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The calcium-dependent protein kinase CPK28 negatively regulates the BIK1-mediated PAMP-induced calcium burst

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Plants are protected from microbial infection by a robust immune system. Two of the earliest responses mediated by surface-localized immune receptors include an increase in cytosolic calcium (Ca^{2+}) and a burst of apoplastic reactive oxygen species (ROS). The Arabidopsis plasma membrane-associated cytoplasmic kinase BIK1 is an immediate convergent substrate of multiple surface-localized immune receptors that is genetically required for the PAMP-induced Ca^{2+} burst and directly regulates ROS production catalyzed by the NADPH oxidase RBOHD. We recently demonstrated that Arabidopsis plants maintain an optimal level of BIK1 through a process of continuous degradation regulated by the Ca^{2+} -dependent protein kinase CPK28. *cpk28* mutants accumulate more BIK1 protein and display enhanced immune signaling, while plants over-expressing CPK28 accumulate less BIK1 protein and display impaired immune signaling. Here, we show that CPK28 additionally contributes to the PAMP-induced Ca^{2+} burst, supporting its role as a negative regulator of BIK1.

Plants perceive pathogen- and damage-associated molecular patterns (PAMPs or DAMPs) through pattern recognition receptors (PRRs) located at the cell surface. All known plant PRRs are plasma membrane-resident receptor kinases or receptor-like proteins that bind ligands outside the cell and transduce the signal inside the cell by forming larger complexes with several additional proteins.¹ One of the earliest responses following PAMP/DAMP perception is an increase in cytosolic calcium (Ca^{2+}), shortly followed by a burst of apoplastic reactive oxygen species (ROS) mediated in Arabidopsis by the NADPH oxidase RBOHD.¹ Downstream immune signal transduction is largely achieved by phosphorylation relays mediated by different subclasses of kinases, including mitogen-activated and Ca^{2+} -dependent protein kinases (MAPKs and CDPKs), leading to transcriptional reprogramming.² Together, these responses constitute PAMP-triggered immunity (PTI), which is thought to be sufficient to protect plants against most microbes.³

The plasma membrane-associated cytoplasmic kinase BIK1 is an immediate convergent substrate of several different PRRs, including FLS2 (which binds bacterial flagellin), EFR (which binds bacterial elongation factor Tu), PEPR1 (which binds

endogenous AtPep peptides), and CERK1 (which binds fungal chitin), and is a key component of the plant immune system.⁴⁻⁸ Moreover, BIK1 is also a substrate of BAK1, an important co-receptor kinase that interacts with and phosphorylates several immune receptors and is required to achieve their full signaling potential.⁹⁻¹⁴ Importantly, plants lacking functional BIK1 and related proteins such as PBL1 (*bik1 pbl1* mutants) are strongly impaired in PTI signaling and are more susceptible to bacterial and fungal pathogens.^{6,8,15,16}

Recent work demonstrated that BIK1 interacts with and phosphorylates the NADPH oxidase RBOHD to enable PAMP-triggered ROS production and consequent stomatal immunity.^{17,18} Although BIK1 phosphorylates RBOHD in a Ca^{2+} -independent manner,^{17,18} signaling through BIK1 is required for an appropriate PAMP-induced cytosolic Ca^{2+} burst.^{18,19} Fluxes in Ca^{2+} can be monitored in living cells using aequorin, a bioluminescent Ca^{2+} -binding protein from jellyfish. Aequorin is a holoprotein composed of the apoenzyme apoaequorin and the luciferin coelenterazine. Thus, if supplied with exogenous coelenterazine, active aequorin can be reconstituted in plant cells transgenically expressing apoaequorin.²⁰ We were interested to test if BIK1 and

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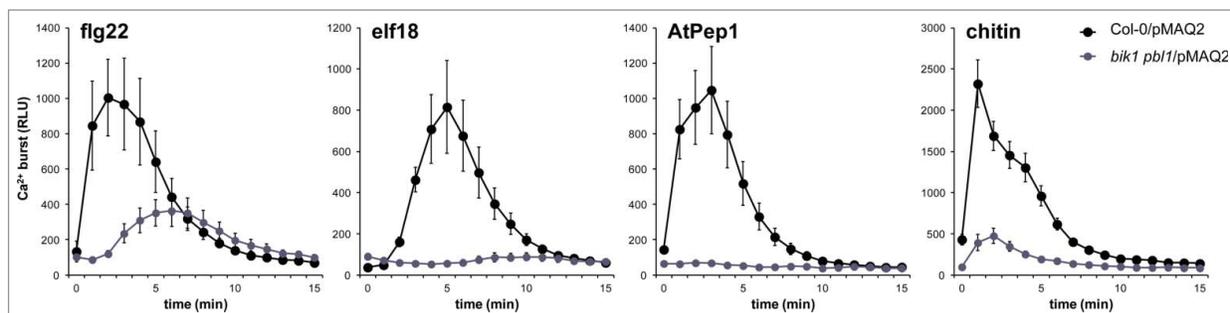


Figure 1. The PAMP-triggered Ca^{2+} burst is altered in *bik1 pbl1* mutants. To normalize expression of the apoaequorin transgene, *bik1 pbl1/pMAQ2* was generated by crossing. Leaf discs were harvested from individual 4-week old plants grown on soil in short-day chambers as described previously²⁹ and were floated in 12.5 μM coelenterazine (LUX Innovate) overnight in the dark prior to treatment with 100 nM flg22 (EZBiolab), 100 nM elf18 (EZBiolab), 1 μM AtPep1 (EZBiolab), or 100 $\mu\text{g/mL}$ chitin (Sigma Aldrich). The induced cytosolic Ca^{2+} burst was measured as relative light units (RLU) using a charge-coupled device camera (Photek Ltd.) as described previously.¹⁷ Values are means \pm standard deviation ($n=8$). Experiments were performed at least 3 times with similar results.

PBL1 are required for the Ca^{2+} burst activated through receptors other than FLS2. We therefore crossed *bik1 pbl1* to a wild-type line expressing cytosolic apoaequorin under the control of the cauliflower mosaic virus 35S promoter (*Col-0/pMAQ2*),²¹ and examined the Ca^{2+} burst in homozygous *bik1 pbl1/pMAQ2* plants after treatment with several elicitors. In agreement with what was previously shown for flg22 treatment in *bik1* mutants,¹⁸ we found that the Ca^{2+} burst was severely impaired in *bik1 pbl1* mutants after treatment with flg22, elf18, AtPep1, or chitin (Fig. 1). During the preparation of this manuscript, another study also reported that *bik1 pbl1* mutants are compromised in the flg22-, elf18-, and AtPep1-triggered Ca^{2+} burst.¹⁹ These results are in support of BIK1 and related proteins functioning as key signaling integrators immediately downstream of multiple PRRs.

Although very little is known about the molecular mechanisms underlying the PAMP/DAMP-induced Ca^{2+} burst, it is clear that changes in intracellular $[\text{Ca}^{2+}]$ are prerequisite to many downstream immune responses.²² Ca^{2+} signals are thought to be decoded into cellular responses by Ca^{2+} -binding proteins such as calmodulins, calcineurin B-like proteins, and/or CDPKs.²² Several Ca^{2+} -binding proteins including CDPKs are involved in biotic and abiotic stress responses.²³ For example, western blot and in-gel kinase assays demonstrated that PAMP perception results in phosphorylation and activation of several CDPKs in Arabidopsis.²⁴ In particular, it was recently shown that PAMP treatment results in hyper-phosphorylation and activation of CPK5 and CPK6,²⁵ which are involved in PTI signaling.²⁴ Notably, in addition to the critical Ca^{2+} -independent regulation by BIK1,^{17,18} the NADPH oxidase RBOHD is an in vitro and in vivo phosphorylation target of CPK5 and related Ca^{2+} -dependent protein kinases CPK4, CPK6, and CPK11.^{17,25} Furthermore, RBOHD itself contains 2 cytoplasmic Ca^{2+} -binding EF-hands²⁶ and PAMP-triggered ROS production is Ca^{2+} -dependent,^{17,27,28} indicating a very tight interplay between ROS and Ca^{2+} signaling during PTI.

Adding to the complexity of Ca^{2+} -dependent and Ca^{2+} -independent mechanisms underlying immune responses, we recently

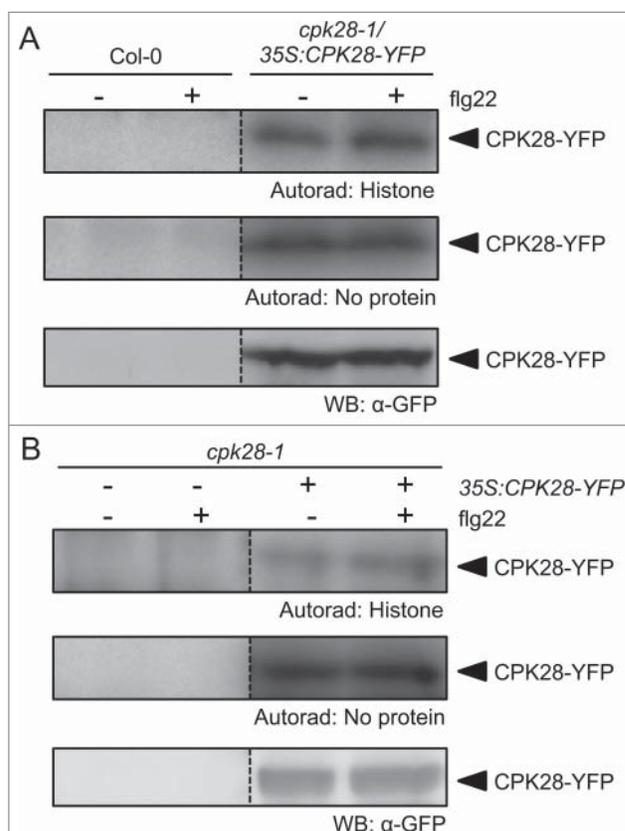


Figure 2. CPK28 is active before and after PAMP treatment. **(A)** Wild-type *Col-0* or functionally complemented *cpk28-1/35S:CPK28-YFP* seedlings were grown in liquid MS media for 2 weeks prior to treatment with water or 100 nM flg22 for 10 minutes as described previously.²⁹ **(B)** Protoplasts were prepared from 5-week-old soil-grown *cpk28-1* plants and transfected with *35S:CPK28-YFP* as described previously.²⁵ Protoplasts were treated 14 hours after transfection with water or 100 nM flg22 for 10 minutes. Total protein extracts were separated by SDS-PAGE and subject to histone in-gel kinase assays (autoradiography), as described previously,²⁵ or western blot (WB: α -GFP), as described previously.³⁰ Results are presented from the same blots; some lanes were removed for clarity as indicated by dashed lines. These assays were repeated at least 3 times with similar results.

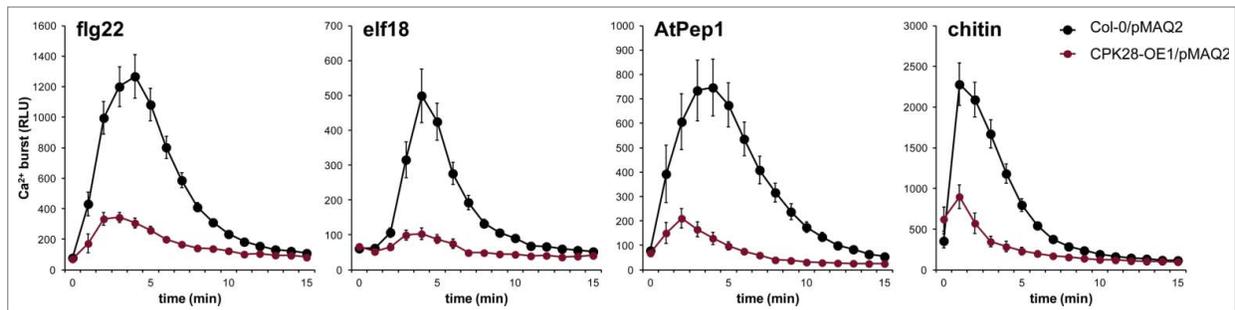


Figure 3. The PAMP-triggered Ca^{2+} burst is altered by *CPK28* over-expression. The PAMP-triggered Ca^{2+} burst in Col-0/pMAQ2 compared to CPK28-OE1/ Experiments were conducted as described in **Figure 1** and were performed at least 3 times with similar results. Values are means \pm standard deviation ($n = 8$).

demonstrated that the Ca^{2+} -dependent protein kinase CPK28 regulates BIK1 turnover to buffer immune signaling.²⁹ Plants over-expressing *CPK28* (CPK28-OE1) accumulate lower levels of BIK1 protein compared to wild-type plants and are strongly impaired in PAMP-triggered responses and anti-bacterial immunity.²⁹ Biochemical characterization demonstrated that CPK28 kinase activity is Ca^{2+} -dependent.³⁰ However, the effect on BIK1 turnover occurs in both the presence and absence of pathogen elicitors,²⁹ suggesting that CPK28 is active even in the absence of PAMP/DAMP-induced Ca^{2+} fluxes. To test if CPK28 activity is altered by PAMP treatment, we extracted total protein from wild-type and functionally complemented *cpk28-1/35S:CPK28-YFP* plants treated with water or flg22 and conducted in-gel kinase assays using radioactive phosphate (**Fig. 2A**). We additionally transfected protoplasts harvested from *cpk28-1* plants with *35S:CPK28-YFP* and similarly conducted kinase assays treated with or without flg22 (**Fig. 2B**). In both seedlings and protoplasts, CPK28 showed constitutive (auto-) phosphorylating activity in untreated and treated samples. This is in contrast to CPK5 and CPK6, which become strongly phosphorylated after PAMP treatment and thus activated.²⁵ While more accurate methods are required to detail and quantify site-specific phosphorylation changes following PAMP treatment, these results suggest that basal cellular levels of Ca^{2+} are already sufficient for CPK28 activation *in vivo*. Thus, CPK28 activity may not be altered in response to PAMP perception, or

additional mechanisms may be involved beyond PAMP-induced Ca^{2+} -binding.

Our previous work demonstrated that CPK28 negatively regulates BIK1 and is therefore genetically upstream of the PAMP-triggered Ca^{2+} burst. This led us to hypothesize that the PAMP-induced Ca^{2+} burst would be impaired in plants overexpressing *CPK28*. To test this, we crossed CPK28-OE1 with Col-0/pMAQ2 and found that the cytosolic Ca^{2+} burst was indeed strongly reduced in homozygous CPK28-OE1/pMAQ2 plants after treatment with flg22, elf18, AtPep1, or chitin (**Fig. 3**), similar to what we observed in *bik1 pbl1* plants (**Fig. 1**). These results suggest that CPK28 resides in an activated state prior to the PAMP-induced Ca^{2+} burst and further support its role as a negative regulator of BIK1.

In accordance with CPK28 regulating BIK1 turnover, loss-of-function *cpk28-1* mutants accumulate higher levels of BIK1 protein compared to wild-type plants and display strongly enhanced PAMP-triggered responses.²⁹ To test the effect of enhanced accumulation of BIK1 on the PAMP-triggered Ca^{2+} burst, we generated a homozygous *cpk28-1/pMAQ2* line through crossing. We found that treatment with flg22, elf18, AtPep1, or chitin resulted in an enhanced cytosolic Ca^{2+} burst in *cpk28-1/pMAQ2* compared to the wild-type (**Fig. 4**). This supports our previous findings that increased BIK1 accumulation results in increased PAMP-triggered signaling including ROS production.²⁹

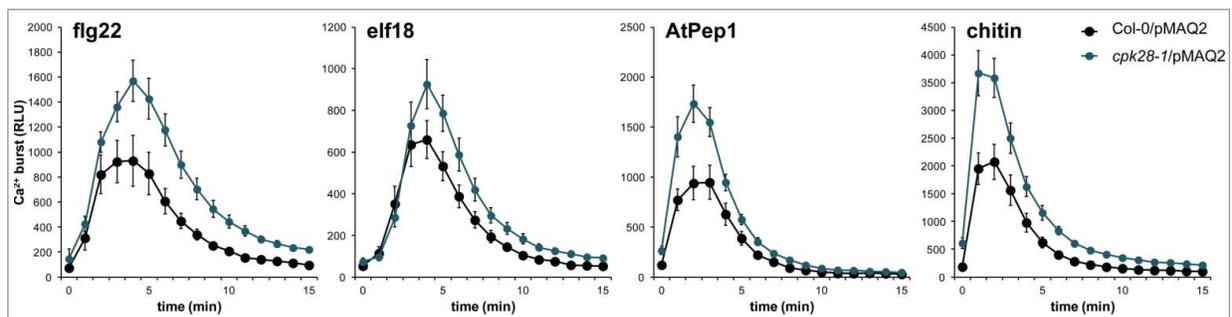


Figure 4. The PAMP-triggered Ca^{2+} burst is altered in *cpk28-1*. Experiments were conducted as described in **Figure 1** and were performed at least 3 times with similar results. Values are means \pm standard deviation ($n=8$).

Secondary messengers such as Ca^{2+} and ROS are rapidly and transiently induced following PAMP perception and contribute to plant immune signaling. Here we show that CPK28 is genetically upstream of the PAMP-induced Ca^{2+} burst, supporting our previous findings that CPK28 is a negative regulator of BIK1. Several immune receptors rely on the signaling integrator BIK1 to relay microbial perception at the cell surface to intracellular responses.^{5,6,8} While BIK1 is positively regulated by phosphorylation mediated by PRRs,⁵⁻⁸ our previous work suggests that BIK1 is also negatively regulated by phosphorylation mediated by CPK28.²⁹ This raises the intriguing possibility that residue-specific or quantitative differences contribute to the 2 states. We propose that CPK28-mediated phosphorylation of BIK1 influences its ubiquitination by a currently unknown E3 ligase, resulting in its degradation. Identification of ubiquitination and CPK28-mediated phosphorylation sites on BIK1 will allow us to directly test this hypothesis. Moreover, future work to unravel the molecular mechanisms underlying the PAMP-induced Ca^{2+} burst and subsequent signaling events will enhance our understanding of this important branch of the plant immune system.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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