. The PR1 expression level in leaves inoculated with Pto strains (OD600 = 0.001) or mock was determined by qRT-PCR at 24 hpi. Bars represent means and standard errors of two biological replicates calculated using a mixed linear model. The vertical axis shows the log2 expression level relative to Actin2 (At2g18780). Asterisks indicate significant differences from mock (P<0.01, two-tailed t-tests).

(TIF)

Figure S2 Expression patterns after inoculation with Pto hrcC and Pto EV are similar while that with Pto AvrRpt2 is distinctive in terms of SID2-dependency. (A) A heatmap of pathogen-regulated genes. Leaves were collected at 24 hpi with the indicated Pto strains (OD600 = 0.001) or mock and mRNA profile analysis was performed using a NimbleGen Array. Genes whose expression was up- or down-regulated (q values<0.01 and more than 2 fold
change) in any samples compared to mock were selected (5361 genes). The log2 ratios compared to mock for the selected genes were subjected to agglomerative hierarchical clustering analysis. Green indicates negative values, red indicates positive values and black indicates zero. (B) A normalized heatmap. Expression changes after inoculation with Pto hrcC, Pto EV and Pto AvrRpt2 were compared using linear regression. Based on the regression coefficients, the log2 ratios of Pto hrcC and Pto AvrRpt2 samples were weighted by factors of 1.96 and 0.76, respectively, to normalize the overall level of induction/suppression among the treatments.

(TIF)

**Figure S3** PR1 induction is independent of SA during AvrRpt2-ETI. Seedlings of transgenic lines carrying the estradiol-inducible AvrRpt2 transgene in a sid2 background (XVE-AvrRpt2/sid2) were treated with different concentrations of estradiol for 24 hours in a liquid medium. The PR1 expression level was determined by qRT-PCR. Bars represent means and standard errors of two biological replicates calculated using a mixed linear model. The vertical axis shows the log2 expression level relative to Actin2 (At2g18780). Asterisks indicate significant differences from mock (P<0.01, two-tailed t-tests).

(TIF)

**Figure S4** The MAPks activated in a sustained manner during AvrRpt2- and AvrRps4-ETI were MPK3 and MPK6. Leaves of Col, mpk3 and mpk6 plants were infiltrated with Pto AvrRpt2 (A) or Pto AvrRps4 (B) (OD<sub>600</sub> = 0.01) and samples were collected at the indicated time points. Activated MAPks were detected by immunoblot using anti-p44/42 MAPK antibody. Ponceau S stained blots are shown for loading controls. Experiments were conducted twice with similar results.

(TIF)

**Figure S5** Sustained MAPK activation is independent of SA. Leaves of Col and sid2 plants were infiltrated with Pto EV (A) or Pto AvrRpt2 (B) at an inoculation dose of OD<sub>600</sub> = 0.01 and samples were collected at the indicated time points. Activated MAPks were detected by immunoblot using anti-p44/42 MAPK antibody. Ponceau S stained blots are shown as loading controls. Experiments were conducted twice with similar results.

(TIF)

**Figure S6** Sustained MAPK activation by MKK4DD and MKK5DD expression. Leaves of DEX-MKK4DD (MKK4DD) or -MKK5DD (MKK5DD) were treated with 2 μM DEX for the indicated time points and activated MAPks were detected by immunoblot using anti-p44/42 MAPK antibody. Ponceau S stained blots are shown as loading controls. Experiments were conducted twice with similar results.

(TIF)

**Figure S7** Induction of FRK1 by flg22 does not require SA. Leaves of Col or sid2 plants were infiltrated with 1 μM flg22 or water and samples were collected at the indicated time points. The expression level of FRK1 was determined by qRT-PCR. Bars represent means and standard errors of at least two biological replicates calculated using a mixed linear model. The vertical axis is the log2 expression level relative to Actin2 (At2g18780). Asterisks indicate significant differences from mock (P<0.01, two-tailed t-tests).

(TIF)

**Figure S8** MPK3 seems to be required for SA-independent PR1 induction conferred by forced MKK3 activation while MPK6 is dispensable. Leaves of transgenic plants carrying DEX-inducible MKK4DD (4DD) or MKK5DD (5DD) (Col, mpk3 or mpk6 background) were infiltrated with 2 μM DEX (DEX) or 0.1% ethanol (mock) and samples were collected at 24 hpi. The expression levels of PR1 (A) and MKK4 or MKK5 (B) were determined by qRT-PCR. Bars represent means and standard errors of two biological replicates calculated using a mixed linear model. The vertical axis is the log2 expression level relative to Actin2 (At2g18780). Asterisks indicate significant differences from mock (P<0.01, two-tailed t-tests).

(TIF)

**Figure S9** Expression patterns in DEX-inducible MKK4DD transgenic plants are very similar in Col and sid2. Leaves of transgenic plants carrying DEX-inducible MKK4DD in Col or sid2 backgrounds were infiltrated with 2 μM DEX or 0.1% ethanol and samples were collected at 24 hpi. mRNA profile analysis was performed as described in Figure 2. Genes whose expression was up-regulated or down-regulated (q-values<0.01 and more than 4 fold change) in DEX-treated samples compared to mock were selected (4743 genes). The log2 ratios (DEX/mock) for the 4743 genes were subjected to agglomerative hierarchical clustering analysis as in Figure 2. Green indicates negative values, red indicates positive values and black indicates zero.

(TIF)

**Figure S10** Expression patterns of genes representing three clusters. Leaves of Col or sid2 plants were infiltrated with Pto EV, Pto AvrRpt2 (OD<sub>600</sub> = 0.001) or water (mock) and samples were collected at 24 hpi. Leaves of transgenic plants carrying DEX-inducible AtMKK4DD in a sid2 background were infiltrated with 2 μM DEX or 0.1% ethanol and samples were collected at 24 hpi. The expression levels of PR1 (At2g14610) (A), Chitinase (At1g02360) (B) and CHS (At5g13930) (C), which represent Clusters I, II, and III, respectively, were determined by qRT-PCR. Bars represent means and standard errors of three biological replicates calculated using a mixed linear model. The vertical axis is the log2 expression level relative to Actin2 (At2g18780). Asterisks indicate significant differences from mock (P<0.01, two-tailed t-tests).

(TIF)

**Figure S11** AvrRpm1-ETI. (A) The bacterial counts of Pto EV (left panel) or AvrRpm1 (right panel) (inoculation dose, OD<sub>600</sub> = 0.0001) at 0 or 2 dpi in leaves of the indicated genotypes were measured. Bars represent means and standard errors of three independent experiments with at least 4 or 12 biological replicates for 0 dpi or 2 dpi, respectively. Statistically significant differences are indicated by different letters per strain per dpi (P<0.01, two-tailed t-tests). (B) The signaling allocations for AvrRpm1-ETI shown in (A, 2 dpi) were estimated for MPK3 and SID2 (left panel) or MPK6 and SID2 (right panel). Bars represent means and standard errors determined using a mixed linear model. Asterisks indicate significant effects or interaction (P<0.01).

(TIF)

**Table S1** Expression levels of the SA-responsive 187 genes and cluster information in Figure 2.

(XLSX)

**Table S2** Expression levels of the genes in Figure S2.

(XLSX)

**Table S3** Expression levels of the genes in Figure S9.

(XLSX)

**Table S4** Primers used in this study.

(DOCX)
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Author Contributions

Conceived and designed the experiments: KT. Performed the experiments: KT AM GB DI. Analyzed the data: KT AM FK. Contributed reagents/materials/analysis tools: CJB YT MS JG. Wrote the paper: KT JG FK.

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