Phosphatidic Acid Regulates BZR1 Activity and Brassinosteroid Signal of *Arabidopsis*

Dear Editor,

Brassinosteroid (BR) is an important hormone and plays crucial roles in plant growth and development (Kim and Wang, 2010). Genetics studies identify many components involving in BR signaling, including transcript factor BZR1 (BRASSINAZOLE RESISTANT 1). BZR1 is dephosphorylated (He et al., 2005) to regulate expression of target genes. A single amino acid mutation in BZR1 PEST domain results in enhanced binding and dephosphorylation by PP2A (PROTEIN PHOSPHATASE 2A; Tang et al., 2011), leading to constitutively activated BZR1 and enhanced BR signal in gain-of-function mutant *bzr1-1D*. Although BR signal is well characterized in *Arabidopsis*, how the components of BR signaling transduction pathway are regulated needs further illustrations.

Recent studies showed signaling molecule phosphatidic acid (PA), which is mainly produced by Phospholipase D (PLD) and Diacylglycerol (DAG) kinase (DGK) (Wang et al., 2006), plays key roles in plant responses to environmental stimulus and vesicular trafficking regulation (Testerink and Munnik, 2011). PA exerts functions by altering membrane structure or binding target proteins, and interaction with PA affects the activity or subcellular location of PA-binding proteins (Zhang et al., 2004). PLD-derived PA increases membrane PP2A activity while it decreases total PP2A activity through binding PP2AA1 subunit (Gao et al., 2013).

Binding of PA-PP2AA1 subunit results in decreased PP2A activity in cytoplasm; we thus investigate whether PA regulates BZR1 activity using transgenic line, W2C, which harbors the pBZR1::BZR1::CFP construct. Western blot analysis showed the ratio of dephosphorylated BZR1 was decreased after PA treatment (Figure 1A, BR treatment as positive control), indicating decreased BZR1 activity. qRT–PCR analysis of BZR1 activity marker genes *CPD* (*CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARF*) and *DWF4* (*DWARF4*) further confirmed the reduced BZR1 activity (Supplemental Figure 1A). Photographic and statistical analysis of hypocotyls growth in dark showed BR treatment resulted in curved and shortened hypocotyls of wild-type and *bzr1-1D* seedlings (Figure 1B, left); however, co-treatment of BR and PA did not lead to significant difference of wild-type, while PA treatment could inhibit the enhanced BR response of *bzr1-1D* (Figure 1B, left), suggesting PA negatively regulated BR response of *bzr1-1D*. Interestingly, the hypocotyl elongation of *bzr1-1D* and wild-type displayed a shift under different treatments. *bzr1-1D* presented shorter hypocotyls under BR treatment; however, the suppression of *bzr1-1D* hypocotyl elongation was less than that of wild-type under treatment with PA and BR (Supplemental Figure 1B), demonstrating that PA inhibition of BR response was more severe in *bzr1-1D* than wild-type.

PLD-derived PA binds PP2AA1 subunit (Gao et al., 2013), suggesting BZR1 activity may be affected by PLD-derived PA. Considering multiple isoforms of *PLD*, specific inhibitor 1-butanol was applied to study PA effect. 1-butanol treatment (0.6% or 0.8%, v/v) resulted in the higher ratio of dephosphorylated BZR1, while 2-butanol (0.8%, v/v, as control) had no significant effects (Figure 1A). Being consistent, RT–PCR analysis confirmed decreased transcription of *CPD* and *DWF4* under 1-butanol treatment, revealing increased BZR1 activity (Supplemental Figure 1C). Further physiological analysis showed 1-butanol reduced hypocotyl elongation and *bzr1-1D* presented shorter hypocotyls than wild-type (Figure 1B, middle), indicating *bzr1-1D* was more sensitive to 1-butanol. Under co-treatment of BR and 1-butanol, the inhibited hypocotyl growth was intensified (Figure 1B, middle), indicating 1-butanol treatment induced BR signaling.

Propranolol (PPL) inhibits the activity of phosphatidate phosphohydrolase enzyme, and PPL treatment results in activated PLD and accumulated PA of animal cell. In plant, PPL treatment induces tobacco pollen tube elongation, which is consistent as the PA effect. Analysis by applying PPL showed PPL treatment obviously reduced BZR1 activity by increasing phosphorylated BZR1 (Figure 1A) and increased transcription of BZR1 suppressing genes *CPD* and *DWF4* (Supplemental Figure 1D). PPL treatment also suppressed hypocotyl elongation of wild-type and *bzr1-1D* to a similar degree, while supplementation with BR led to shortened and curved hypocotyls in *bzr1-1D* but not wild-type (Figure 1B, right), demonstrating PPL might counteract BR response by accumulating PA.

PP2AA1 is a PA-binding protein (Testerink et al., 2004), as well as a member of the scaffold subunit of PP2A phosphatase. PP2AB subunit can bind to BZR1 PEST domain and dephosphorylates BZR1 to activate BR signaling (Tang et al., 2011). We investigated whether and how PP2AA1 subunit participated in BR signaling using PP2AA1 loss-of-function mutant *pp2aa1* (also known as *rcn1*). qRT–PCR analysis showed increased

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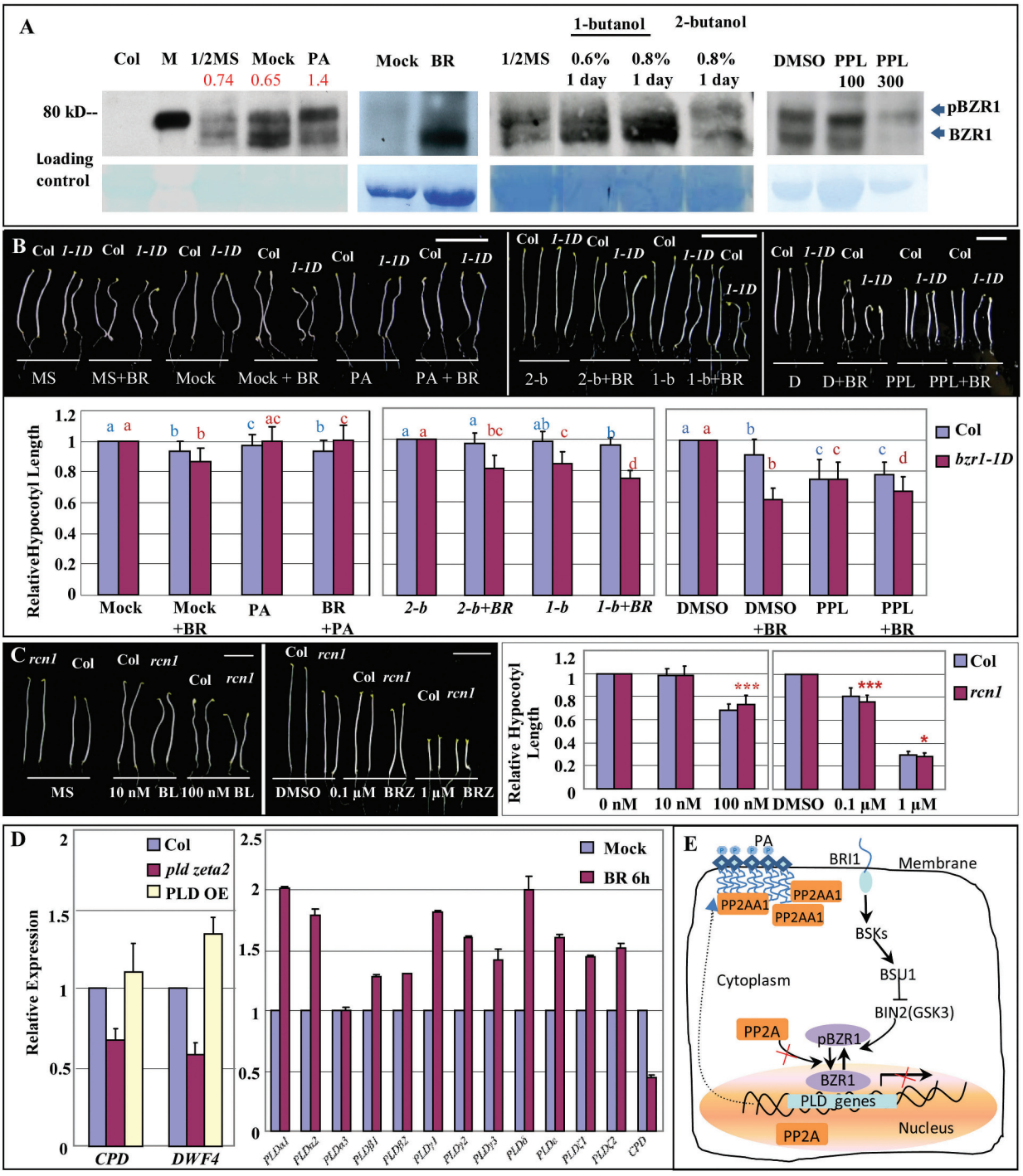

Figure 1. Phosphatidic Acid Regulates AtBZR1 Activity and Brassinosteroid Signal in a PP2A-mediated Way.

(A) Western-Blot analysis detected the ratio of dephosphorylation/phosphorylation BZR1 (BZR1/pBZR1) after PA treatment (left, number in red color calculated by measuring with image pro plu), BR treatment (positive control), 1-butanol treatment (middle), and PPL treatment (right, negative control labeled as DMSO). Rubisco bands in bottom panel show the equal loading. **(B)** BR response of Col and *bzr1-1D* (labeled as *1-1D*) after PA treatment (left), 1-butanol treatment (middle, labeled 1-b), and Propanolol treatment (labeled PPL). Bar = 1 cm. Hypocotyl lengths were measured and relative length were shown as average ± SD (n = 60). Statistical analysis were performed by a one-way AVONA module and Turkey multiple comparison methods in Statsitical Product and Service Solutions (SPSS). **(C)** BR and BRZ responses of Col and *rcn1*. Bar = 1 cm. Hypocotyl lengths were measured and relative length is shown as average ± SD (right, n = 40). Statistical analysis were performed by using t-test analysis (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(D)** qRT-PCR analysis of the expression of BZR1 target genes in *pldζ2* mutant and PLDζ2 OE transgenic plant (left). qRT-PCR analysis of *PLD* genes after BR treatment (right). Means were calculated from three biological samples, and each biological sample was examined in triplicate. Error bars indicate SD. **(E)** Hypothetical model of PA effects in regulation of BR signal.

CPD and *DWF4* expressions in *rcn1* (Supplemental Figure 1E), indicating BZR1 activity reduced in *rcn1*. Phenotypic analysis showed *rcn1* seedlings were less sensitive to BR and more sensitive to BRZ (Figure 1C), confirming decreased BR signal of *rcn1*. These results indicated that PP2AA1 subunit positively regulated BZR1 activity and BR signal.

The transcription level of *CPD* and *DWF4* showed increased BZR1 activity in *pldζ2* mutant; and *DWF4* expression also showed decreased BZR1 activity in PLDζ2-OE transgenic line (Figure 1D, left), confirming PLD-derived PA level regulated BZR1 activity. We did not consider DGK-derived PA regulates BZR1 activity and BR signal because R59022 (the specific inhibitor of DGK) treatment did not alert BZR1 dephosphorylation status (Supplemental Figure 2A), although DGK also affected PA level and R59022 influenced *CPD*/*DWF4* expressions (Supplemental Figure 2B). In addition, 12 *PLDs* and 7 *DGKs* expressions (Figure 1D, right, and Supplemental Figure 2C) were induced by BR treatment (except for PLDα3), suggesting BR might stimulate the cellular PA level.

Our study provided evidence that PA decreased BZR1 dephosphorylation to inhibit BZR1 activity and BR signal/response by regulating PP2A activity. PP2A was reported to dephosphorylate BRI1 in membrane to suppress BR signal (Wu et al., 2011), but to dephosphorylate BZR1 in cytoplasm and activate BR signal (Tang et al., 2011). Our results were closer to the latter one, which might due to the same ecotype.

In sum, we hypothesize PP2A dephosphorylates BZR1 and actives BR signal in cytoplasm; when PA level enhances after stimulation of unknown signals (environmental stimuli or hormones), PP2AA1 subunit is bound and recruited to membrane by PA and reduced cytoplasmic PP2A activity decreases BZR1 activity and BR signal. *PLDs* are direct targets of BZR1 (Sun et al., 2010) and are induced by BR (Figure 1D, right), suggesting that BR may stimulate PA level to affect PP2A activity, thus suppressing the BR signal itself in a feedback mechanism (Figure 1E). There is the other possibility that PP2A is recruited in membrane by PA; cytoplasmic PP2A activity, BZR1 activity, and BR signal remain at a low level. When unknown signals release PP2AA1 from membrane, increased cytoplasmic PP2A dephosphorylates BZR1 and actives BR signal. Then BZR1 induces *PLDs* and accumulates PA level, recruits PP2A to membrane, and suppresses BR signal again.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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