ROPGAP-dependent interaction between brassinosteroid and ROP2-GTPase signaling controls pavement cell shape in *Arabidopsis*

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SUMMARY

The epidermal pavement cell shape in *Arabidopsis* is driven by chemical and mechanical cues that direct partitioning mechanisms required for the establishment of the lobe- and indentation-defining polar sites. Brassinosteroid (BR) hormones regulate pavement cell morphogenesis, but the underlying mechanism remains unclear. Here, we identified two PLECKSTRIN HOMOLOGY GTPase ACTIVATING proteins (PHGAPs) as substrates of the GSK3-like kinase BR-INSENSITIVE2 (BIN2). The *phgap1*/*phgap2* mutant displayed severe epidermal cell shape phenotypes and the PHGAPs were markedly enriched in the anticlinal face of the pavement cell indenting regions. BIN2 phosphorylation of PHGAPs was required for their stability and polarization. BIN2 inhibition activated ROP2-GTPase signaling specifically in the lobes because of PHGAP degradation, while the PHGAPs restrained ROP2 activity in the indentations. Hence, we connect BR and ROP2-GTPase signaling pathways via the regulation of PHGAPs and put forward the importance of spatiotemporal control of BR signaling for pavement cell interdigitation.
INTRODUCTION

The plant steroidal hormones, brassinosteroids (BRs), are indispensable for growth and development\(^1\). They control cell division and elongation in the root\(^2\,^3\), photomorphogenesis\(^6\), stomatal development\(^7\,^8\,^9\), xylem differentiation\(^11\), reproduction\(^12\,^13\) and responses to different stresses\(^14\,^15\). The *Arabidopsis thaliana* SHAGGY/GSK3-like kinase (*AtSK*) BR-INSENSITIVE2 (BIN2) functions as a negative BR signaling regulator\(^17\,^18\). In the absence of BRs, BIN2 phosphorylates and inactivates the transcription factor BRASSINAZOLE-RESISTANT1 (BZR1)\(^19\) and its homologue BRI1-EMS-SUPPRESSOR1 (BES1)/BZR2\(^20\), both controlling the BR-responsive gene expression. BIN2 activity is directly controlled by phosphatase, BRI1 SUPPRESSOR1 (BSU1)\(^18\) and the F-box protein KINK SUPPRESSED IN BZR1-1D (KIB1)\(^21\). BSU1 dephosphorylates and deactivates BIN2, whereas KIB1 recruitment blocks the BIN2 interaction with BZR1 and BES1/BZR2 and further promotes the BIN2 ubiquitination and degradation. BR perception in the apoplast by the plasma membrane-localized receptor complex, comprising the main receptor BR INSENSITIVE1 (BRI1) and the coreceptor BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1), triggers a downstream phosphorylation cascade that inactivates and degrades BIN2\(^1\), with the accumulation of dephosphorylated BZR1 and BES1/BZR2 in the nucleus as consequence, followed by either induction or repression of the BR-responsive gene expression\(^1\).

The Rho-related GTPase from Plants (ROP) are important molecular switches essential for different cell polarity-related processes, such as root hair development\(^22\,^23\), pollen tube growth\(^24\,^25\) and pavement cell interdigitation\(^26\,^29\). Among the known ROPs in *Arabidopsis*\(^31\), ROP2 and ROP6 are the main players that control pavement cell interdigitation\(^26\,^27\). ROP2 preferentially localizes at the tip of the pavement cell lobe and promotes lobe formation by activating the ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN4 (RIC4)-mediated assembly of fine cortical actin microfilaments (MFs) that are necessary for bulge formation\(^26\,^27\). Antagonistically, ROP6 localizes predominantly at the indentation region and restricts lateral expansion through RIC1-mediated bundling and transverse alignment of cortical microtubules (MTs) at the neck region\(^26\,^27\). The active ROP2 suppresses the RIC1-mediated function in the MT organization, which, in turn, inhibits the ROP2-RIC4 interaction\(^27\). Expression of a constitutively active ROP2 (CA-rop2) in *Arabidopsis* causes delocalized distribution of the fine actin MFs throughout the cell cortex and, simultaneously, inhibits the bundling of the transverse cortical MTs across the neck, thus eliminating interdigitation and generating regularly shaped pavement cells\(^26\). ROPs cycle between a GDP-bound inactive state
and a GTP-bound active state in a strictly regulated manner\textsuperscript{32}. Plant-specific guanine nucleotide exchange factors (ROPGEFs) function as ROP GTPase activators that catalyze the GDP-to-GTP exchange\textsuperscript{33,34}, whereas two classes of ROP GTPase-activating proteins (ROPGAPs) have been shown to promote the GTP hydrolysis of ROPs, the Cdc42/Rac-interactive binding (CRIB) motif-containing ROPGAPs\textsuperscript{35} and the pleckstrin homology (PH) domain-containing PHGAPs\textsuperscript{36}.

BRs affect leaf epidermis in *Arabidopsis* by controlling stomatal development\textsuperscript{7-10} and pavement cell growth and morphology\textsuperscript{37,38}. Loss-of-function BR biosynthesis or signaling mutants exhibit smaller epidermal pavement cells with fewer lobes and less complexity than the wild type\textsuperscript{37,38}. By contrast, the triple BIN2 knockout mutant and its homologues BIN2-LIKE1 (BIL1) and BIL2, *bin2-3bil1bil2* had larger pavement cells with more lobes\textsuperscript{38}, suggesting a role for BRs in promoting pavement cell expansion and interdigitation. However, the exact molecular mechanisms by which BRs regulate epidermal cell morphology are still unclear.

Here, we identified the PLECKSTRIN HOMOLOGY GTPase-ACTIVATING PROTEINS (PHGAPs), PHGAP1 and PHGAP2, previously reported to regulate cell division in the root meristem of *Arabidopsis*\textsuperscript{39,40} as novel BIN2 substrates. The PHGAP2 is similar to ROP ENHANCER1 (REN1)\textsuperscript{36} and it was previously named RENGAP3 (REN3)\textsuperscript{39}, whereas the PHGAP1 is also known as RENGAP2 (REN2)\textsuperscript{39}. The pavement cell shape was severely compromised in the *phgap1phgap2* double mutant and the PHGAPs were noticeably enriched in the anticlinal face of pavement cell indentations. The BIN2-mediated phosphorylation of PHGAPs controlled their polarization and protein abundance, whereas BIN2 inhibition led to PHGAP hypophosphorylation and degradation. BRs activated ROP2 through a phosphorylation-dependent destabilization of PHGAPs. Thus, our study proposes a so far unknown mechanism for the regulation of epidermal pavement cell shape by BRs in plants and connects the BR and ROP2-GTPase signaling pathways via the regulation of the PHGAPs.

RESULTS

**BIN2 interacts with PHGAP1 and PHGAP2**

Previously, we had identified the BIN2 interactome in the stomatal lineage of *Arabidopsis* by immunoprecipitation coupled to mass spectrometry (IP-MS) of 3-day-old *bin2-3* seedlings expressing a GSgreen-tagged BIN2 driven by the *TOO MANY MOUTHS* (*TMM*) promoter (*TMM*pro:*BIN2-GSgreen/bin2-3*)\textsuperscript{10}. The ROP GTPase activating protein PHGAP2/REN3 (AT5G19390) was found as a putative BIN2 interactor\textsuperscript{10} and it was significantly enriched in the
repeat experiment (Data S1). To validate this interaction, we carried out a co-immunoprecipitation (Co-IP) assay with transgenic *Arabidopsis* plants co-expressing the GFP-tagged BIN2 genomic fragment with the mCherry-tagged genomic fragment of either PHGAP2 or its close homologue PHGAP1/REN2 (AT5G12150), all driven by their native promoters at 5 days post germination (dpg). Both PHGAP1 and PHGAP2 co-immunoprecipitated with BIN2 (Figure 1A). Similarly, BIN2-HA co-purified with each GFP-PHGAP1 and GFP-PHGAP2 in tobacco (*Nicotiana benthamiana*) leaf epidermis when transiently co-expressed (Figure 1B). To investigate whether the interaction between BIN2 and PHGAPs is direct we employed yeast two-hybrid system (Figures 1C). The GAL4-binding domain fused with BIN2 (BD-BIN2) was transformed together with the GAL4 activation domain fused with either PHGAP1 or PHGAP2 (AD-PHGAP1 and AD-PHGAP2, respectively) in yeast. The known interaction between BIN2 and KIB1 served as a positive control and the combination of BD-KIB1 with AD-PHGAP1 as a negative control. Yeast grew in all tested combinations, except for the negative control and for the combinations co-expressing either BD-BIN2 with free AD or each AD-PHGAP1, AD-PHGAP2 and AD-KIB1 with free BD (Figures 1C), suggesting that BIN2 interacts directly with PHGAP1 and PHGAP2. As confirmation, a ratiometric bimolecular fluorescence complementation (rBiFC) assay was carried out (Figures 1D and 1E). Tobacco epidermal cells were transiently transformed with constructs allowing the expression of either BIN2-cYFP or BIN2-nYFP with the corresponding nYFP-PHGAP1 and cYFP-PHGAP2 and of the red fluorescent protein (RFP) as expression control. The combinations of nYFP-ROP2 with cYFP-PHGAP2 and of KIB1-nYFP with cYFP-PHGAP2 were used as positive and negative controls, respectively. As expected, both combinations, nYFP-PHGAP1 and BIN2-cYFP and BIN2-nYFP and cYFP-PHGAP2 significantly recovered the YFP signal in comparison with the negative control, indicating that PHGAP1 and PHGAP2 interact directly with BIN2 in vivo. Altogether, PHGAP1 and PHGAP2 are previously unknown interactors of BIN2.

**PHGAP1 and PHGAP2 control pavement cell shape in Arabidopsis**

PHGAP1 and PHGAP2 are implicated in the control of cell division plane orientation in the *Arabidopsis* root meristem, but their function in leaves has not been investigated. To this end, we analyzed the abaxial cotyledon epidermis of the *Arabidopsis* *phgap1* and *phgap2* single mutants and *phgap1phgap2* double mutant at 5 dpg (Figure 2A). No obvious phenotype was observed in the *phgap1* mutant, but the epidermal pavement cell shape was mildly defective in the *phgap2* mutant and severely impaired the *phgap1phgap2* double mutant, resembling the constitutively active ROP2 (*CA-rop2*) mutant. Quantification of the pavement cell circularity
and of the lobe counts (i.e. lobe numbers per cell)\textsuperscript{42,43} revealed an increase in circularity and a decrease in lobe numbers in \textit{phgap2} and especially in \textit{phgap1phgap2}, similarly as for the \textit{CAtrop2} mutant (Figures 2D and 2E). The observed phenotypes hinted at a role for PHGAP1 and PHGAP2 in maintenance of the pavement cell shape.

To establish the expression pattern of PHGAP1 and PHGAP2 in the cotyledon epidermis, we fused the genomic \textit{PHGAP1} and \textit{PHGAP2} fragments N-terminally to GFP and expressed them in the \textit{phgap1phgap2} double mutant from their native promoters. The cotyledon epidermis of several independent homozygous transgenic lines was examined (Figure S1). The observed phenotypes hinted at a role for PHGAP1 and PHGAP2 in maintenance of the pavement cell shape.

Next, we checked the localization of GFP-PHGAP1 and GFP-PHGAP2 in the abaxial cotyledon epidermis of seedlings at 5 dpg (Figures 2E, 2F, S1E and S2A). GFP-PHGAP1 and GFP-PHGAP2 were ubiquitously expressed in the cotyledon epidermis, although GFP-PHGAP1 was somewhat less abundant (Figure S2A). The GFP-PHGAP2 was highly enriched in the stomatal lineage, particularly in meristemoids and guard cells (Figure S2A). Reporter lines, in which the promoters of the two \textit{PHGAP} genes were fused to a nuclear localization sequence (\textit{NLS})-GFP, had gene expression patterns similar to those of the GFP-tagged proteins (Figure S2B). Remarkably, both GFP-PHGAP1 and GFP-PHGAP2 distinctively localized in the plasma membrane of the cotyledon pavement cells (Figures 2F, 2G, S1E and S2A). After a 0.8 M mannitol-induced plasmolysis to separate complementary lobes from indentations, it became apparent that GFP-PHGAP2 was enriched along the anticlinal cell face of the indentations, but not of the lobes (Figure 2J). Markedly, this localization pattern occurred after 2 dpg (Figure S3A), coinciding with the pavement cell lobe initiation\textsuperscript{42,44}. The mutant complementation analyses revealed that the indentation enrichment of GFP-PHGAP2 was evident only in lines with native expression levels of the transgene (lines #3.1.1 and #1.1.1) (Figures 2F, 2G, S1B and S1E). In contrast, when the expression levels of GFP-PHGAP2 were
increased (lines #1.5.1 and #5.4.20), the anticlinal cell face localization pattern was no longer visible and the pavement cell phenotype in the phgap1phgap2 mutant was not complemented (Figures 2F, 2G, S1B and S1E). Taken together, the phenotypic analysis revealed that PHGAP1 and PHGAP2 functioned in the epidermal cell lobe formation that correlated with the specific enrichment of the proteins in indentations along the anticlinal cell face.

**BIN2 phosphorylates PHGAP1 and PHGAP2 in vitro**

To examine if BIN2 regulates the function of PHGAP1 and PHGAP2, we first tested whether BIN2 can phosphorylate PHGAP1 and PHGAP2 in vitro. Kinase assays with bacterially produced recombinant GST-BIN2 and HIS-MBP-PHGAP1 or HIS-MBP-PHGAP2 were carried out. GST-BIN2 combined with HIS-MBP was used as a negative control. Both HIS-MBP-PHGAP1 and HIS-MBP-PHGAP2 were strongly phosphorylated by the GST-BIN2, whereas the HIS-MBP protein was not (Figure 3A). Further MS analysis identified 6 and 13 phosphorylated residues in PHGAP1 and PHGAP2, respectively (Data S1). Five and eight residues in PHGAP1 and PHGAP2, respectively, had previously been reported in the PhosPhAt 4.0 database (http://phosphat.uni-hohenheim.de) of which two residues in each protein overlapped with the in vitro identified (Figure 3B and Data S1). Substitutions of 8 and 19 residues with alanine (A) in PHGAP1 [PHGAP1(8A)] and PHGAP2 [PHGAP2(19A)], respectively, markedly reduced the phosphorylation by BIN2, but did not abolish it (Figures 3C and 3D), implying that more amino acids in PHGAP1 and PHGAP2 might be phosphorylated by BIN2. As the identified phosphorylation sites were mainly found in the C-terminal parts of PHGAP1 and PHGAP2 (Figure 3B), we explored whether the N-terminal part of the proteins that includes the important PH and GAP domains were also subjected to phosphorylation by BIN2 in vitro. To this end, we generated truncated PHGAP1(1-380) and PHGAP2(1-420) versions containing the PH and GAP domains only and repeated the in vitro kinase assay (Figures 3C and 3D). The PHGAP1(1-380) and PHGAP2(1-420) phosphorylation by BIN2 was notably reduced, but still not abolished when compared with the full-length proteins. Altogether, our results indicate that BIN2 mostly phosphorylates the C-terminal parts of PHGAP1 and PHGAP2, although we cannot rule out the contribution of the N terminal region to the PHGAP phosphorylation.

**Phosphorylation of PHGAP2 by BIN2 led to its stabilization and polarization**

To evaluate the importance of the PHGAP2 phosphorylation by BIN2 in vivo, we introduced a GFP-tagged genomic fragment of PHGAP2(19A) into the phgap1phgap2 double mutant driven
by its native promoter. Three GFP-PHGAP2(19A)/phgap1phgap2 plants, each expressing the transgene at a level similar to the corresponding GFP-PHGAP2/phgap1phgap2 plants, were assessed (Figures 2B-2E and S1B). Whereas the GFP-PHGAP2 fully complemented the epidermal cell phenotype of the phgap1phgap2 mutant at native levels (line #3.1.1) (Figures 2B-2E and S1A-S1D), the GFP-PHGAP2(19A) expressed at the same levels (line #5.1.1) complemented the phgap1phgap2 mutant only partially (Figures 2B-2E). Compared with the GFP-PHGAP2/phgap1phgap2 plants (line #3.1.1), the GFP-PHGAP2(19A) expressing plants (line #5.1.1) displayed a significant increase in the pavement cell circularity together with reduced lobe numbers. In contrast to the GFP-PHGAP2 overexpressing plants (line #1.5.1), the phgap1phgap2 complementation with higher GFP-PHGAP2(19A) levels (line #3.12.5) generated less compromised phenotypes (Figures 2B-2E). The pavement cells in GFP-PHGAP2(19A) overexpression plants had a significantly decreased circularity and an increased lobe number, similarly to the wild type or to phgap1phgap2 mutant complemented with native GFP-PHGAP2 levels (line #3.1.1). Examination of the GFP-PHGAP2(19A) localization in the cotyledon epidermis at 5 dpg (Figures 2H and 2I) revealed that, although the anticlinal cell face localization of GFP-PHGAP2(19A) in the indentation tips was retained, the overall protein abundance of PHGAP2(19A) was lower than that of the GFP-PHGAP2 at similar transcript levels (Figures 2K).

In support of these observations, we examined the protein amounts of GFP-PHGAP2 when the BIN2 activity was inhibited. To this end, the PHGAP2_pro:GFP-PHGAP2/phgap1phgap2 seedlings (line #7.8.1) were treated with either brassinolide (BL) (100 nM), the most active BR, or with the plant-specific GSK3 kinase inhibitor, bikinin (BIK) (50 µM) in a time course manner (Figure S4A). All experiments were done in the presence of cycloheximide (CHX) to block de novo protein synthesis. As expected BL and BIK both accelerated the turnover of GFP-PHGAP2 compared with the mock control, albeit BIK caused a more pronounced effect, thus confirming that the PHGAP2 phosphorylation by BIN2 governs its protein stability. Furthermore, we tested whether blocking the BIN2 kinase activity would affect the anticlinal cell face localization of GFP-PHGAP2. Consequently, PHGAP2_pro:GFP-gPHGAP2/phgap1phgap2 (line #3.1.1) and PHGAP2_pro:GFP-gPHGAP2(19A)/phgap1phgap2 (line #5.1.1) seedlings at 3 dpg were treated with BL (100 nM) and BIK (50 µM) in liquid media for 16 h (Figures 4A and 4B). To evaluate the effect of exogenous BL and BIK on the indentation enrichment of GFP-PHGAP2, pavement cell wall undulations were subdivided into indentation region and outside region based on the full width at half-maximal lobe height, and the relative indentation enrichment (RIE) value of GFP-PHGAP2 (average GFP intensity in the
indentation region / average GFP intensity in the outside region) was measured. Quantification results showed that exogenous BL significantly decreased the number of undulations with GFP-PHGAP2 enrichment in the indentation regions (peak localization, RIE value of GFP-PHGAP2 > 1.5), whereas the undulations with partial indentation enrichment (partial peak localization, RIE value of GFP-PHGAP2 within 1.2 - 1.5) or totally diffused localization in indentation regions (diffuse localization, RIE value of GFP-PHGAP2 < 1.2) were significantly higher than that with the mock treatment. The indentation tip enrichment localization pattern of GFP-PHGAP2(19A) was more sensitive to exogenous BL than that of GFP-PHGAP2. Moreover, BIK had a dramatic effect, because the peak localization pattern of both GFP-PHGAP2 and GFP-PHGAP2(19A) was almost abolished and only partial peak or diffuse localization patterns were observed. Similarly to exogenous BL, the effect of BIK was more pronounced for GFP-PHGAP2(19A). Taken together, these data indicate that the PHGAP2 phosphorylation by BIN2 is essential for maintaining optimal protein levels and for retaining its polarized anticlinal cell face localization in the pavement cell indentations.

**BRs activated ROP2 through phosphorylation-mediated destabilization of PHGAPs**

Because PHGAPs interact with ROP2 in vivo and the expression pattern of GFP-ROP2 in the cotyledon epidermis was similar to that of GFP-PHGAP2 (Figure S2C), we hypothesized that the reduced PHGAP2 protein stability caused by the BIN2 inhibition would activate ROP2. To test this hypothesis, we examined the ROP2 activity after BL and BIK treatment by using the RIC1 binding-based assay, in which RIC1 specifically binds the active form of ROP2. In this experiment, the GTP-bound active ROP2 was pulled down with RIC1-conjugated beads. The synthetic auxin 1-naphthaleneacetic acid (NAA), previously reported as a strong ROP2 activator, was used as a positive control (Figure 4C). As expected, ROP2 was activated by both BL and BIK in the Col-0 wild type. The same assay was used to test whether the BL- and BIK-induced ROP2 activation depended on PHGAP1 and PHGAP2. To this end, a time-course analysis of the ROP2 activity was carried out for Col-0 and the phgap1phgap2 mutant with exogenous BL and BIK (Figure 4D). Prolonged treatment with BL enhanced the ROP2 activation in Col-0, whereas BIK strongly activated ROP2 even at a low concentration (10 µM). However, the ROP2 activity in the phgap1phgap2 mutant was already higher than that in Col-0 and was insensitive to both BL and BIK treatments (Figure 4D). Because of the PHGAP enrichment in the pavement cell indentations, where ROP6 is active, we tested whether exogenous BL or BIK can also activate ROP6 and, if so, depends on PHGAP (Figures S3B). Surprisingly, both BL and BIK activated ROP6 equally in the wild type and in the
phgap1phgap2 mutant. Altogether, our data suggest that exogenous BRs trigger both ROP2 and ROP6, but that only the ROP2 activation depended on the PHGAP function.

**BR signaling controls epidermal pavement cell shape**

We next evaluated if inhibition of BIN2 activity with exogenous BRs or BIK affects the epidermal pavement cell shape. Wild type seedlings were treated with increasing concentrations of BL (0.2 nM, 1 nM, 10 nM, and 1 µM) and with 50 µM BIK by germinating and growing seeds directly in liquid medium containing the chemicals for 5 days (Figures 5A and 5B). BL affected the pavement cell shape in a dose-dependent manner. Low concentrations of BL (0.2 nM and 1 nM) slightly promoted pavement cell interdigititation and generated cells with lower circularity and higher lobe number whereas concentrations of BL of 10 nM and higher inhibited pavement cell interdigitation, increased the circularity and reduced the lobe number of the pavement cells. Notably, 1 µM BL and 50 µM BIK treatments both severely compromised pavement cell interdigitation that resembled the epidermal phenotype of phgap1phgap2 mutant (Figures 5A and 5B). As expected, the epidermal cells in phgap1phgap2 mutant were insensitive to exogenous BL even when applied at high concentrations (Figures 5A and 5B).

To provide additional genetic evidence for the function of BIN2 and homologs in pavement cell shape, the abaxial epidermis of AtSK quadruple (atsk13RNAi/bin2/bil1/bil2, noted as atskquad) and sextuple (atsk11RNAi/atsk12RNAi/atsk13RNAi/bin2/bil1/bil2, noted as atskext) mutants was examined at 5 dpg. Similar to the previously described bin2/bil1/bil2 triple mutant, the atskquad exhibited higher complexity with significantly lower cell circularity and higher lobe numbers when compared with that of the wild type in Wassilewskija (WS) background (Figures 6A and 6B). Because the atskext mutant is seedling lethal, the phenotype of the plants was analyzed in the T1 generation (Figures S4A and S4B). In contrast to atskquad, the atskext mutant presented epidermal pavement cells with higher circularity and less lobe numbers (Figures S4A, S4B and S4C).

Since eight AtSKs are expressed in the cotyledon epidermis of Arabidopsis, we used CRISPR to simultaneously target AtSK11, AtSK12, AtSK13, AtSK21/BIN2, AtSK22/BIL1, AtSK23/BIL2, AtSK32 and AtSK42, in both Col-0 and the phgap1phgap2 mutant complemented with the PHGAP2pro::GFP-gPHGAP2 construct (line #3.1.1). A pair of guide RNAs (gRNA) for each of the eight AtSK was introduced into a single, ubiquitously expressed CRISPR-associated protein 9 (Cas9) construct (RPS5Apro::Cas9-P2A-mCherry;8AtSK-1;8AtSK-2). The T-DNA vector carrying the Cas9 cassette contained also a fluorescent seed marker for seed sorting. The respective transgenic lines were designated as atskext (RPS5Apro::Cas9-P2A-
mCherry;8AtSK-1;8AtSK-2/Col-0) and GFP-PHGAP2/atsk\textsuperscript{octu} (RPS5A\textsubscript{pro}:Cas9-P2A-mCherry;8AtSK-1;8AtSK-2/PHGAP2\textsubscript{pro}:GFP-gPHGAP2/phgap1phgap2). Due to the seedling lethality of the atsk\textsuperscript{octu} plants, the abaxial cotyledon epidermis and the localization pattern of GFP-PHGAP2 were examined in the T1 generation at 5 dpg (Figure S5). Approximately 15% of the examined T1 atsk\textsuperscript{octu} mutants in the two experiments displayed seedling defects with elongated hypocotyls and short or no roots (Figure S5A). Sequencing analysis of two individual T1 mutants of each genotype revealed a high genome-editing efficiency for all targeted AtSK genes (Figure S5B and Data S2). The epidermis of the atsk\textsuperscript{octu} plants presented severe pavement cell shape defects that resembled phgap1phgap2 and CA-rop2 mutants or treatments with high concentration of BL (1 μM) and BIK (50 μM) (Figures 6A-6C and S5C). Moreover, the GFP-PHGAP2 enrichment in the pavement cell indentations was noticeably abolished in the GFP-PHGAP2/atsk\textsuperscript{octu} plants and the phenotype of the phgap1phgap2 mutant was no longer complemented with the PHGAP2\textsubscript{pro}:GFP-gPHGAP2 construct, when compared with PHGAP2\textsubscript{pro}:GFP-gPHGAP2/phgap1phgap2 plants (line #3.1.1) (Figures 6D, 6E, S5D and S5E).

To overcome the seedling lethality caused by the higher-order atsk mutants, we utilized a CRISPR tissue-specific knockout system (CRISPR-TSKO)\textsuperscript{49} by targeting the eight AtSK genes using an epidermis-specific promoter MERISTEM LAYER1 (ML1)\textsuperscript{50}. The ML1\textsubscript{pro}:CAS9-P2A-mCherry;GFP-1 construct was capable of knocking out the GFP target gene in the cotyledon epidermis of Col-0 plants expressing a nuclear-localized GFP and β-glucuronidase (GUS) fusion protein driven by a ubiquitous promoter of HISTONE THREE-RELATED5 (HTR5) (HTR5\textsubscript{pro}:NLS-GFP-GUS/Col-0)\textsuperscript{49} (Figure S6). Consequently, we expressed the ML1\textsubscript{pro}:Cas9-P2A-mCherry;8AtSK-1;8AtSK-2 construct in the wild type (Col-0) and in the PHGAP2\textsubscript{pro}:GFP-gPHGAP2/phgap1phgap2 plants (line #3.1.1). The epidermal phenotype was examined in seedlings of the T2 generation (Figures 6A-6D). Similarly to atsk\textsuperscript{octu} (RPS5A\textsubscript{pro}:Cas9-P2A-mCherry;8AtSK-1;8AtSK-2), the atsk\textsuperscript{octu} (pML1\textsubscript{pro}:Cas9-P2A-mCherry;8AtSK-1;8AtSK-2) displayed epidermal pavement cells with an increased circularity and fewer lobe numbers, albeit the phenotypes were weaker (Figures 6A-6C). Notably, the GFP-PGHAP2 polarization in the indentations was compromised (Figure 6D and 6E). Altogether, our results provide strong genetic evidence that BR signaling and AtSKs control the pavement cell shape through PHGAPs.

**DISCUSSION**
The leaf epidermal phenotypes observed in various BR loss-of-function Arabidopsis mutants\textsuperscript{37,38} imply that the BR hormones function in pavement cell morphogenesis, but the exact mechanisms are not fully elucidated. Here, we discovered a previously unknown interaction between the BR and ROP2 GTPase signaling pathways that controls pavement cell shape in Arabidopsis. The ROPGAPs, PHGAP1 and PHGAP2, known to reside on the cell division plane in the Arabidopsis root\textsuperscript{39,40}, were identified as interactors of the negative BR signaling regulator, BIN2, in the leaf epidermis. Remarkably, the double phgap1phgap2 Arabidopsis mutant presented severe pavement cell shape phenotypes in the abaxial cotyledon epidermis, hinting at a function for these ROPGAPs in pavement cell morphogenesis. Notably, the PHGAPs displayed a distinct localization pattern, because they accumulated clearly in the pavement cell indentations, but not in the lobes. The evident enrichment of PHGAP1 and PHGAP2 along the anticlinal face of the pavement cell indentations coincided with the lobe/indentation formation (2 to 3 dpg)\textsuperscript{42,44}, supporting the hypothesis that PHGAPs are recruited to either initiate undulations or to define and maintain cell polarity throughout the lobe/indentation formation.

BIN2 phosphorylated the PHGAPs in vitro and most of the identified phosphorylation sites resided in the C-terminal part of the proteins, in which both predicted coiled-coil domains are located, implying that phosphorylation as such might not influence the GAP activity directly, but that it could affect it through changes in the protein localization. Indeed, PHGAP deletion mutants lacking the non-catalytic domains were unable to complement the phgap1phgap2 mutant and did not properly localize in pavement cells\textsuperscript{51}. Similarly, the C-terminal part of the close PHGAP homologue, REN1, containing coiled-coil domains and active in continuous tip growth, was not essential for the GAP activity, but was needed for the apical REN1 localization in the pollen tubes\textsuperscript{36}.

Post-translational regulation of GAPs had previously not been reported in plants, whereas in mammalian cells a number of GAPs are known to be regulated by phosphorylation that can affect either their GAP activity or subcellular localization. For example, the RhoGTPase-activating protein (RhoGAP) DELETED IN LIVER CANCER1 (DLC1) is phosphorylated by the protein kinase A, which induces its homodimerization and increases its GAP activity\textsuperscript{52}. DLC1 is also activated through phosphorylation by the cyclin-dependent kinase 5 (CDK5) that relieves its inactive conformation\textsuperscript{53}. Likewise, the GAP activity of MgcRACGAP, previously known as a GAP for Rac/Cdc42, is functionally converted into a RhoGAP through phosphorylation by the Aurora B kinase\textsuperscript{54}. In some cases, phosphorylation regulates the subcellular localization of the GAPs. For instance, the Rho GAP ARHGAP19 that controls cell
elongation and cytokinesis during lymphocyte mitosis is phosphorylated sequentially by the RhoA-activated kinases ROCK1 and ROCK2 and by CDK1, which prevents ARHGAP19 from relocating to the plasma membrane during prophase and metaphase, thus allowing RhoA activation. The ROCK phosphorylation of the Rac-specific GAP, FilGAP, stimulates its Rac GAP activity and switches its subcellular localization from the actin filaments to the cytoplasm. In our study, mutations of 19 putative BIN2 phosphorylation sites in PHGAP2 into alanine (A) reduced the in vitro phosphorylation by BIN2 to approximately 50% of that of the wild type PHGAP2. Complementation analysis of phgap1phgap2 with PHGAP2(19A) revealed that the phosphorylation-deficient PHGAP2 can only partially restore the pavement cell shape phenotype of the mutant because of the overall decreased protein amounts when expressed at the same levels of transcription as the wild type PHGAP2. Similarly, overexpression of PHGAP2(19A) did not compromise the pavement cell phenotype compared to the overexpression of GFP-PHGAP2. No obvious differences in the polarized anticlinal cell face localization patterns were detected between PHGAP2(19A) and PHGAP2. Nevertheless, the PHGAP2 and PHGAP2(19A) polarization was abolished by exogenous BRs and BIK, both BIN2 activity inhibitors and markedly the PHGAP2(19A) localization was hypersensitive to the two treatments. These results imply that possibly more BIN2-phosphorylation sites are required for the subcellular localization of PHGAP2. As expected, BRs and BIK also reduced the total PHGAP protein levels, with the impact of BIK more pronounced. Hence, BIN2 activity is necessary for maintaining adequate PHGAP protein levels and polarized localization in the pavement cell indentations. However, BIN2 might not affect the anticlinal cell wall localization of PHGAP2 by direct phosphorylation. It has been shown that the indentation enrichment of PHGAPs is dependent on MTs, whose protein stability is regulated by BIN2 phosphorylation. It remains to be determined if BIN2 regulates the indentation enrichment of PHGAP2 through phosphorylation and stabilization of MTs.

Both PHGAP1 and PHGAP2 interacted with several ROPs in rBIFC experiments in tobacco, including ROP2 and ROP6 proteins that antagonistically control lobe outgrowth and indentation, respectively. Exogenous BRs and BIK could trigger both ROP2 and ROP6, with only the ROP2 activation being dependent on the PHGAPs. Therefore, PHGAPs might specifically target ROP2, but not ROP6. Several additional observations corroborated this conclusion. BRs affected pavement cell shape in a concentration-dependent manner. Low (<1 nM) and high (>10 nM) levels of BL promoted and inhibited pavement cell interdigitation, respectively. Excessive BRs possibly over activated ROP2, thus negatively affecting pavement cell interdigitation and mimicking the constitutively active ROP2 mutant. The last also
resembled the phenotypes of *phgap1phgap2*, of higher-order *atsk* mutants and that of BIK-treated wild type plants. Because the AtSKs are highly redundant, the *atsk*quad mutant resembled treatment with low concentration of BL that promoted pavement cell interdigitation. In contrast, the epidermal phenotype of the higher order mutants, *atsksexu* and *atskocta*, mimicked treatments with high concentration of BL that inhibited pavement cell interdigitation due to over activation of ROP2. Interestingly, the concentration-dependent effect of BRs was similar to that of auxin, which also promoted or reduced lobe formation at low (10-50 nM) and high (>100 nM) concentrations, respectively. Because BR and auxin signaling activate each other and act synergistically in multiple plant developmental processes, we can speculate that this could also be the case for their roles in pavement cell shape formation. Additionally, PHGAP1 and PHGAP2 interacted with ROP2, but not with ROP6, in both yeast and *Arabidopsis* cells and bacterially expressed RIC1 could bind more activated ROP2, but not ROP6, in the *phgap1phgap2* mutant. The reason for the PHGAP enrichment in the pavement cell indentations, in which ROP6 locally activates RIC1 to restrict cell outgrowth, might be that PHGAPs are required to limit the ROP2 activity in the indentation region. Indeed, this hypothesis is plausible, because the GFP-ROP2 was ubiquitously distributed in the plasma membrane of the abaxial epidermis (Figure S2C), albeit more abundantly in the stomatal lineage cells.

BIN2 and homologues act as negative regulators of BR signaling. BR hormones inactivate BIN2, ultimately resulting in PHGAP hypophosphorylation and degradation followed by ROP2 activation. To promote pavement cell interdigitation, ROP2 has to remain active in the lobe region, but inactive in the cell indentations, in which the PHGAPs are enriched. This differential regulation through the PHGAPs can be realized only when the BR signaling is locally triggered in the lobes, but remains inactive in the indentations (Figure 6E). Regulation of BR signaling in such a spatiotemporal manner can be achieved either by local activation in the lobe region due to changes in the cell wall properties or by insulating the BIN2 activity in the indentation regions through interaction with scaffolds. The two scenarios are plausible, because mechanisms that integrate cell wall and BR signaling exist and both BIN2 and PHGAPs bind scaffolds that control their localization and activity. This model is in line with the already proposed function for BRs in regulating the pavement cell shape through BIN2 binding-induced cortical MT stabilization. Pavement cell lobe initiation had been linked to differences in cell wall stiffness producing locally confined tensile stresses that lead to alignment of MTs, which in turn guide deposition of cellulose fibers and differential expansion. The accumulation of transfacial MTs in the anticlinal-periclinal cell boundary
was recently correlated with tensile stress patterns, signifying the importance of MT-cellulose system along the anticlinal walls to generate morphogenetic response\textsuperscript{65}. Interestingly, PHGAPs associate with MTs via their C-terminal domain either directly or indirectly (via additional interactors) in the indentations\textsuperscript{51}. One possible scenario could involve the MT-targeted IQ67 DOMAIN proteins some of which interact with either PHGAPs or GSK\textsuperscript{340,66}. Although it is still unclear how MTs, PHGAPs and BIN2 interact, the MT stabilization in the indentations will also require that BIN2 remains active.

Increasing evidence implies that auxin along with cell wall components and mechanical stresses control pavement cell shape\textsuperscript{67,68}. Such signals activate plasma membrane-localized receptors as FERONIA, the TRANSMEMBRANE KINASE1 and the leucine-rich repeat extensins that impinge on the cortical MT-organizing ROP\textsuperscript{67,68}. The mechanisms behind the ROP6 signaling activation by BRs are still unclear. The question remains to be addressed whether the BR signaling interferes with auxin or with any of the signaling pathways to regulate ROP6.

In summary, our study reveals a novel ROPGAP-dependent interaction between BR and ROP GTPase signaling that controls pavement cell shape and puts forward a new model in which high BR signaling levels might promote lobe outgrowth, while active BIN2 along the MTs might restrict ROP2 activity via the PHGAPs.

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**AUTHOR CONTRIBUTIONS**
C.Z., A.H. and E.R. initiated the project and designed experiments. C.Z. performed most of the experiments. T.L. and S.M. shared materials and performed image analysis. W.T. and T.X. carried out the ROP activation assays. S.Z. and I.D.S. performed the phosphorylation site analysis, D.E. and G.D.J. performed the IP-MS and analyzed data, T.B.J. guided the CRISPR work. C.Z. and E.R. wrote the manuscript. All authors revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. BIN2 interacts with PHGAP 1 and PHGAP2
(A) BIN2 co-immunoprecipitated with PHGAP1 and PHGAP2 in Arabidopsis seedlings co-expressing BIN2<sub>pro</sub>:gBIN2-GFP (line #27.7) with either PHGAP1<sub>pro</sub>:mCherry(mCh)-gPHGAP1 (line #3.8) or PHGAP2<sub>pro</sub>:mCh-gPHGAP2 (line #5.1) at 5 days post germination. Wild type seedlings expressing only the BIN2<sub>pro</sub>:gBIN2-GFP (line #27.7) were used as a negative control. Total proteins were extracted (Input) and immunoprecipitated (IP) using GFP beads. The immunoblot (IB) was done with α-GFP and α-RFP antibodies.

(B) BIN2 co-immunoprecipitated with PHGAP1 and PHGAP2 in the tobacco leaf epidermis. The 35S<sub>pro</sub>:BIN2-HA construct was transiently co-expressed with either 35S<sub>pro</sub>:GFP-PHGAP1, 35S<sub>pro</sub>:GFP-PHGAP2 or 35S<sub>pro</sub>:GFP (as a negative control). Proteins were extracted (Input) and immunoprecipitated (IP) by means of GFP beads. The immunoblot (IB) was done with α-GFP and α-HA antibodies.

(C) BIN2 interacted with PHGAP1 and PHGAP2 in the yeast two-hybrid assay. The yeast cells were cultured on SD medium without leucine (L), tryptophan (T) and histidine (H). BIN2 was fused with GAL4-BD and PHGAP1, PHGAP2 and KIB1 with GAL4-AD. Yeast containing either BD-BIN2 and AD-KIB1 or AD-PHGAP1 and BD-KIB1 were used as positive and negative controls, respectively. AD-PHGAP1, AD-PHGAP2, AD-KIB1 and AD each did not interact with the BD. BD, binding domain; AD, activation domain.

(D) Interaction between BIN2, PHGAP1 and PHGAP2 in tobacco leaf epidermis detected by the ratiometric bimolecular fluorescence complementation (rBiFC) assay. Interactions between ROP2 and PHGAP2 and between KIB1 and PHGAP2 were used as positive and negative
controls, respectively. Scale bar, 50 µm.

(E) Quantification of rBiFC in (C).

Data are shown as individual value plots in columns and with whiskers representing means and SD. Significant differences between were determined with single-factor ANOVA compared to the negative KIB1-PHGAP2 control. ***P < 0.001. All experiments were repeated independently three times. n, number of cells.

See also Data S1

Figure 2. PHGAP1 and PHGAP2 control pavement cell morphogenesis

(A and B) Abaxial cotyledon epidermis of (A) Arabidopsis Col-0, phgap1, phgap2, phgap1phgap2, CA-rop2 and (B) phgap1phgap2 mutant complemented with PHGAP2pro:GFP-gPHGAP2 (lines #3.1.1, #1.1.1 and #1.5.1) or with PHGAP2pro::GFP-gPHGAP2(19A) (lines #5.1.1, #3.7.2, and #3.12.5) at 5 days post germination (dpg). The variation in cell circularity is shown with a colour code. Scale bar, 100 µm.

(C) Expression of PHGAP2 in PHGAP2pro::GFP-gPHGAP2/phgap1phgap2 and PHGAP2pro::GFP-gPHGAP2(19A)/phgap1phgap2 seedlings at 5 dpg measured by qRT-PCR. Three biological replicates were analyzed and three technical repeats were done per sample. ACTIN2 was used as reference gene and transcript levels were normalized to the endogenous expression in the wild type (Col-0). Bars represent SD.

(D and E) Quantification of cell circularity (D) and lobe number (E) per cell of images show in (A) and (B). Cells ≥ 1400 µm² from 5-6 individual cotyledons were segmented and measured with the ‘PaCeQuant’ Image J plugin. Data are shown as individual value plots in columns with whiskers representing means and SD. n, number of cells used for measurements. Significant differences were determined with single-factor ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significant difference.

(F to I) Fluorescence intensity images of protein localization of GFP-PHGAP2 (F) and GFP-PHGAP2(19A) (H) in the abaxial cotyledon epidermis at 5 dpg. Scale bar, 50 µm (F,H). Asterisks mark the representative cells used for 3D reconstruction of the anticlinal face of cells (G,I). White arrows point to the representative anticlinal cell face curves, which were straightened and used to create the plot profile. Scale bar, 20 µm (G,I).

(J) PHGAP2 enrichment in the indentation along anticlinal cell face, but not in the lobe. Plasmolysis with 0.8M mannitol was performed to distinguish the complementary lobe and indentation in the two adjacent cells. Scale bar, 5 µm.
(K) Protein abundance of GFP-PHGAP2 and GFP-PHGAP2(19A) in lines described (B). Western blot (WB) was performed with α-GFP and α-Tubulin (Tub) antibodies. Numbers below the blot show the relative band intensities of the GFP/Tub (bottom) and ± SD (top). 4 biological replicates were performed (n=4). See also Figures S1-S2

Figure 3. BIN2 phosphorylates PHGAP1 and PHGAP2 in vitro
(A) In vitro phosphorylation of PHGAP1 and PHGAP2 by BIN2. Recombinant GST-BIN2 was used as a kinase and HIS-MBP-PHGAP1, HIS-MBP-PHGAP2 or HIS-MBP (negative control) were used as substrates.

(B) Schematic PHGAP1 and PHGAP2 proteins. Red dots, BIN2 phosphorylation sites identified in vitro; green dots, BIN2 phosphorylation sites identified in PhosPhAt 4.0; blue dots, BIN2 phosphorylation sites identified in vitro and found in PhosPhAt 4.0. All noted amino acids were mutated into alanine (A). Dotted lines indicate truncated proteins. PH, pleckstrin homology domains; RhoGAP, Rho GTPase-activating protein; CC, coiled-coil domains; aa, amino acids.

(C and D) In vitro BIN2 kinase assays with (c) PHGAP1(8A) and truncated PHGAP1(1-380) and (D) PHGAP2(19A) and truncated PHGAP2(1-420). Tag-free recombinant BIN2 was used. Recombinant HIS-MBP-PHGAP1, HIS-MBP-PHGAP1(8A), HIS-MBP-PHGAP1(1-380), HIS-MBP-PHGAP2, HIS-MBP-PHGAP2(19A) and HIS-MBP-PHGAP2(1-420) were used as substrates. CBB, Coomassie brilliant blue.
See also Data S1

Figure 4. BRs stimulate ROP2 activity through PHGAPs degradation
(A) Fluorescence intensity images of GFP-PHGAP2. The GFP-PHGAP2 enrichment in the pavement cell indentations was abolished after treatment with brassinolide (BL) and bikinin (BIK). Three-Day-old phgap1phgap2 seedlings expressing PHGAP2:GFP-gPHGAP2 were treated with 100 nM BL, 50 µM BIK and mock (DMSO) for 16 h. Scale bars (top), 50 µm. Asterisks mark the cells used for 3D reconstruction to show the anticlinal cell faces. Scale bar (bottom), 20 µm.

(B) Quantification of the images of (A). Pavement cell wall undulations were subdivided into indentation region and outside region based on the full width at half-maximal lobe height (FWHM). Relative indentation enrichment (RIE) value of GFP-PHGAP2 of undulations (average GFP florescence intensity in indentation region/ average GFP florescence intensity in
outside regions) from 5 individual images were measured. Undulations were classified into 3 categories based on their RIE value of GFP-PHGAP2. Peak, RIE of GFP-PHGAP2 > 1.5; Partial peak, RIE of GFP-PHGAP2 within 1.2 - 1.5; Diffuse, RIE of GFP-PHGAP2 < 1.2. Bars represent SD.

(C) ROP2 was activated by exogenous BL and BIK. Protoplasts from 4-week-old wild type Col-0 leaves were treated with different concentrations of BL, BIK and 1-naphthaleneacetic acid (NAA) for 10 or 30 min. Total ROP2 or GTP-bond active ROP2 that was pulled down with GST-RIC1-conjugated beads were detected by western blot with anti-ROP2 antibody. Numbers below the blot show the relative band intensities of active ROP2/total ROP2 normalized with mock (bottom), ± SD (middle) and P-value from a Student’s t-test compared to 0 h (top). 4 biological replicates were performed (n=4).

(D) ROP2 was over activated in phgap1phgap2 plants and it was insensitive to exogenous BL and BIK. Numbers below the blot show the relative band intensities of active ROP2/total ROP2 normalized with 0 h (bottom), ± SD (middle) and P-value from a Student’s t-test compared to 0 h (top). 3 biological replicates were performed (n=3).

See also Figures S2-S3

Figure 5. BRs affect pavement cell development in a dose-dependent manner
(A) Abaxial cotyledon epidermis of 5-day-old Arabidopsis wild type Col-0 and phgap1phgap2 mutant plants germinated and grown in liquid media containing different concentrations of brassinolide (BL) and bikinin (BIK). The variation in cell circularity is shown with a color code. Scale bar, 100 μm.

(B) Quantification of the images shown (A). Circularity and lobe count of pavement cells (\( \geq 1400 \mu m^2 \)) from 5-6 individual cotyledons were measured with the ‘PaCeQuant’ Image J plugin. n, number of cells analyzed. Data are shown as individual value plots in columns and with whiskers representing means and SD. Significant differences were determined with single-factor ANOVA compared to DMSO treatment. *P < 0.05, **P < 0.01, ***P < 0.001. ns, no significant difference.

Figure 6. AtSK genes are required for epidermal cell development
(A) Abaxial cotyledon epidermis of 5-day-old Arabidopsis wild type Wassilewskija (WS), Col-0, atskquad, atskext (line #5) and two atskoctu mutants. The atskoctu mutants were generated by knocking out AtSK11, AtSK12, AtSK13, AtSK21, AtSK22, AtSK23, AtSK32 and AtSK42 by the Cas9 driven by the RPS5A and ML1 promoters in Col-0 denoted as atskoctu (RPS5Apro:Cas9-
P2A-mCherry(mCh);8AtSK-1;8AtSK-2(Col-0) and atsk\textsuperscript{octu} (ML1\textsubscript{pro}:Cas9-P2A-mCh;8AtSK-1;8AtSK-2(Col-0), respectively. The phenotypes of atsk\textsuperscript{sex} and atsk\textsuperscript{octu} (RPS5A\textsubscript{pro}:Cas9-P2A-mCh;8AtSK-1;8AtSK-2(Col-0) mutants were analyzed in the T1 generation. The variation in cell circularity is shown with a color code. Scale bar, 100 µm.

(B and C) Quantification of the images shown (A). Circularity (B) and lobe count (C) of pavement cells (≥ 1400 µm\textsuperscript{2}) from 5-6 individual cotyledons were measured with the ‘PaCeQuant’ Image J plugin. n, number of cells analyzed. Data are shown as individual value plots in columns and whiskers representing means and SD. Significant differences were determined with single-factor ANOVA compared to the corresponding wild types. ***P < 0.001.

(D) Knocking out eight AtSKs by the Cas9 driven by the RPS5A and ML1 promoters in PHGAP2\textsubscript{pro}:GFP-gPHGAP2/phgap1phgap2 plants abolished the indentation enrichment of PHGAP2 and compromised the complementation of the phgap1phgap2 mutant phenotype. Fluorescence intensity images of GFP-PHGAP2 are shown. Scale bar, 50 µm.

(E) Quantification of the images of (D). Undulations were classified into peak, partial peak and diffuse based on their relative indentation enrichment (RIE) value of GFP-PHGAP2.

(F) To promote pavement cell interdigitation, ROP2 has to remain active in the lobe region, but inactive in the cell indentations, in which the PHGAPs are enriched. This differential regulation through the PHGAPs can be realized only when the BR signaling is locally activated in the lobes, while remaining inactive in the indentations.

See also Figures S4-S6 and Data S2

**STAR METHODS**

**RESOURCE AVAILABILITY**

**Lead contact**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Eugenia Russinova (eurus@psb.vib-ugent.be)

**Materials availability**

Plasmids and transgenic *Arabidopsis* generated in this study are available from the Lead Contact upon request.

**Data and code availability**
- Data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

*Arabidopsis thaliana* transgenic and mutant lines were used to performed experiments. The *Arabidopsis* seeds were surface sterilize with chlorine gas and sown on half-strength Murashige and Skoog (1/2 MS) agar plates without sucrose or in liquid 1/2 MS without sucrose and stratified at 4°C under dark conditions for 2 days or 1 day, respectively. Germinated seeds were grown at 22°C and with a 16-h light (110 μmol m⁻² s⁻¹)/8-h dark photoperiod for 2, 3, 5 or 7 days in walk-in chambers with LED light bulbs (OSRAM L36W/840). When germinated and grown in liquid medium, seeds were shaken at 100 rpm. BL (OlChemIm, Ltd.) or BIK (synthesized in-house) were added to the liquid medium at the indicated concentrations. Wild type tobacco (*Nicotiana benthamiana*) plants were grown in the greenhouse under a 14-h light (93 μmol m⁻² s⁻¹)/10-h dark regime at 25°C.

**METHOD DETAILS**

**Molecular Cloning**

cDNA or genomic DNA from *Arabidopsis thaliana* (L.) Heynh. Columbia-0 (Col-0) were used as templates for PCR cloning. Promoters of *PHGAP1* (1489 bp) and *PHGAP2* (1460 bp), genomic (g) fragments of *gPHGAP1* (5708 bp) and *gPHGAP2* (6745 bp) or cDNAs of *PHGAP1* (2484 bp) and *PHGAP2* (2613 bp) were amplified by PCR with the primers listed in Table S1. cDNA and gDNA fragments of *PHGAP1*(8A) and *PHGAP2*(19A) were generated by DNA synthesis based on the coding sequence of *PHGAP1* (*AT5G12150*, NM_121253) and *PHGAP2* (*AT5G19390*, NM_180711), respectively and by assembly of exons amplified from their cDNAs and introns amplified from their gDNA by means of the Gibson assembly system, respectively. The *PHGAP1* or *PHGAP2* promoters were recombined in the pMK7S*NFm14GW vector⁶⁹ that contains a NLSGFP-GUS expression cassette to generate *PHGAP1* <sup>pro</sup>:NLSGFP-GUS and *PHGAP2*<sup>pro</sup>:NLSGFP-GUS. The constructs were then transformed into Col-0 plants. *PHGAP1* and *PHGAP2* promoters were also combined with *GFP* and the gDNAs of *PHGAP1* and *PHGAP1*(8A) or *PHGAP2* and *PHGAP2*(19A) into the pH7m34GW backbone vector⁷⁰ by means of the MultiSite Gateway system to generate the *PHGAP1*<sup>pro</sup>:GFP-*gPHGAP1*,
**PHGAP1 pro:** GFP-gPHGAP1(8A), **PHGAP2 pro:** GFP-gPHGAP2 or **PHGAP2 pro:** GFP-gPHGAP2(19A) constructs. These constructs were then transformed into phgap1phgap2 plants. **PHGAP1 pro:** mCherry-gPHGAP1 and **PHGAP2 pro:** mCherry-gPHGAP2 were assembled in the pB7m34GW backbone vector\(^7\) with a similar cloning strategy and then transformed into BIN2 pro:BIN2-GFP/Col-0 plants\(^1\).

For transient expression in tobacco, the cDNAs of **PHGAP1** and **PHGAP2** were cloned into pDONR221, whereafter pDONR221-PHGAP1 and pDONR221-PHGAP2 were recombined in pK7WGF2\(^7\) that contained the 35S promoter and N-terminal GFP tag to generate the 35S pro:GFP-PHGAP1 and 35S pro:GFP-PHGAP2 constructs, respectively. pDONR221-BIN2\(^10\) was recombined with the 35S promoter and HA tag into the pK7m34GW vector\(^7\) to generate 35S pro:BIN2-HA. For the yeast two-hybrid (Y2H) experiment, KIB1 cDNA was synthesized and cloned into pDONR221, whereafter pDONR221-KIB1 and the above-described pDONR221-PHGAP1 and pDONR221-PHGAP2 were recombined with the GAL4 activation domain (AD) in pDEST22, whereas pDONR221-BIN2 was recombined with the GAL4-binding domain (BD) in pDEST32.

For the rBiFC\(^41\), the ROP2 cDNA was amplified by PCR and cloned into pDONR-p3p2. The cDNAs of **PHGAP1** and **KIB1** were also cloned into pDONR-p3p2, whereas the cDNAs of **PHGAP2** and **BIN2** were cloned into pDONR-p1p4. To generate pBIFC-nYFP-ROP2+cYFP-PHGAP2, pBIFC-KIB1-nYFP+cYFP-PHGAP2 or pBIFC-BIN2-nYFP+cYFP-PHGAP2, pDONR-p3p2-ROP2, pDONR-p3p2-KIB1 or pDONR-p3p2-BIN2\(^10\) were recombined with pDONR-p1p4-PHGAP2 into pBiFC-2in1-NN or pBiFC-2in1-CN\(^41\) and to generate pBIFC-nYFP-PHGAP1+BIN2-cYFP, pDONR-p3p2-PHGAP1 was recombined with pDONR-p1p4-BIN2 into pBiFC-2in1-NC\(^41\).

For protein purification, the GST-BIN2 in pGEX6P1\(^10\) and MBP-RIC1 in pMAL21 constructs\(^47\) have been described previously. PHGAP1(1-380) and PHGAP2(1-420) were amplified from PHGAP1 or PHGAP2 cDNAs by PCR and cloned into pDONR221. The cDNAs of PHGAP1(8A) and PHGAP2(19A) were also cloned into pDONR221 and pDONR221-PHGAP1, pDONR221-PHGAP1(1-380), pDONR221-PHGAP1(8A), pDONR221-PHGAP2, pDONR221-PHGAP2(1-420) and pDONR221-PHGAP2(19A) were recombined into the bacterial expression vector pDEST-HISMBP\(^73\) containing a tac promoter and the HISMBP tag.

For generation of the CRISPR constructs, gRNAs targeting AtSK11, AtSK12, AtSK13, AtSK21, AtSK22, AtSK23, AtSK32 and AtSK42 were designed with Geneious Prime 2019.1 (https://www.geneious.com). For each gene, two gRNAs were designed to ensure successful gene knockout. Primers are listed in Table S1. The gRNA pairs were cloned into the entry
vectors pGG-A-AtU6-26-BbsI-ccdB-BbsI-B, pGG-B-AtU6-26-BbsI-ccdB-BbsI-C, pGG-C-AtU6-26-BbsI-ccdB-BbsI-D, pGG-D-AtU6-26-BbsI-ccdB-BbsI-E, pGG-E-AtU6-26-BbsI-ccdB-BbsI-F or pGG-F-AtU6-26-BbsI-ccdB-BbsI-G as previously described49. Five pairs of gRNA expression cassettes together with pGG-F-AarI-SacB-AarI-G-G vector were assembled into a CRISPR destination vector of pFASTRK-RPS5AP-AtCas9-NLS-P2A-mCherry-G7T-A-Cmr-ccdB-G72 using a Golden Gate reaction. The assembled CRISPR expression vector was pre-digested with the restriction enzyme AarI and then recombined with the three pairs of gRNA expression cassettes and a linker vector of pGG-D-linkerII-G. The resulting constructs were verified by sequencing and restriction digest. For CRISPR-TSKO in the epidermis, the ML1 promoter was cloned into the entry pGGA000 vector and then recombined with pGG-B-linker-C, pGG-C-Cas9PTA-D, pGG-D-P2A-mCherry-NLS-E, pGG-E-NOST-F and pGG-F-AarI-SacB-AarI-G-G into the destination vector pFASTRK-AG using Golden Gate reaction to generate the TSKO destination vector. The eight pairs of gRNA expression cassettes were assembled into the TSKO destination vector following the same strategy as described above. The CRISPR expression constructs were transformed into both wild type (Col-0) and PHGAP2pro:GFP-gPHGAP2/phgap1/phgap2 (line #3.1.1) plants. To test whether ML1 promoter is capable of driving Cas9 protein and induce gene knockout, a ML1pro:Cas9-P2A-mCherry;GFP-1 construct was generated by the recombination of pGG-A-ML1-B with pFASTR-Bsai-Cmr-ccdB-Bsai-Cas9-P2A-mCherry-G7T-AtU6-GFP-1 vector and transformed into pHTR5:NLS-GFP-GUS/Col-0 plants49.

**Generation of transgenic plants**

Stable transgenic lines were generated by using the standard Agrobacterium tumefaciens (strain C58C1)-mediated transformation74 in the phgap1/phgap2 mutant or Col-0. The transformants were screened by germinating on agar plates containing 10 mg/l BASTA or 20 mg/l Hygromycin according to the plant resistance marker in the binary vector. For the constructs generated with Golden Gate system, selection of transformants was done using fluorescence stereo microscope based on the fluorescent seed marker.

**Quantitative analysis of epidermal pavement cell phenotypes**

For the epidermal pavement cell shape analysis, seedlings at 5 dpg were incubated in 30-50 µM FM4-64 (Sigma-Aldrich) for 30 min with vacuum application to promote penetration of the dye into the cotyledon epidermal layer. Abaxial cotyledons were imaged with a confocal microscope (Leica SP8). A 0.3-mm² area in the middle part of cotyledon was segmented and
quantified with the Image J plugin ‘PaCeQuant’. Circularity levels and lobe counts (total number of lobes along a cell contour) of pavement cells larger than 1400 µm² from 5-8 individual seedlings were analysed.

Confocal Microscopy
Confocal images were acquired with confocal microscope (Leica SP8) with implementation of the gating system for autofluorescence removal. The excitation and detection window settings for GFP and the FM4-64 dye were 488 nm and 515 nm and emission at 500–530 nm and at 570–670 nm, respectively. The 3D images were constructed with the ‘Imaris’ software (imaris.oxinst.com). To induce plasmolysis seedlings were treated with 0.8 M mannitol for 5 min before imaging.

Quantitative RT–PCR
RNA was extracted from approximately 100 mg seedlings at 5 dpg with the RNeasy mini kit according to the manufacturer’s instructions (Qiagen). The cDNA library was generated using 16 µL (100 ng) total RNA and 4 µL qScript cDNA SuperMix (Quantabio) according to manufacturer’s protocol. The resulting cDNA was diluted 1:5 with nuclease-free water. qPCR was run for three technical and biological replicates with 5 µL volumes containing 2.5 µL SYBR green I qPCR master mix (Roche), 0.5 µL diluted cDNA and 2 µL mixed forward and reverse primers (0.5 µM) in a LightCycler® 480 machine (Roche). The cycling conditions were as follows: 95°C, 10 min (preincubation); 95°C, 10 s, 60°C, 15 s, and 72°C, 15 s (45 cycles of amplification); 95°C, 1 s, and 65°C, 1 s (melting curve); 40°C, 10 s (cooling). The integrity of the PCR amplicon was confirmed by melt curve analysis. ACTIN2 (AT3G18780) was used as reference gene and transcript levels were normalized to the endogenous expression in the wild type. The used primers are listed in Table S1.

Co-immunoprecipitation assay (co-IP)
For co-IP in Arabidopsis seedlings co-expressing BIN2pro: gBIN2-GFP with either PHGAPIpro: mCherry-gPHGAPI or PHGAP2pro: mCherry-gPHGAP2 were germinated and grown on 1/2 MS without sucrose; seedlings were harvested at 5 dpg and ground to powder with liquid nitrogen. The fine powder material was resuspended in extraction buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% [v/v] NP-40 and complete EDTA-free protease inhibitor cocktail (Roche)]. The extracts were centrifuged at 20,000 g at 4°C for 15 min, the supernatant was transferred to a new 2-ml tube and spun for an additional 15 min. After centrifugation, the
supernatants were incubated with GFP trap magnetic agarose (Chromotek) for 1 h at 4°C. After incubation, the beads were washed three times with 1 ml extraction buffer, and then with 80 µl elution buffer containing 1× sample loading buffer (LDS; Thermo Fisher) and 1× sample reducing agent (Thermo Fisher) and boiled for 10 min at 75°C. Samples were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by western blot with α-GFP-HRP antibody (Miltenyi Biotec, 1:5000) for GFP-tagged proteins, and with α-RFP antibody (chromotek, 1:2500) followed by α-mouse-HRP antibody (GE Healthcare, 1:10000) for mCherry-tagged proteins. The blot membrane was incubated with primary (α-GFP-HRP and α-RFP) or secondary antibodies (α-mouse-HRP) diluted in tris-buffered saline (TBS) buffer containing 5% skim milk (Thermo Scientific) and 0.1% TWEEN 20 (Sigma-Aldrich) for 1 h at room temperature. For co-IP in tobacco, Agrobacterium tumefaciens (C58C1) cultures expressing constructs of 35Spro:BIN2-HA, 35Spro:GFP-HGAP1, 35Spro:GFP-PHGAP2 or 35Spro:GFP were resuspended in infiltration buffer [10 mM MgCl2; 10 mM MES pH 5.6; 0.1 mM acetoxyringone] for 2 h at room temperature. Re-suspended cultures were mixed together with certain combinations at a final OD600 = 0.5 and infiltrated into tobacco epidermal layers. Tobacco leaves were harvested 3 days after infiltration, ground to powder with liquid nitrogen and resuspended in extraction buffer (150 mM Tris-HCl, pH 7.5; 150 mM NaCl; 10% [v/v] glycerol; 10 mM EDTA; 1% [v/v] NP-40; 10 mM dithiothreitol; 0.5% (w/v) polyvinylpolyprrolidone and complete EDTA-free protease inhibitor cocktail (Roche)] for 30 min at 4°C. The extracts were centrifuged at 18,000 g at 4°C for 20 min and the supernatant was passed through a 40-µm cell filter. The supernatants were incubated with GFP trap magnetic agarose (Chromotek) for 1 h at 4°C. Beads were washed three times with 1 ml of wash buffer [20 mM Tris-HCl pH 7.5; 150 mM NaCl; 0.5% (v/v) NP-40] and then with 80 µl elution buffer containing 1× sample loading buffer (LDS; Thermo Fisher) and 1× sample reducing agent (Thermo Fisher) and boiled for 10 min at 75°C. Samples were then separated by SDS-PAGE and analyzed by western blot with α-GFP-HRP antibody (Miltenyi Biotec) for GFP-tagged proteins, and with α-HA antibody (Sigma-Aldrich) followed by α-mouse-HRP antibody (GE Healthcare) for HA-tagged proteins.

**Yeast two-hybrid (Y2H) assay**

Constructs used for the Y2H experiment were generated as described above. Plasmids with certain combinations were co-transformed into the yeast strain PJ694A by the LiAc-mediated yeast transformation method. Positive yeast transformants were screened on SD-Leu-Trp medium. Protein interactions were tested on the selective medium SD-Leu-Trp-His.
rBiFC assay

Agrobacterium-mediated transformation was used to express the rBiFC constructs transiently in tobacco leaves as described previously\(^{41}\). Imaging was carried out 3 days after infiltration, and the images were taken with identical confocal settings. For the measurement, cell PM was selected with the selection brush tool in ImageJ 1.53c, and the average fluorescence signal intensity of YFP and RFP was measured. The YFP/RFP ratios were calculated to evaluate the strength of interaction\(^{41}\).

In vitro kinase assay and in-gel trypsin digestion for mass spectrometry

HIS-MBP-tagged proteins used in the *in vitro* kinase assays were purified with amylose resin (NEB), whereas GST-tagged proteins were purified with glutathione-sepharose 4B beads (GE-Healthcare). Depending on the experiment, the GST tag was cleaved off or not with PreScission Protease (GE-Healthcare). The *in vitro* kinase assay was carried out by mixing either GST-BIN2 or tag-free BIN2 with the substrate proteins of HISMBP-PHGAP1, HISMBP-PHGAP1(8A), HISMBP-PHGAP1(1-380), HISMBP-PHGAP2, HISMBP-PHGAP2(19A) or HISMBP-PHGAP2(1-420) in 30 µl reaction buffer [5 µCi (\(\gamma\)-\(^{32}\)P) hot ATP; 50 mM Tris-HCl, pH 7.5; 50 mM KCl; 15 mM MgCl\(_2\) and 50 µM cold ATP], and incubated at 25°C for 1 h. The reaction was stopped by adding 11.5 µl of 4× LDS and 4.6 µl 10× sample-reducing agent and boiled at 95°C for 2 min. For the analysis of the phosphorylation sites by mass spectrometry, an *in vitro* kinase assay with cold ATP was carried out following the same steps, but with cold instead of hot ATP. At the end of the reaction, samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by Coomassie blue staining and in-gel trypsin digestion as described previously\(^{10}\). The digested peptides were purified in OMIX C18 Pipette Tips (Agilent Technologies) and were subsequently applied to a liquid chromatography-tandem mass spectrometry system as described previously\(^{10}\).

Western blot analysis

For the comparison of the GFP-PHGAP2 protein abundance between the PHGAP2/phgap1phgap2 and PHGAP2(19A)/phgap1phgap2 plants, seedlings were grown on 1/2 MS without sucrose for 5 days, harvested and frozen in liquid nitrogen. For the CHX chase assay of GFP-PHGAP2 treated with BL and BIK, seedlings at 5 dpg of \(PHGAP2_{pro:GFP-gPPHGAP2/phgap1phgap2}\) grown on 1/2 MS without sucrose were transferred to liquid 1/2 MS medium without sucrose for 2 h and supplemented with 100 µM CHX (Sigma-Aldrich),
DMSO (0.1% [v/v]) and with 100 nM BL or 50 µM BIK for 0, 1, 3, 6 or 12 h. After treatment, seedlings were harvested and frozen in liquid nitrogen. The frozen materials were ground into powder with liquid nitrogen and resuspended in extraction buffer [50 mM Tris-HCl pH 7.5; 150 mM NaCl; 1% [v/v] NP-40 and complete EDTA-free protease inhibitor cocktail (Roche)] for 30 min on ice. The extracts were centrifuged at 20,000 g at 4°C for 15 min, the supernatant was transferred to a new 2-mL tube and spun for an additional 15 min. The supernatant was mixed with 1× sample-loading buffer (LDS; Thermo Fisher) and 1× sample reducing agent (Thermo Fisher) and boiled for 10 min at 75°C. The PHGAP2 protein abundance was detected by western blot with anti-GFP antibody (Miltenyi Biotec) and normalized to the western blot-detected tubulin with α-tubulin (Sigma-Aldrich) and α-mouse-HRP antibodies (GE Healthcare).

**Quantitative image analysis**

Seedlings at 3 dpg of PHGAP2<sub>pro:</sub>GFP-gPPHGAP2/phgap1phgap2 or PHGAP2<sub>pro:</sub>GFP-gPPHGAP2(19A)/phgap1phgap2 grown on 1/2 MS without sucrose were transferred to liquid 1/2 MS medium containing DMSO (0.1% [v/v]) and 100 nM BL or 50 µM BIK for 16 h at 21°C with mild shaking at 100 rpm. Abaxial cotyledons were imaged with confocal microscope (Leica SP8) after treatment. To evaluate the effect of exogenous BL and BIK on the indentation enrichment of GFP-PHGAP2, pavement cell wall undulations were subdivided into indentation region and outside region based on the full width at half-maximal lobe height, and the relative indentation enrichment value of GFP-PHGAP2 (average GFP intensity in the indentation region / average GFP intensity in the outside region) was measured. Undulations were separated into peak (average GFP intensity in the indentation region / average GFP intensity in the outside region > 1.5), partial peak (average GFP intensity in the indentation region / average GFP intensity in the outside region within 1.2-1.5) and diffuse (average GFP intensity in the indentation region / average GFP intensity in the outside region < 1.2), and the proportions of different types of undulations were quantified.

**ROP2 and ROP6 activity assays in protoplasts**

Protoplasts were isolated from 4-week-old leaves of Col-0 or the phgap1phgap2 mutant as described previously<sup>47</sup>. Isolated protoplasts were treated with different concentrations of BL, BIK or NAA and various durations. The ROP2 or ROP6 activity assays were done as described previously<sup>47</sup>. In brief, either active ROP2 or ROP6 was pulled down by MBP-RIC1-conjugated agarose beads from the protoplast total protein extracts and detected by western blot with anti-ROP2 or anti-ROP6 antibodies.
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were carried out in Excel. Statistically significant differences were determined with a single-factor analysis of variance (ANOVA) test. Quantification of rBiFC and all cell shape measurements were presented as individual value plots in columns and with whiskers representing means and SD. Asterisks indicated the $P$ value $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Data S1. Identification of BIN2 interactome in the stomatal lineage of Arabidopsis by IP-MS and the identification of PHGAP1 and PHGAP2 phosphorylation sites. Related to Figure 1 and Figure 3.

A Significantly enriched proteins in BIN2 pull downs using $TMM_{pro}$:BIN2-GSgreen/bin2-3 seedlings vs control GFP pull down using $35S_{pro}$:GFP/Col-0 seedlings. LC-MS/MS data acquired on a Q Exactive. Analysis with MaxQuant and Perseus. FDR=0.01, S0=1.

B List of peptides identified by LC-MS/MS in the $TMM_{pro}$:BIN2-Gsgreen/bin2-3 IP.

C Phosphosite analysis for PHGAP1 and PHGAP2 by LC-MS/MS. Data acquired on a Q Exactive. Analysis with MaxQuant.

D MaxQuant search parameters for BIN2 IP-MS and PHGAP1 and PHGAP2 phosphorylation-sites identification

Data S2. CRISPR analysis. Related to Figure S5 and Figure 6.

A Sequencing analysis of 5dpg atsk$^{octu}$ and GFP-PHGAP2/atsk$^{octu}$ plants in T1 generation. Sanger trace data were analyzed using online software "ICE analysis" (https://ice.synthego.com/#/). Indel %, percentage of the pool with non-wild type sequence. Knockout score, ICE KO score. R indicates the Pearson correlation coefficient.

B Relative contribution of each edited sequence. Indel, Insertion/deletion mutations. [g1] and [g2] represent guide RNA1 and guide RNA2, respectively. Indel 0 indicates the wild type sequence. Black vertical lines in the sequences represent the cut sites.

REFERENCES


