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

Cécile Segonzac[1,†], Alberto P Macho[1], Maite Sanmartín[2], Vardis Ntoukakis[1,§], José Juan Sánchez-Serrano[2] & Cyril Zipfel[1,*]

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'η/ζ 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.

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,

1 The Sainsbury Laboratory, Norwich Research Park, Norwich, UK
2 Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología, CSIC, Madrid, Spain
    *Corresponding author. Tel: +44 1603 450056; Fax: +44 1603 450011; E-mail: cyril.zipfel@tsl.ac.uk
    †Current address: Institute of Agriculture and Environment, Massey University, Palmerston North, New Zealand
    §Current address: School of Life Sciences, University of Warwick, Coventry, UK

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 steps. 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; MacKintosh *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'η/ζ 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
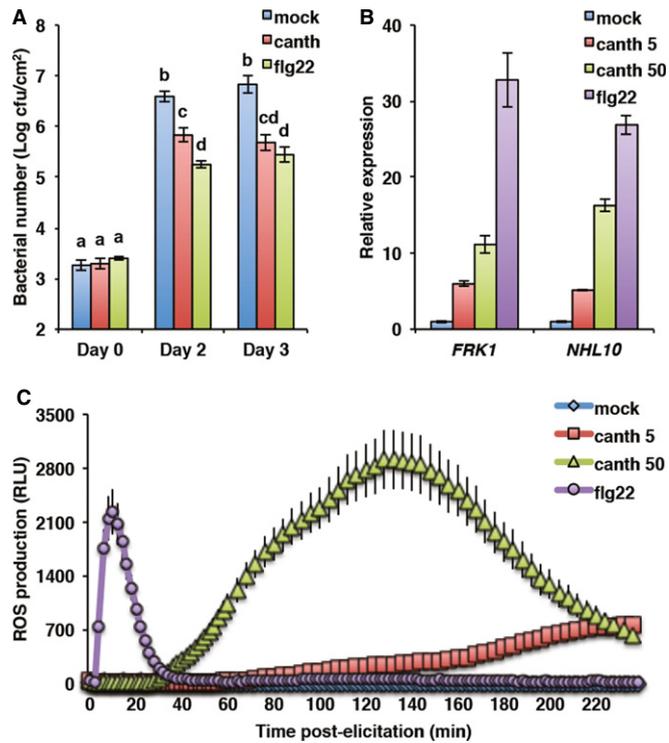
Figure 1. Treatment with the PP2A inhibitor cantharidin activates PAMP-triggered immunity.

A PP2A chemical inhibition protects *Arabidopsis* from bacterial infection. Wild-type Col-0 plants were pre-treated 24 h with mock, 1 μM flg22, or 50 μM cantharidin. Bacterial number was determined at the indicated time following *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 inoculation. Values presented are average of four biological repeats ± SE. Values labeled with different letters are statistically different as established by a one-way ANOVA ($P < 0.05$). cfu, colony-forming units.

B PP2A inhibits flg22-induced gene expression. Accumulation of marker gene transcripts *FRK1* (At2g19190) and *NHL10* (At2g35980) was assessed by qRT-PCR in Col-0 seedlings 1 h after treatment with mock, 100 nM flg22, 5 μM, or 50 μM cantharidin. Values are average of three biological repeats ± SE presented as fold induction compared with mock-treated samples.

C PP2A chemical inhibition triggers oxidative burst. ROS production was measured as relative luminescence units (RLU) in Col-0 in response to mock, 100 nM flg22, 5 μM, and 50 μM cantharidin. Values presented are average of three biological repeats ± SE.

cytoplasmic kinase BIK1, as measured by the mobility shift of BIK1 in a transgenic *BIK1_pro:BIK1-HA* line (Fig 2A). This observation does not necessary 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; Lin *et al*, 2014). Notably, we found that cantharidin-triggered ROS production was strongly reduced in the null *bak1-4* and *bik1 pbl1* mutants, while it was unaffected in *fls2* and completely absent in *rbohD* (Fig 2B). These data demonstrate that PP2A acts upstream or at the level of BAK1 and/or BIK1.

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-elicited 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 insertional 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'η*, and *pp2a-b'ζ* mutants by testing flg22-induced protection against bacterial infection. In this assay, *pp2a-a1*, *pp2a-c4*, and *pp2a-b'η* 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 to a lesser extent *pp2a-b'η* and *pp2a-b'ζ* were more resistant to spray-infection by *Pto* DC3000 (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 the Ws 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'η*, and *pp2a-b'ζ* 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
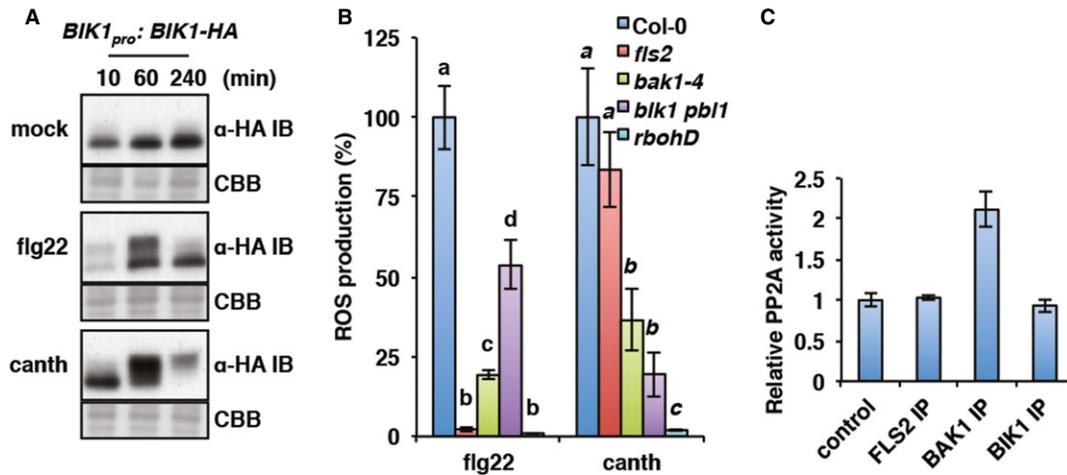
Figure 2. PP2A associates with BAK1 *in planta.*

A   PP2A chemical inhibition triggers BIK1 activation. BIK1 phosphorylation status was detected by anti-HA immunoblot in *BIK1pro:BIK1-HA* seedlings treated with mock, 100 nM flg22, or 50 μM cantharidin for the indicated time. Coomassie Brilliant Blue staining of the membrane (CBB) is shown to assess equal protein loading.

B   Cantharidin-triggered oxidative burst is impaired in mutants of PRR complex components. ROS production in response to 100 nM flg22 or 50 μM cantharidin was measured in Col-0, *fls2*, *bak1-4*, *bik1 pbl1*, and *rbohD*. Values are average of three biological repeats ± SE presented as percentage of Col-0 response. Values labeled with different letters (regular and italic for flg22 and cantharidin treatment respectively) are statistically different as established by a one-way ANOVA ($P < 0.05$).

C   PP2A activity is constitutively associated with BAK1. PP2A activity in un-elicited *BIK1pro:BIK1-HA* seedlings was measured by colorimetry on protein extracts enriched with anti-FLS2, anti-BAK1, or anti-HA antibodies. PP2A activity relative to background detected in non-enriched protein extracts in absence of antibodies (control) is presented as average of three biological repeats ± SE.

Source data are available online for this figure.

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.
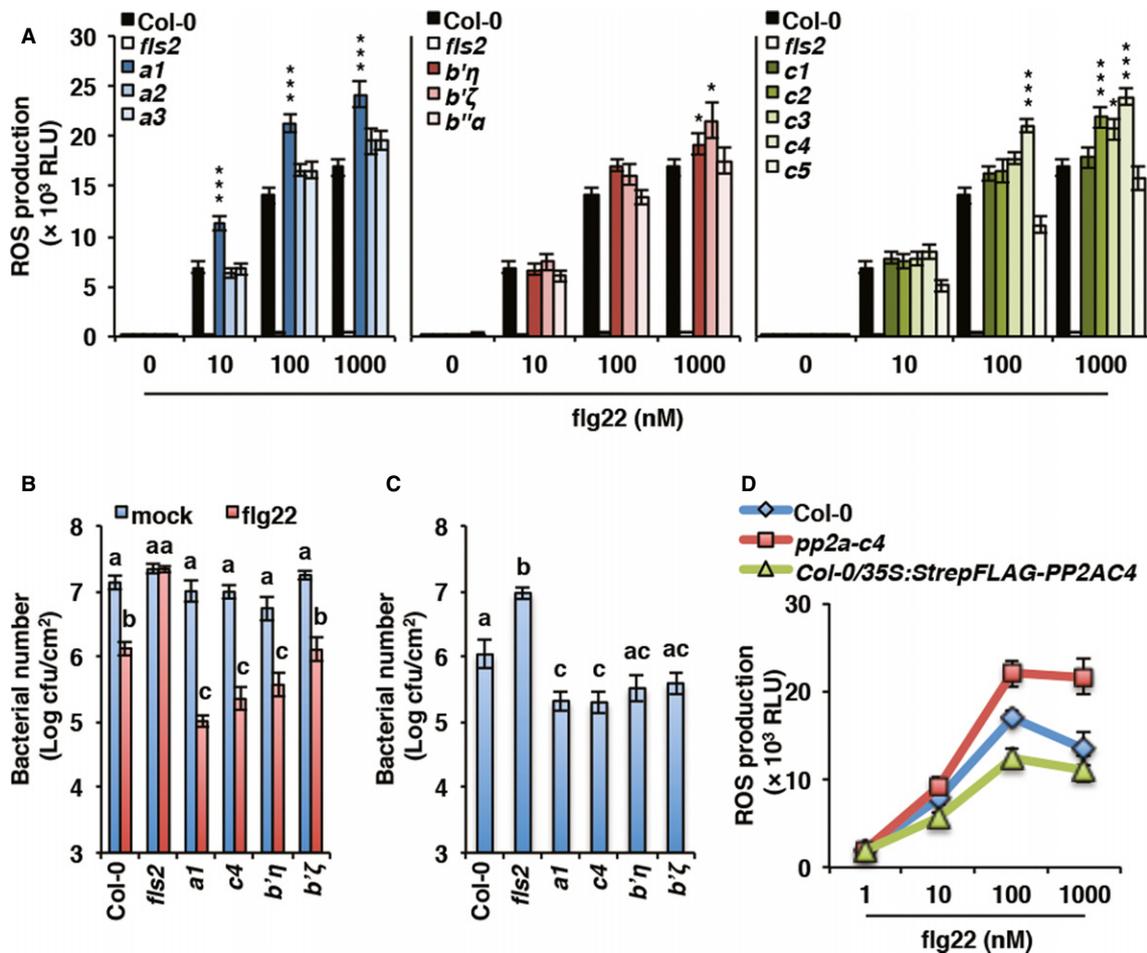
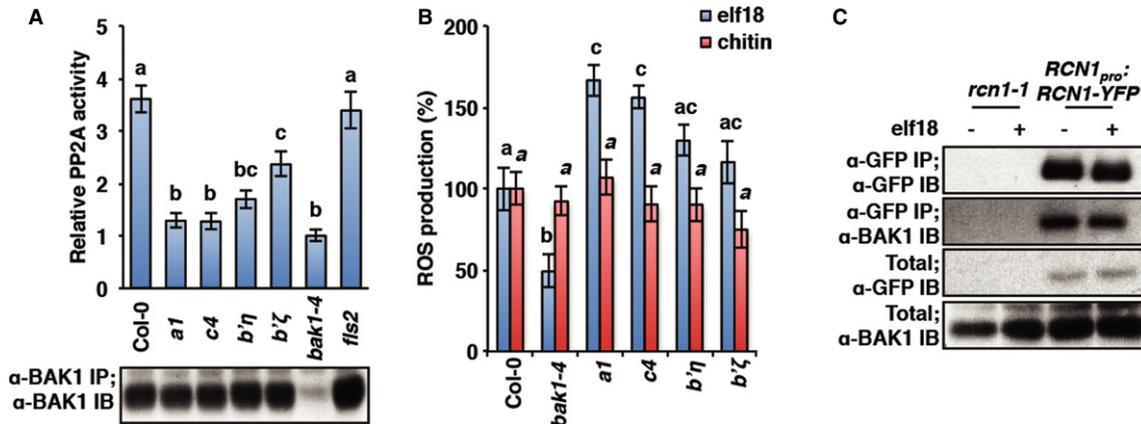**Figure 3. The PP2A subunits A1, B'η, B'ζ, and C4 regulate PTI signaling.**

A   Specific PP2A subunits regulate flg22-triggered oxidative burst. ROS production in response to increasing flg22 concentration was measured as relative luminescence units (RLU) in *pp2a* mutant lines compared to wild-type Col-0. Values presented are average of three biological repeats ± SE. Values labeled with asterisk are statistically different from Col-0 as established by one-way ANOVA (*$P < 0.01$ and ***$P < 0.001$).

B   Specific PP2A subunits control flg22-mediated restriction of bacterial growth. Induced resistance to *Pto* DC3000 was determined 2 days post-infection in *pp2a* mutant lines compared to Col-0 following 24 h mock or 100 nM flg22 treatment. Values presented are average of four biological repeats ± SE. Values labeled with different letters are statistically different as established by a one-way ANOVA ($P < 0.01$). cfu, colony-forming units.

C   Specific PP2A subunits control *Arabidopsis* susceptibility to bacterial infection. Susceptibility to *Pto* DC3000 was determined 2 days after spray-inoculation in *pp2a* mutant lines compared to Col-0. Values are presented as average of four biological repeats ± SE. Values labeled with different letters are statistically different as established by a one-way ANOVA ($P < 0.01$). cfu, colony-forming units.

D   Over-expression of *PP2A-C4* inhibits PAMP-triggered ROS burst. ROS production in response to increasing flg22 concentration was measured as relative luminescence units (RLU) in 5-week-old *pp2a-c4* mutant and $35S_{pro}$:*StrepFlag-PP2AC4* lines compared to Col-0. Values presented are average of three biological repeats ± SE.

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'η/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'η/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

Figure 4. The PP2A subunits A1, B'η, B'ζ, and C4 are part of a constitutive BAK1 complex.

A BAK1-associated PP2A activity is impaired in *pp2a* mutants. PP2A activity (top) was detected in Col-0 and *pp2a* mutant protein extracts enriched with anti-BAK1 antibodies. PP2A activity is presented relative to the activity detected in *bak1-4* protein extract as average of three biological repeats ± SE. Values labeled with different letters are statistically different as established by one-way ANOVA ($P < 0.01$). Equal amount of immunoprecipitated BAK1 (bottom) was assessed by immunoblot in the same samples.

B Specific PP2A subunits regulate BAK1-dependent PTI. ROS production in response to 100 nM elf18 or 1 mg/ml chitin was measured in *pp2a* mutant lines. Values presented are average of three biological repeats ± SE. Values labeled with different letters (regular and italic for elf18 and chitin treatment respectively) are statistically different as established by a one-way ANOVA ($P < 0.01$).

C PP2A-A1 (RCN1) subunit constitutively interacts with BAK1. PP2A/BAK1 interaction was detected by co-immunoprecipitation in *rcn1-1* or *rcn1-1/RCN1_{pro}:RCN1-YFP* protein extracts enriched on GFP-Trap beads. Seedlings were treated with water (−) or 100 nM elf18 (+) for 5 min.

Source data are available online for this figure.

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 subfamily 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 co-receptor 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; Schwessinger *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'η (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'η*, and *pp2a-b'ζ* mutants 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
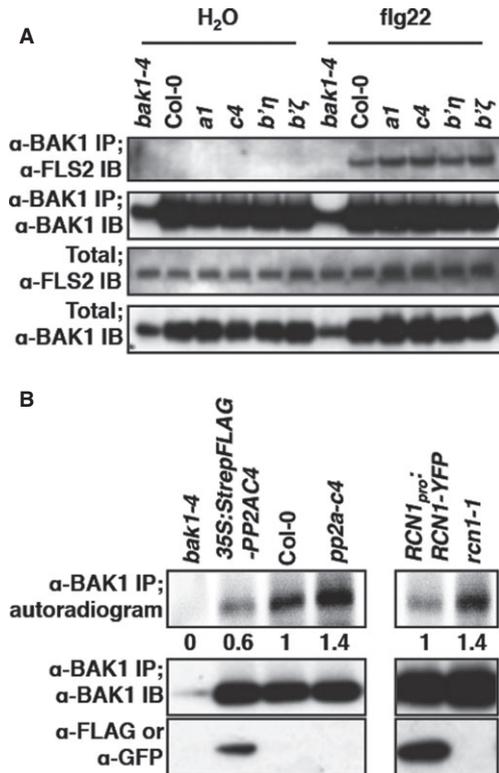
Figure 5. PP2A controls BAK1 activation *in planta*.

A   *pp2a* mutants do not exhibit constitutive ligand-independent FLS2/BAK1 heteromerization. Ligand-dependent FLS2/BAK1 heteromerization was detected by co-immunoprecipitation and immunoblotting on *pp2a* mutant protein extracts enriched with anti-BAK1 antibodies, following 5 min of treatment with water or 100 nM flg22.

B   BAK1 is hyper-activated in *pp2a* mutants. BAK1 kinase activity (top) was detected by incorporation of ^{32}P on protein extracts enriched with anti-BAK1 antibodies. BAK1 activity is shown as relative band intensity below the autoradiogram. Equal amount of immunoprecipitated BAK1 (middle) and presence of tagged proteins (bottom) was assessed by immunoblot in the same samples.

Source data are available online for this figure.

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 carboxymethyltransferase SBI1 that methylates PP2A-C subunits (Wu *et al*, 2011). This methylation shifts the pool of PP2A toward the plasma membrane potentially leading to dephosphorylation and degradation of BR-activated BRI1 (Wu *et al*, 2011). Whether BRI1 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 BRI1 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 BRI1 (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; Duchefa) 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
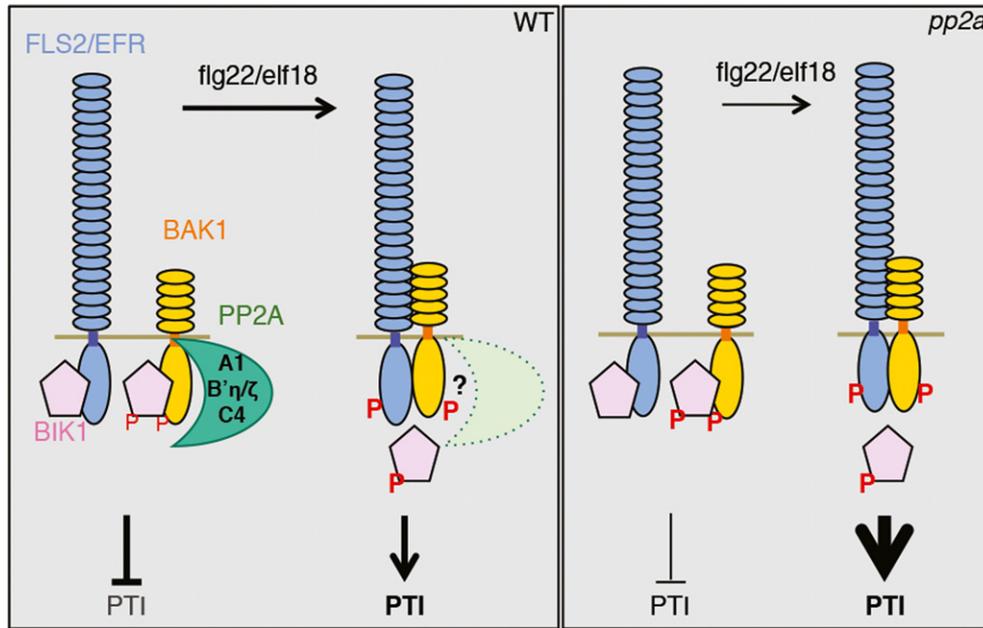
Figure 6.   Model depicting the negative regulation of BAK1 activation by PP2A.

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 ($2^{-\Delta\Delta C_t}$). 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, 2.5 mM Na_3VO_4, 50 nM calyculin A, 1 mM PMSF, 10 mM NaF, 5 mM Na_2MoO_4]. 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, 100 mM 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 $MgCl_2$, 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 $[^{32}P]\gamma$-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).

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 mg/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

Author contributions

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

Conflict of interest

The authors declare that they have no conflict of interest.

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