Negative control of BAK1 by protein phosphatase 2A during plant innate immunity

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Abstract

Recognition of pathogen-associated molecular patterns (PAMPs) by surface-localized pattern-recognition receptors (PRRs) activates plant innate immunity, mainly through activation of numerous protein kinases. Appropriate induction of immune responses must be tightly regulated, as many of the kinases involved have an intrinsic high activity and are also regulated by other external and endogenous stimuli. Previous evidences suggest that PAMP-triggered immunity (PTI) is under constant negative regulation by protein phosphatases but the underlying molecular mechanisms remain unknown. Here, we show that protein Ser/Thr phosphatase type 2A (PP2A) controls the activation of PRR complexes by modulating the phosphostatus of the co-receptor and positive regulator BAK1. A potential PP2A holoenzyme composed of the subunits A1, C4, and B′γζ/ inhibits immune responses triggered by several PAMPs and anti-bacterial immunity. PP2A constitutively associates with BAK1 in planta. Impairment in this PP2A-based regulation leads to increased steady-state BAK1 phosphorylation, which can poise enhanced immune responses. This work identifies PP2A as an important negative regulator of plant innate immunity that controls BAK1 activation in surface-localized immune receptor complexes.

Keywords innate immunity; negative regulation; phosphatase; receptor kinase

Introduction

Recognition of pathogen-associated molecular patterns (PAMPs) by surface-localized pattern-recognition receptors (PRRs) is central to the establishment of innate immunity (Ronald & Beutler, 2010). However, the appropriate timing and intensity of innate immune responses must be tightly controlled. The negative regulation of PRR-triggered immunity (PTI) starts to be well understood in mammals (Kondo et al, 2012; Sasai & Yamamoto, 2013). In contrast, hardly anything is known in plants where over-activation of immune receptors can have a dramatic impact on growth.

Plant PRRs are surface-localized receptor kinases (RKs) or receptor-like proteins (RLPs) (Monaghan & Zipfel, 2012; Schwessinger & Ronald, 2012). These PRRs require dynamic association with regulatory kinases within plasma membrane-localized immune receptor complexes to initiate signaling (Monaghan & Zipfel, 2012). Notably, the regulatory leucine-rich repeat (LRR)-RK BAK1 (also named SERK3) is a key immune component that acts as a co-receptor required for the function of several LRR-containing PRRs (Monaghan & Zipfel, 2012; Santiago et al, 2013; Sun et al, 2013a,b; Liebrand et al, 2014).

The best-studied PRR-BAK1 complexes involve the Arabidopsis LRR-RKs FLS2 and EFR, which are the receptors for bacterial flagellin (or the derived immunogenic peptide flg22) and for elongation factor Tu (EF-Tu) (or the derived immunogenic peptide elf18), respectively (Monaghan & Zipfel, 2012; Schwessinger & Ronald, 2012). FLS2 and EFR form a ligand-induced complex with BAK1 leading to rapid phosphorylation of both proteins (Chinchilla et al, 2007; Heese et al, 2007; Schulze et al, 2010; Roux et al, 2011; Schwessinger et al, 2011; Sun et al, 2013b). In turn, downstream cytoplasmic kinases such as BIK1 (and its closest paralog PBL1) or BSK1 are phosphorylated and dissociate from the PRR-BAK1 complex (Lu et al, 2010; Zhang et al, 2010; Shi et al, 2013; Xu et al, 2013; Lin et al, 2014). These dynamic interactions and phosphorylation events lead to the activation of immune responses, including production of reactive oxygen species (ROS) by the NADPH oxidase RBOHD, activation of mitogen-activated protein kinase (MAPK) cascade, transcriptional reprogramming, and immunity to pathogens (Kadota et al, 2014; Li et al, 2014a; Macho & Zipfel, 2014).

However, mechanisms controlling the activation of PRR-BAK1 complexes prior to or upon ligand perception are still poorly understood. Recently, the identification of the LRR-RK BIR2 has highlighted a mechanism that limits the formation of the FLS2-BAK1 complex in absence of elicitation (Halter et al, 2014). In addition,
the E3-ubiquitin ligases PUB12 and PUB13 were shown to regulate the degradation of the ligand-bound FLS2 after BAK1 activation, most likely to enable the replenishment of ligand-free receptor at the plasma membrane (Robatzek et al., 2006; Lu et al., 2011; Smith et al., 2014).

Phosphorylation is a reversible post-translational modification. Protein phosphatases have been implicated in the negative regulation of immune signaling at diverse stages. Several members of the Ser/Thr protein phosphatase type 2C (PP2C) family are involved in stress signaling and biotic responses (Schweighofer et al., 2004). For example, in Arabidopsis, the PP2C AP2C1 is a negative regulator of the MAPKs MPK4 and MPK6 and modulates the levels of the hormones jasmonic acid and ethylene during immunity (Schweighofer et al., 2007). In addition, MPK3 and MPK6 are also dephosphorylated by the Arabidopsis dual specificity phosphatases (DSPs) MKP1 and MKP2 during PTI or oxidative stress in Arabidopsis (Lee & Ellis, 2007; Anderson et al., 2011). Plant Ser/Thr protein phosphatase type 2A (PP2A) holoenzymes are equally involved in development and responses to external stimuli or hormones. Similarly to their metazoan counterparts, plant PP2A are trimeric holoenzymes composed of a conserved catalytic subunit (‘C’), associated via a scaffold or hook subunit (‘A’) to one of many regulatory subunits (B, B’, or B”) that determine localization and substrate specificity (Uhrig et al., 2013). As such, different combinations of these subunits can potentially generate a wealth of different holoenzymes regulating distinct specific processes (Virshup & Shenolikar, 2009). Several plant PP2A substrates have been identified such as the auxin receptor PIN1, the blue light receptor Phot2, the ethylene biosynthetic enzyme ACS6 or the transcription factor BZR1, which regulates brassinosteroid (BR) responses (Michniewicz et al., 2007; Tseng & Briggs, 2010; Skottke et al., 2011; Tang et al., 2011). Interestingly, the PP2A-B’γ subunit may act as a negative regulator of immune responses under low light condition or day length-dependent oxidative stress (Trotta et al., 2011; Li et al., 2014b), and silencing of the PP2A-C subunits in Nicotiana benthamiana leads to enhanced immune responses (He et al., 2004). However, PP2A substrates involved in immunity are currently unknown.

Given the major role played by protein kinases in early PTI signaling, it is likely that protein phosphatases negatively regulate early immune components. Yet, so far, only a few examples of protein phosphatases acting at the level of plant PRR complexes are known. The PP2C KAPP interacts with the FLS2 cytoplasmic domain in yeast two-hybrid assays, and KAPP over-expression leads to flg22-insensitivity (Gomez-Gomez et al., 2001). However, no data are available on the effect of KAPP on FLS2 activity, and the specificity of KAPP is questionable since it was also reported to interact with many RKs (Ding et al., 2007). In rice, the PP2C XB15 interacts with and dephosphorylates the PRR XA21 leading to the negative regulation of XA21-dependent immunity (Park et al., 2008). RKs acting as PRR often belong to the non-RD (where RD refer to conserved Arg and Asp residues in the kinase subdomain VIb) kinase family and exhibit weak kinase activities (Dardick et al., 2012). In contrast, PRR-associated kinases, such as BAK1 and BIK1, generally have stronger kinase activity and can be involved in diverse plant signaling pathways (Wang et al., 2008; Lu et al., 2010; Zhang et al., 2010; Cheng et al., 2011; Schwessinger et al., 2011; Yan et al., 2012; Lin et al., 2013). Thus, a tight control of these kinases is required to avoid their activation in the absence of the appropriate stimulus, as well as to ensure optimal outputs upon ligand-induced activation. Yet, no protein phosphatase negatively regulating PRR complexes in Arabidopsis or other plant species has been identified, despite previous evidences suggesting that PTI is under constant negative regulation by various protein phosphatases (Felix et al., 1994; MacIntosh et al., 1994; Chandra & Low, 1995; Suzuki & Shinshi, 1995). In this study, we directly investigated the possible involvement of PP2A in the negative regulation of early PTI signaling. We used pharmacological, reverse-genetic and biochemical approaches to reveal that a specific PP2A holoenzyme potentially composed of the subunits A1, C4, and B’γ/c negatively regulates the steady-state activity of the co-receptor BAK1 during immunity. Our results illustrate a novel function for a plant PP2A by controlling the activity of a common regulatory receptor kinase.

Results

Protein phosphatase 2A inhibits immune signaling

Previous evidences suggest that PTI is under constant negative regulation by protein phosphatases, including PP2A (Felix et al., 1994; Chandra & Low, 1995; Suzuki & Shinshi, 1995). However, the exact identity of the phosphatase(s) and the mechanisms involved remain elusive. We first tested whether PP2A is involved in regulating PTI in Arabidopsis. We used the well-established specific PP2A inhibitor cantharidin (Li & Casida, 1992; Bajsa et al., 2011) to investigate the effect of PP2A activity on PTI. PAMP-induced resistance restricts by more than tenfold the growth of the phytopathogenic bacterium Pseudomonas syringae pv. tomato (Pto) DC3000 (Zipfel et al., 2004). Strikingly, chemical inhibition of PP2A by cantharidin induced a comparable level of resistance to Pto DC3000 in mature wild-type (WT) Col-0 plants to that observed upon pre-treatment with flg22 (Fig 1A). Importantly, cantharidin treatment did not trigger any visible symptoms by itself under the same conditions used for the pre-treatment experiments (Supplementary Fig S1A). This clearly suggests that PP2A negatively regulates immunity in Arabidopsis.

To identify at which point of the PTI signaling network is PP2A acting, we tested the effect of cantharidin treatment on early PTI events. Cantharidin by itself induced the accumulation of PTI marker transcripts such as FRK1 and NHL10 in axenic Col-0 seedlings (Fig 1B). Also, cantharidin triggered a dose-dependent ROS production in mature Col-0 leaves, which had similar characteristics but was slower than that observed upon flg22 treatment (Fig 1C). This may be explained by different diffusion rate across the cell wall and/or dynamics of action between flg22 and cantharidin. Together, these data suggest that PP2A acts early in PTI signaling at the level of the PRR complex, or that PP2A-dependent processes control several downstream signaling components, such as the NADPH oxidase RBOHD or the cytoplasmic kinases involved in the responses measured.

PP2A associates with BAK1 in planta

To determine if PP2A acts directly at the level of the PRR complex, we tested if PP2A chemical inhibition is sufficient to induce PRR complex activation. Remarkably, cantharidin treatment alone triggered the phosphorylation of the receptor complex-associated
cytoplasmic kinase BIK1, as measured by the mobility shift of BIK1 in a transgenic BIK1pro:BIK1-HA line (Fig 2A). This observation does not necessarily imply that PP2A targets BIK1. Indeed, BIK1 is phosphorylated by activated FLS2 and BAK1 upon flg22 perception (Lu et al, 2010; Zhang et al, 2010; Shi et al, 2013; Xu et al, 2013; Lin et al, 2014).

To identify which kinase from the PRR complex is associated with PP2A, we measured the PP2A activity present in FLS2, BAK1, and BIK1 immunoprecipitates from un-elicted seedlings. After subtraction of the background activity observed in non-enriched extract, a significant PP2A activity could be detected in complex

with BAK1, but not with FLS2 or BIK1 (Fig 2C). Moreover, the BAK1-associated PP2A activity was abolished by cantharidin treatment in vitro (Supplementary Fig S1B). These results indicate that PP2A associates constitutively with BAK1 in planta. The observations that cantharidin treatment is sufficient to induce BIK1 phosphorylation (Fig 2A) and that PP2A does not associate with BIK1 (Fig 2C) are consistent with BAK1 acting upstream of BIK1 (Lu et al, 2010; Zhang et al, 2010; Shi et al, 2013; Xu et al, 2013; Lin et al, 2014).

Specific PP2A subunits negatively regulate PAMP-triggered immunity

The key to understand the regulation of distinct cellular processes by PP2A is the identification of specific subunits composing PP2A holoenzymes (Shi, 2009; Virshup & Shenolikar, 2009). Of particular interest is the identification of the ‘B’ subunits that provide localization and substrate specificity. Thus, we conducted a reverse-genetic approach to identify PP2A subunits responsible for the negative regulation of early PTI signaling. The Arabidopsis genome encodes five isoforms of the catalytic ‘C’ subunit, three isoforms of the hook ‘A’ subunit, and up to 18 isoforms of the ‘B’ regulatory subunit, allowing the potential formation of multiple highly specific trimeric enzymes (Farkas et al, 2007). Expression of the ‘C’ and ‘A’ genes is globally ubiquitous and constant, whereas expression of several ‘B’ genes, mostly in the B subgroup, is altered by PAMP perception and/or biotic stresses (Supplementary Fig S2).

Despite the high homology between the catalytic isoforms and expected functional redundancy, single pp2a-c mutants yet display informative phenotype (Ballesteros et al, 2013). We obtained and characterized insertion mutant lines for the 5 ‘C’ and 3 ‘A’ genes, as well as for the 3 ‘B’ genes whose transcript accumulation is induced by biotic stress (Supplementary Figs S2 and S3). We postulate that given the negative role of PP2A in PTI, loss-of-function mutants in relevant subunits should be hyper-responsive to PAMPs. Using ROS production in response to increasing flg22 dose as a screening assay, we identified several pp2a mutants that show higher ROS production than Col-0 (Fig 3A).

We further characterized flg22 responsiveness in the pp2a-a1, pp2a-c4, pp2a-b’-n, and pp2a-b’-c’ mutants by testing flg22-induced protection against bacterial infection. In this assay, pp2a-a1, pp2a-c4, and pp2a-b’-n allowed significantly less growth of Pto DC3000 than Col-0 after pre-treatment with a low dose of flg22 (Fig 3B), demonstrating flg22 hypersensitivity in these lines. Consistent with an increased PAMP sensitivity, pp2a-a1, pp2a-c4, and pp2a-b’-n allowed significantly less growth of Pto DC3000 than Col-0 after pre-treatment with a lower dose of flg22 (Fig 3C). Additionally, we observed a reduced flg22-triggered ROS production in plants over-expressing PP2A-C4 (Fig 3D). Moreover, complementation of the rcn1-1 (an A1 subunit mutant) by RCN1 expression suppressed the exaggerated elf18 response (Supplementary Fig S4). We used elf18 for this assay as rcn1-1 is theWs ecotype background, which is a natural fls2 mutant (Zipfel et al, 2004). Finally, it is noteworthy that the flg22 hypersensitivity observed in pp2a-a1, pp2a-c4, pp2a-b’-n, and pp2a-b’-c’ mutant lines did not seem associated with constitutive immune responses, as suggested by the normal development of the plants when grown in soil and the absence of immune transcripts...
over-accumulation in un-elicited seedlings compared to WT (Supplementary Fig S5).

As BAK1 is also involved in BR signaling (Zhu et al, 2013; Liebrand et al, 2014), we tested whether the PP2A subunits identified in our study could also play a role in BR-triggered responses. Interestingly, the pp2a-a1 and pp2a-c4 mutants appeared slightly hypersensitive to exogenous BR treatment as measured by expression of the BR marker genes CPD and SAUR-AC1 (Supplementary Fig S6).

Specific PP2A subunits are part of a constitutive BAK1 complex

To test if the subunits identified by reverse-genetics are indeed part of the BAK1-associated PP2A holoenzyme, we assessed the impact of the corresponding mutations on BAK1-associated PP2A activity. We found that this activity in the pp2a-a1, pp2a-c4, and pp2a-b’-η mutants was similar to that in the null bak1-4 mutant (Fig 4A), indicating that the A1, C4, and B’-η subunits constitute the core of the PP2A holoenzyme associated with BAK1 in planta. The reduced BAK1-associated PP2A activity (Fig 4A) together with the increased flg22-induced ROS production and resistance to Pto DC3000 (Fig 3A and C) observed in the pp2a-b’-ζ mutant suggest that this subunit may also be part of the holoenzyme.

Importantly, in accordance with a specific association of PP2A with BAK1 (Fig 2C), we found that the pp2a-a1, pp2a-c4, and to a lesser extent pp2a-b’-η and pp2a-b’-ζ mutants were also hypersensitive to elf18, but not to chitin (Fig 4B) whose responsiveness is BAK1-independent (Shan et al, 2008; Ranf et al, 2011).

In Arabidopsis seedlings, PP2A subunit A1 (RCN1) participates in most of PP2A activity (Deruere et al, 1999). We therefore tested the interaction between BAK1 and RCN1 by co-immunoprecipitation before and after elicitation with elf18. Consistent with Figs 2C and 4A, BAK1 was detected in the RCN1 immunoprecipitate (using the rcn1-1/RCN1pro:RCN1-YFP transgenic line) independently of elf18 treatment (Fig 4C). Interestingly, we found that, while PP2A does not dissociate from BAK1 upon elicitation (Fig 4C and Supplementary Fig S7), BAK1-associated PP2A activity rapidly decreases by ~50% upon flg22 treatment (Supplementary Fig S7B). Altogether, these data suggest that PP2A is constitutively associated with BAK1 and that the holoenzyme activity is attenuated upon ligand binding to the receptor complex.

PP2A negatively controls BAK1 phosphorylation status

Lastly, we investigated the impact of the PP2A association on BAK1 accumulation, its ligand-induced complex formation with FLS2 and its activation. The pp2a-a1, pp2a-c4, pp2a-b’-η, and pp2a-b’-ζ mutants accumulated similar amount of BAK1 protein as WT (Figs 4A and 5A). Furthermore, co-immunoprecipitation experiments showed that flg22-induced complex formation with FLS2 is not affected in any of the pp2a mutants tested (Fig 5A).

We next analyzed BAK1 steady-state phosphorylation status, as BAK1 kinase activity is mainly controlled by its phosphorylation status (Oh et al, 2010). Interestingly, BAK1 kinase activity was increased by ~40% in the pp2a-c4 mutant line and conversely reduced by ~60% in the PP2A-C4 over-expressing line (Fig 5B). A similar enhanced BAK1 activity was also observed in the rcn1-1 line compared to the rcn1-1/RCN1pro:RCN1-YFP complemented line (Fig 5B). These results unveil a regulatory role of the C4 catalytic and A1 hook subunits in regulating BAK1 basal phosphorylation status.
Discussion

PAMP perception by LRR-containing PRRs leads to the rapid recruitment of the co-receptor BAK1 that acts as key mediator of immune signaling. Our findings suggest that PP2A negatively regulates the basal activity of BAK1 in the absence of stimulus, ultimately determining the intensity of the eventual PTI responses upon PAMP perception (Fig 6). In the absence of elicitation, a PP2A potentially composed of the subunits A1, B’γ/ζ, and C4 is associated with BAK1 and maintains low basal kinase activity. Upon ligand perception, PP2A most likely remains associated with BAK1 but its activity is rapidly attenuated, which ultimately allows increase in BAK1 kinase activity and consequent immune receptor activation. The primed immune responses observed in pp2a mutants could be due to increased phosphorylation upon elicitation of PRRs and/or PRR substrates, such as BIK1 and BSK1 (Lu et al., 2010; Zhang et al., 2010; Shi et al., 2013). Also, the enhanced BAK1 basal kinase activity in pp2a mutants may accelerate the release of BIR2 from BAK1, as this dissociation is BAK1 kinase activity-dependent (Halter et al., 2014), allowing a faster formation of an active BAK1-PRR immune complex upon low elicitation.

Interestingly, mutants in A1, C4 (which belongs to the subfamily II of catalytic subunits), or B’γ/ζ subunits did not show any signs of constitutive immune responses and rather exhibit increased responsiveness to flg22 and elf18 (Fig 3 and Supplementary Fig S5). This is consistent with the notion that the ‘primed’ BAK1 in these mutants...
still needs to form a ligand-induced complex with FLS2/EFR to activate downstream immune signaling. However, this is in contrast with the constitutive immune responses observed upon continuous cantharidin treatment (Fig 1), or when knocking-down the subclass I of catalytic subunits in Nicotiana benthamiana or PP2A-B’γ in Arabidopsis (He et al, 2004; Trotta et al, 2011), which suggests that other specific PP2A holoenzymes involving distinct subunits control additional steps of immune signaling. The composition of these heterocomplexes and their cellular target(s) remain to be determined.

The LRR-RKs BAK1 and the paralogous SERK proteins have emerged recently as key regulator of multiple pathways triggered by LRR-containing RKs and RLPs (Liebrand et al, 2014). Notably, BAK1 seems to exist in pre-formed complexes with ligand-binding receptors, which would explain the extremely rapid complex stabilization upon ligand binding (Schulze et al, 2010; Bucherl et al, 2013). Consistently, BAK1 was recently shown to act as a coreceptor for the LRR-RKs FLS2 and BRI1 (which is the BR receptor) forming direct interactions with both the ligand-bound receptors and the ligands (Santiago et al, 2013; Sun et al, 2013a,b). BAK1 is a constitutively highly active RD kinase capable of both auto- and trans-phosphorylation (Wang et al, 2008; Cheng et al, 2011; Schuessinger et al, 2011; Yan et al, 2012) and can spontaneously fold into an active kinase even in the absence of cellular context (Aan den Toorn et al, 2012). Thus, mechanisms that keep BAK1 activity under control must exist, although they are still poorly defined.

BRI1, another strong RD kinase, is negatively regulated by a combination of intramolecular inhibition, phosphorylation, and binding of inhibitory proteins such as BKI1 and PP2A (Wang et al, 2005, 2008; Wang & Chory, 2006; Jaillais et al, 2011; Oh et al, 2011, 2012; Wu et al, 2011). Recently, the C-terminal tail of BAK1 has been shown to regulate its activity both negatively and positively, and complex formation with BRI1 was proposed to relieve the inhibitory action of BAK1 C-terminal region (Oh et al, 2014).

Our work reveals PP2A as the first known inhibitory protein for BAK1. We show that PP2A constitutively associates with BAK1, but not with FLS2 or BIK1 in planta (Fig 2). The fact that the BAK1-associated PP2A activity is abrogated in insertional mutants of the subunits A1, C4, and B’γ (Fig 4) suggests that these proteins constitute the core of the PP2A holoenzyme that associates with BAK1. This is further substantiated by the observation that BAK1 basal phosphorylation status is increased in the pp2a-c4 and rcn1-1 mutants, while it is reduced in a transgenic line over-expressing the C4 subunit (Fig 5). Interestingly, the pp2a-a1, pp2a-c4, pp2a-b’γ, and pp2a-b’γ variants displayed a similar amount of BAK1 protein as WT (Figs 4A and 5A), which is in contrast to what has been observed previously with BRI1 whose degradation positively correlates with PP2A (Wu et al, 2011). Notably, the PP2A holoenzyme regulating BRI1 (whose exact composition is still unknown) does not affect BAK1 levels (Wu et al, 2011) further illustrating the specific roles played by distinct heteromeric PP2A enzymes.

The mechanisms by which PP2A negatively affects BAK1 phosphorylation status are, however, still unclear. An obvious possibility is that PP2A dephosphorylates important residues on BAK1. This hypotheses will be tested in future work, but the identification of in vivo BAK1 phosphosites playing roles in immunity is currently technically challenging due to the inhibitory impact of C-terminal immunological tags on BAK1 (Ntoukakis et al, 2011) and the poor protein coverage obtained by mass spectrometry after enrichment using native BAK1 antibodies (data not shown).

Interestingly, we observed that BAK1-associated PP2A activity is reduced by ~50% within 2 min after flg22 treatment (Supplementary Fig S7B). We postulate that this inhibition is required to enable full
immune signaling strength upon PAMP perception. PP2A activity can be regulated by post-translational modifications, such as phosphorylation, methylation or ubiquitination, ultimately affecting PP2A complex formation, stability or subcellular localization (Janssens et al., 2008; Virshup & Shenolikar, 2009). For example, BR perception leads to increased expression of the leucine carboxyl-methyltransferase SB11 that methylates PP2A-C subunits (Wu et al., 2011). This methylation shifts the pool of PP2A toward the plasma membrane potential leading to dephosphorylation and degradation of BR-activated BR1 (Wu et al., 2011). Whether BR1 is indeed a PP2A substrate remains to be determined.

We could not find any clear evidence for dissociation of the BAK1-RCN1 complex or for degradation of the A1 or C subunits upon PAMP treatment (Fig 4C, Supplementary Fig S7 and data not shown). It will be interesting in the future to decipher the exact mechanisms underlying the inhibition of PP2A activity in response to PAMP perception.

Of note, PP2A has been previously implicated in both the negative and positive regulation of BR signaling at two distinct levels (Di Rubbo et al., 2011). PP2A can dephosphorylate the BR-activated LRR-RK BR1 leading to its degradation (Wu et al., 2011). In addition, a PP2A enzyme potentially comprising the subunits A1, B’α, and B’β positively regulates BR-triggered responses by dephosphorylating the key transcriptional regulator BZR1, which enables its release from cytoplasmic 14-3-3 proteins and its transfer to the nucleus (Tang et al., 2011).

Interestingly, we found that the pp2a-a1 and pp2a-c4 mutants are slightly hypersensitive to exogenous BR treatment as measured by expression of BR marker genes (Supplementary Fig S6). However, we cannot completely exclude the possibility that the effect of the pp2a-a1 mutation may also be due to its previously described impact on the degradation of the ligand-activated BR receptor BR1 (Wu et al., 2011). Importantly, our data suggest enhanced BR responsiveness in pp2a mutants, ruling out that the enhanced PTI responses in these lines are a direct consequence of the antagonism between BR and PTI signaling (Albrecht et al., 2012; Belkhadir et al., 2012; Lozano-Duran et al., 2013; Fan et al., 2014; Malinovsky et al., 2014).

In summary, our work reveals an important regulatory mechanism that fine-tunes PRR complex activation during innate immunity and illustrates a novel function of PP2A in the regulation of receptor kinase-based pathways. Given the central role of BAK1 in multiple receptor kinase complexes involved in immunity and other cellular processes, our findings have broad implications to understand and engineer plant adaptation to environmental stresses. Moreover, this work further illustrates how distinct PP2A holoenzymes have evolved to regulate multiple cellular processes.

Materials and Methods

Plant materials and growth conditions

The fls2, bak1-4, and bik1 pbl1 mutants have been described previously (Zipfel et al., 2004; Chinchilla et al., 2007; Zhang et al., 2010). The pp2a mutant lines used in this study are described in Supplementary Fig S3. Primers used to genotype the pp2a-b mutants are listed in Supplementary Table S1.

Arabidopsis plants used for ROS production measurement and infection assays were grown in soil at 21°C with a 10-h photoperiod. For Arabidopsis sterile seedlings, seeds were surface-sterilized and germinated on plates containing Murashige-Skoog medium (including vitamins; Duchela) and 1% sucrose supplemented with 0.8% agar for the first 5 days at 22°C and with a 16-h photoperiod. Seedlings were then pricked out in liquid Murashige-Skoog medium supplemented with 1% sucrose.

Chemicals and elicitors

Elicitor peptides flg22 and elf18 were ordered from Peptron. Phosphatase inhibitor cantharidin was obtained from Enzo Life Sciences.

Measurement of ROS generation

Oxidative burst measurement was performed as described previously (Albrecht et al., 2012). ROS was elicited with cantharidin, flg22, or elf18, and mock elicitation was included in all experiments as

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negative control. Twelve leaf disks from 5-week-old plants were used for each condition. Luminescence was measured over time with a high-resolution photon counting system (HRPCS218; Photek).

RNA isolation and quantitative RT-PCR

Total RNA was prepared from 2-week-old seedlings grown in liquid medium. Total RNA was extracted using TRI reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were treated with Turbo DNA-free DNase (Ambion) and quantified with a NanoDrop spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized from 5 μg of RNA by using SuperScript RNA H-Reverse Transcriptase (Invitrogen) and an oligo(dT) primer, according to the manufacturer’s instructions. cDNA were amplified in triplicate by quantitative PCR by using SYBR Green JumpStart Taq ReadyMix (Sigma) and the PTC-200 Peltier Thermal Cycler (MJ Research). The relative expression values were determined by using U-box gene (At5g15400) as reference and the comparative Ct method \((2^{-\Delta\Delta Ct})\). Primers used for quantitative PCR are listed in Supplementary Table S2.

Induced resistance and susceptibility to bacteria

Induced resistance assays were realized as described previously (Albrecht et al., 2012). Briefly, water, flg22, or cantharidin were infiltrated with a needleless syringe into leaves of 5-week-old Arabidopsis plants. After 24 h, the same leaves were syringe-infiltrated with \(10^5\) cfu/ml of Pto DC3000. Bacterial growth was determined 2 days after inoculation by plating serial dilutions of leaf extracts on L agar medium supplemented with appropriate antibiotics. To test susceptibility to Pto DC3000, 5-week-old plants were sprayed with a suspension of Pto DC3000 \(10^8\) cfu/ml supplemented with 0.04% Silwett L-77 (Lehle seeds). Bacterial growth was determined 2 and 3 days after inoculation.

Protein extraction and immunoprecipitation

Protein extraction and immunoprecipitation using Arabidopsis seedlings were performed as described below (Kinase assay) and previously (Schwessinger et al., 2011).

Kinase assay

Two-week-old seedlings were treated with 1 μM flg22 or 1 μM elf18 and ground in liquid nitrogen. Proteins were extracted with 0.5 volume/weight of buffer [50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 10% glycerol; 1 mM EDTA; 5 mM DTT; 1% (vol/vol) protease inhibitor cocktail (Sigma); 1% (vol/vol) Nonidet P-40] and 50 nM calyculin A, 1 mM PMSF, 10 mM NaF, 5 mM Na2MoO4]. Samples were centrifuged 20 min at 4°C at 20,000 g. Supernatants were filtered and adjusted to 2–3 mg/ml protein; extracts were incubated with gentle agitation for 2 h at 4°C in the presence of 20 μl TrueBlot anti-rabbit Ig IP beads (eBioscience) and 15 μl anti-BAK1 antibodies. Beads were washed twice with washing buffer 1 (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 M NaCl, 1% Nonidet P-40) and once with washing buffer 2 (20 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1 M NaCl, 1% Nonidet P-40). Anti-BAK1 immunoprecipitates were washed once with kinase buffer (20 mM Tris–HCl, pH 7.5, 15 mM MgCl2, 5 mM EDTA, 1 mM DTT). Immunoprecipitates were finally incubated 30 min at 30°C and under vigorous shaking with 30 μl of kinase buffer supplemented with radioactive \(^{32}\)P-ATP (183 kBq; Perkin-Elmer). The reactions were stopped by addition of 10 μl of NuPAGE 4× LDS sample buffer (Invitrogen) in presence of 1× reducing agent and denatured for 10 min at 70°C. Proteins were separated by SDS/PAGE 10% and analyzed by Western blot by using rabbit polyclonal anti-BAK1 antibodies. The membranes were subjected to autoradiography by using a FLA5000 PhosphorImager (Fuji).

Figure 6. Model depicting the negative regulation of BAK1 activation by PP2A.
Phosphatase assay

PP2A phosphatase activity present in immunoprecipitates was measured using a non-radioactive molybdate dye-based phosphatase assay kit (Promega) according to the manufacturer’s instructions. The synthetic phosphopeptide, RRA[pT]VA, was used as the substrate. The reaction mixture (50 μl) contained PP2A buffer, 100 μM phosphopeptide substrate, and 3 μg/ml protein extract immunoprecipitated with anti-FLS2, anti-BAK1, or anti-HA antibodies. The reactions were incubated at 37°C for 1 h and stopped by adding 50 μl molybdate dye-additive. A standard curve for absorbance at 600 nm was prepared using 0, 2, 4, 10, 20, and 40 pmol inorganic phosphate solution. The phosphate released by the samples was then determined by extrapolating the absorbance at 600 nm against this standard curve.

Statistical analysis

All experiments were conducted in triplicate. Statistical significances based on one-way ANOVA analyses were determined with Prism 5.01 software (GraphPad).

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

CS and C2 designed research; CS, APM, and VN performed research; MS and jSS contributed new materials; CS, APM, and VN analyzed data; CS and C2 wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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