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Antagonistic SnRK2 and PID kinases' action on auxin transport–mediated root gravitropism

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Plants have evolved sophisticated mechanisms to adapt to environmental changes, with root gravitropism playing a pivotal role in nutrient and water acquisition. Our study reveals that SnRK2 kinases (SnRK2.2 and SnRK2.3) are critical regulators of root gravitropism through their direct phosphorylation of the auxin transporter PIN2 at S259. We demonstrate that SnRK2s-mediated phosphorylation modulates both the polar localization and transport activity of PIN2. Importantly, SnRK2s function antagonistically to the AGCVIII kinase PID, which phosphorylates PIN2 at a distinct site (S258), establishing a regulatory balance essential for adaptive root growth. Structural modeling and phosphorylation assays further suggest that SnRK2s-mediated phosphorylation at S259 sterically hinders access of PID to S258, providing a mechanistic basis for their antagonistic relationship. These findings uncover a novel regulatory mechanism, by which plants fine-tune root developmental programs to adapt to environmental stimuli, highlighting the evolutionary significance of multilayered kinase-mediated regulation in plant adaptation.

kinases | auxin transport | root gravitropism

Plants have developed complex mechanisms to cope with environmental fluctuations. A key adaptive mechanism is the gravitropic response of plant roots, enabling deeper soil penetration for essential water and nutrient acquisition (1, 2). The root gravitropism is mediated by polar auxin transport through PIN-FORMED (PIN) exporters (3). Although phosphorylation modifications regulate PIN function (3–5), the involved kinases and their mechanisms remain incompletely understood, suggesting a greater diversity of regulatory components than presently identified.

We screened Arabidopsis kinase–deficient mutants and found *sucrose non-fermenting-1* (*SNF1*)*-related protein kinase 2s* (*SnRK2s*) double mutants (*snrk2.2 snrk2.3*, *snrk2.2 snrk2.6,* and *snrk2.3 snrk2.6*) with impaired gravitropic responses (Fig. 1*A*). SnRK2 kinases are well-characterized components of abscisic acid signaling transduction, with established roles in root hydrotropism (6). Since root hydrotropic and gravitropic responses are mechanistically distinct (6, 7), we sought to investigate the specific role of SnRK2s in modulating root gravitropism.

Root gravitropism requires asymmetric auxin distribution (8). In *snrk2.2 snrk2.3* mutant, auxin redistribution was impaired upon gravistimulation (Fig. 1 *B* and *C*), and both shoot-to-root and root-to-shoot auxin transport were reduced (Fig. 1 *D* and *E*). Given that auxin transport and gravitropic responses are mediated by PIN auxin transporters, particularly PIN2, which exhibits apical (shootward) localization in epidermal cells and basal (rootward) polarization in young cortical cells (9), we examined PIN2 distribution in the mutant. Notably, basal localization of PIN2 in cortical cells and apical localization in epidermal cells were both reduced in *snrk2.2 snrk2.3* roots (Fig. 1 *F–H*). These results suggest SnRK2s are required for PIN2 polar localization, thereby enabling auxin transport and auxin distribution–driven root gravitropism.

To determine whether SnRK2s directly associate with PIN2, we performed coimmunoprecipitation (Co-IP) assays in Arabidopsis protoplasts coexpressing SnRK2.2/2.3-GFP and Flag-tagged PIN2 hydrophilic loop (PIN2HL). Both SnRK2s were specifically coprecipitated with PIN2HL (Fig. 1*I*). This interaction was further validated by firefly luciferase complementation imaging (LCI) and in vitro pull-down assays (Fig. 1 *J* and *K*). In planta, liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis of anti-Flag immunoprecipitates from *Super::SnRK2.3-Flag* roots identified PIN2 peptides (Dataset S1), confirming that SnRK2s physically associate with PIN2 both in vitro and in vivo.

Given that PIN polarity is regulated by kinase-mediated phosphorylation (3–5), we tested whether PIN2 serves as a SnRK2 substrate. In vitro phosphorylation assays revealed that SnRK2.2/2.3 exhibited autophosphorylation activity and efficiently phosphorylated PIN2HL (Fig. 1*L*). In-gel kinase assays confirmed SnRK2 activity under normal growth

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The authors declare no competing interest.

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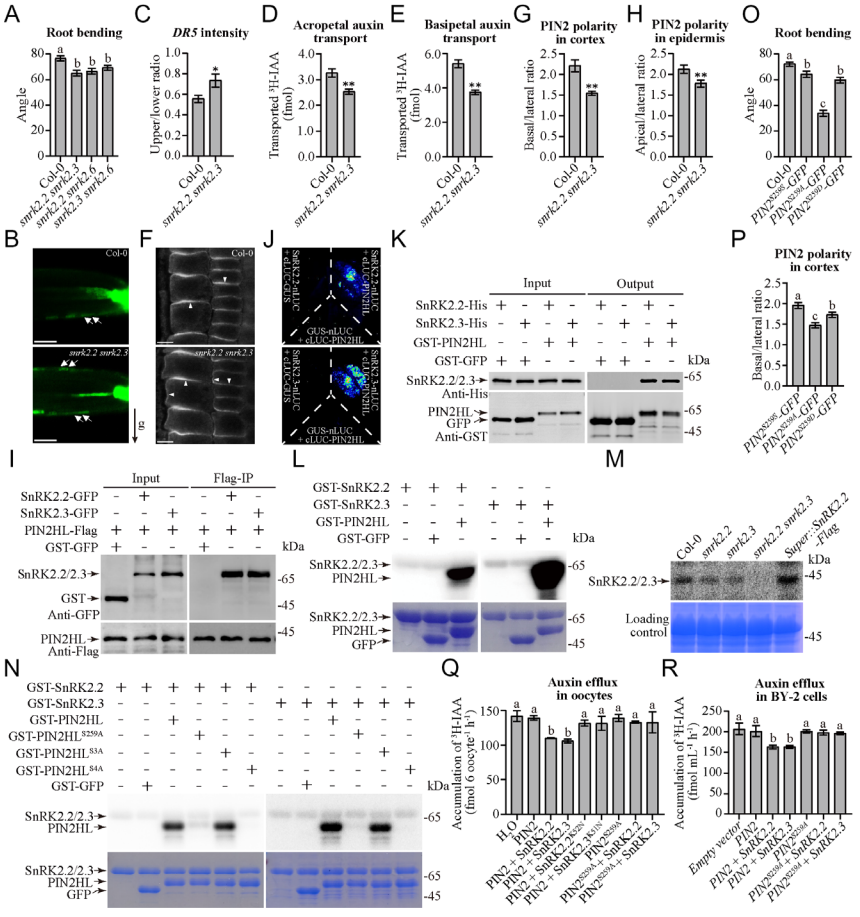

Fig. 1. SnRK2s phosphorylate PIN2 and regulate its function. (*A*) Gravitropic defects in *SnRK2* double mutants ($n \geq 13$ roots). (*B–E*) Impaired auxin translocation (*B* and *C*), acropetal and basipetal transport (*D* and *E*) in *snrk2.2 snrk2.3* roots ($n \geq 8$ roots). Black arrow: gravity vector; white arrows: auxin flow. (Scale bars, 50 μm.) (*F–H*) Disrupted PIN2 polarity in cortex and epidermis of *snrk2.2 snrk2.3* roots ($n \geq 46$ cells). Arrowheads: PIN2 polarity. (Scale bars, 5 μm.) (*I–K*) SnRK2s–PIN2HL interaction by Co-IP (*I*), LCI (*J*), pull-down (*K*). (*L*) In vitro phosphorylation of PIN2HL by SnRK2s. (*M*) In-gel kinase assays of SnRK2s activity. (*N*) S259 identified as the primary PIN2 phosphosite for SnRK2s. (*O* and *P*) Gravitropism (O, $n = 15$ roots) and cortical PIN2 polarity (P, $n \geq 35$ cells) in *PIN2*^{S259A}/*PIN2*^{S259D}*-GFP* lines. (*Q* and *R*) Auxin efflux in oocytes (*Q*) and BY-2 cells (*R*) ($n = 3$ replicates).

conditions (Fig. 1*M*). LC–MS/MS analysis identified four putative phosphorylation sites (S210/S259/S331/S409), which we mutated to non-phosphorylatable Alanine residues: S259A, S210A/S331A/S409A (S3A), or S210A/S259A/S331A/S409A (S4A). Phosphorylation of GST-PIN2HL^{S259A} and GST-PIN2HL^{S4A} was significantly reduced (Fig. 1*N*), indicating S259 being the primary target. Notably, PIN2^{S259A} exacerbated the root agravitropic phenotype in *pin2* mutants and impaired basal cortical localization compared to PIN2^{S259S} (Fig. 1 *O* and *P*). Together with its disrupted apical epidermal polarity (10), these results demonstrate that S259 phosphorylation is critical for correct PIN2 polarity and thus its function.

To determine whether SnRK2s directly activate PIN2-mediated auxin efflux, we conducted transport assays in *Xenopus oocytes* and tobacco BY-2 cells. While PIN2 expression alone showed no auxin efflux activity, coexpression with SnRK2.2/2.3 significantly enhanced transport in oocytes (Fig. 1*Q*). This activation required both kinase activity and PIN2 phosphorylation, as neither kinase-dead SnRK2s with wild-type PIN2 nor wild-type SnRK2s with phosphor-dead PIN2^{S259A} could stimulate auxin efflux (Fig. 1*Q*). Consistently, BY-2 cells coexpressing PIN2 with SnRK2.2/2.3 showed reduced ^{3}H-IAA accumulation (Fig. 1*R*). Collectively, SnRK2s function as activators of PIN2-mediated auxin efflux.

We next investigated potential cooperation between SnRK2s and PINOID (PID), an AGCVIII kinase that can phosphorylate PIN2 at the neighboring S258 and regulate PIN polarity (11–13). While Co-IP, LCI, and pull-down assays confirmed SnRK2s–PID interactions (Fig. 2 *A–C*), in vitro phosphorylation assays showed mutual autophosphorylation but no cross-phosphorylation (Fig. 2 *D* and *E*).

To evaluate SnRK2s' regulation of PID activity, we first transiently expressed *Super::PID-GFP* in wild-type and *snrk2.2 snrk2.3* protoplasts. Notably, PID-mediated PIN2 phosphorylation was enhanced in the mutant (Fig. 2*F*). We next investigated the effect of SnRK2s on the PID activity in heterologous expression systems. Coexpression of SnRK2.2/2.3 with PIN2 and PID in oocytes resulted in significantly higher ^{3}H-IAA accumulation than PIN2 and PID alone (Fig. 2*G*), consistent with PIN2 activity inhibition. Notably, kinase-dead SnRK2s did not inhibit PID-mediated PIN2 activation, and wild-type SnRK2s did not suppress PID-driven activation of the PIN2^{S259A} variant (Fig. 2*G*). Similarly, SnRK2s antagonized PID-dependent PIN2 activation also in BY-2 cells (Fig. 2*H*). Genetic analysis further confirmed this antagonistic relationship, with *SnRK2.2* overexpression delaying *35S::PID*-induced root meristem collapse (Fig. 2*I*). Collectively, these findings suggest that SnRK2s negatively regulate PID activity.

Structural modeling using AlphaFold 3 revealed the molecular basis for SnRK2s–PID antagonism. The PID–ATP–PIN2HL complex showed PIN2HL^{S258} optimally positioned for phosphorylation (Fig. 2*J*), which was stabilized by polar contacts of PIN2HL^{R256} and PIN2HL^{S259} with the adjacent residues in PID catalytic center (Fig. 2 *K* and *L*). Phosphorylation at PIN2HL^{S259} would disrupt this configuration through steric hindrance (Fig. 2*M*), explaining the reduced PID-mediated phosphorylation of PIN2HL^{S259D} versus wild-type PIN2HL or PIN2HL^{S259A} variant (Fig. 2*N*). These results suggest that SnRK2s-mediated PIN2HL^{S259} phosphorylation disrupts the PID-mediated phosphorylation efficiency of PIN2HL^{S258}.

Root gravitropism is an essential adaptive strategy for nutrient acquisition, particularly nitrate (NO_3^-) (1, 2). We analyzed CRISPR/Cas9 knockouts of maize *ZmSnRK2.9* and *ZmSnRK2.10*, orthologs of Arabidopsis *SnRK2.2/2.3* (~74 to 80% similarity) with

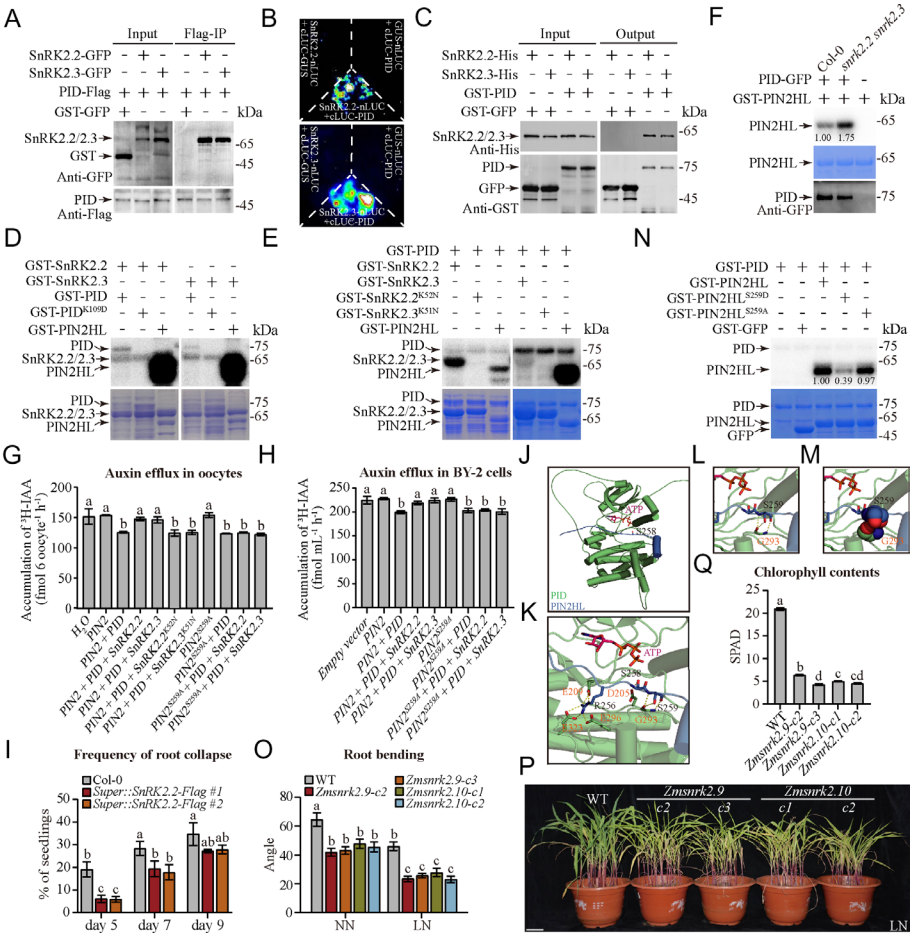

Fig. 2. SnRK2s interact with and inhibit PID activity. (*A*–*C*) SnRK2s–PID interaction by Co-IP (*A*), LCI (*B*), pull-down (*C*). (*D* and *E*) Nonphosphorylation of kinase-dead PID^{K109D} (*D*) or SnRK2.2^{K52N}/SnRK2.3^{K51N} (*E*) by SnRK2s or PID. (*F*) Enhanced PID-mediated phosphorylation in *snrk2.2 snrk2.3* protoplasts. (*G* and *H*) Auxin efflux in oocytes (*G*) and BY-2 cells (*H*) (n = 3 replicates). (*I*) *SnRK2.2* overexpression delayed *35S::PID*-mediated root collapse (n = 3 replicates). (*J*–*M*) Structural model of PID–ATP–PIN-2HL complex. (*N*) Reduced PID phosphorylation of phospho-mimic PIN2HL^{S259D}. (*O*-*Q*) *Zmsnrk2* mutants showing impaired root gravitropism (*O*, n = 8 roots) and accelerated leaf yellowing (*P* and *Q*, n = 16 leaves) under normal- and/or low-NO_3^- conditions. (Scale bars, 10 cm.)

root-enriched expression (14). Under normal conditions, *Zmsnrk2.9* and *Zmsnrk2.10* mutants exhibited impaired gravitropism, with aggravated defects under low NO_3^- (Fig. 2*O*). Concomitantly, these mutants showed enhanced leaf yellowing under NO_3^- limitation compared to wild type (Fig. 2 *P* and *Q*). These results suggest that ZmSnRK2s positively regulate gravitropism and stress adaptation, potentially via root–nutrient coordination, though additional regulatory factors may contribute.

Our findings demonstrate that SnRK2.2/2.3 phosphorylate PIN2 at S259, regulating both its polar localization and transport activity. Importantly, SnRK2s function antagonistically to PID-mediated PIN2 phosphorylation, establishing a regulatory balance essential for maintaining proper root growth. These findings uncover an unexpected mechanism, through which phosphorylation of one residue by one type of kinase will affect regulatory phosphorylation of another residue by a different kinase. In our uncovered case of regulation of PIN2 auxin transporter by PID and SnRK2 kinases, plants fine-tune their root development in response to environmental cues.

Materials and Methods

Arabidopsis Columbia-0 (Col-0) and maize ND101 were used as wild-type controls. Experimental details are provided in *SI Appendix*. Materials and protocols are available upon request.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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