

Arabidopsis Receptor of Activated C Kinase1 Phosphorylation by WITH NO LYSINE8 KINASE^{1[OPEN]}

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Receptor of activated C kinase1 (RACK1) is a versatile scaffold protein that binds to numerous proteins to regulate diverse cellular pathways in mammals. In *Arabidopsis* (*Arabidopsis thaliana*), RACK1 has been shown to regulate plant hormone signaling, stress responses, and multiple processes of growth and development. However, little is known about the molecular mechanism underlying these regulations. Here, we show that an atypical serine (Ser)/threonine (Thr) protein kinase, WITH NO LYSINE8 (WNK8), phosphorylates RACK1. WNK8 physically interacted with and phosphorylated RACK1 proteins at two residues: Ser-122 and Thr-162. Genetic epistasis analysis of *rack1 wnk8* double mutants indicated that RACK1 acts downstream of WNK8 in the glucose responsiveness and flowering pathways. The phosphorylation-dead form, RACK1A^{S122A/T162A}, but not the phosphomimetic form, RACK1A^{S122D/T162E}, rescued the *rack1a* null mutant, implying that phosphorylation at Ser-122 and Thr-162 negatively regulates RACK1A function. The transcript of RACK1A^{S122D/T162E} accumulated at similar levels as those of RACK1^{S122A/T162A}. However, although the steady-state level of the RACK1A^{S122A/T162A} protein was similar to wild-type RACK1A protein, the RACK1A^{S122D/T162E} protein was nearly undetectable, suggesting that phosphorylation affects the stability of RACK1A proteins. Taken together, these results suggest that RACK1 is phosphorylated by WNK8 and that phosphorylation negatively regulates RACK1 function by influencing its protein stability.

Receptor for activated C kinase1 (RACK1) is an evolutionarily conserved scaffold protein that was originally identified as a receptor for activated protein kinase C in mammalian cells (Mochly-Rosen et al., 1991; Ron et al., 1994). Subsequent studies indicated that RACK1 binds many other proteins, and consequently,

RACK1 is now viewed as a versatile scaffold protein that regulates diverse cellular pathways in animals (McCahill et al., 2002; Adams et al., 2011; Ron et al., 2013). For example, human RACK1 scaffolds an ADP ribosylation factor GTPase Accelerating Protein and focal adhesion kinase to neuronal outgrowths to control focal adhesion kinase activity and consequently, cell adhesion (Dwane et al., 2014).

The first plant *RACK1* gene was cloned from tobacco (*Nicotiana tabacum*) Bright Yellow-2 cells as an auxin-induced gene, *arcA* (Ishida et al., 1993). RACK1 homologs are found in all plant species, and both the protein sequences and the crystal structure of RACK1 are highly conserved in plants (Chen et al., 2006b; Guo et al., 2007; Ullah et al., 2008). Like its counterpart in mammals, plant RACK1 protein interacts with nearly 100 proteins that fall into many different functional categories (Guo et al., 2007; Klopffleisch et al., 2011; Olejnik et al., 2011; Kundu et al., 2013). RACK1 is involved in plant hormone signaling (McKhann et al., 1997; Perennes et al., 1999; Chen et al., 2006a, 2006b; Guo et al., 2009a, 2009b; Fennell et al., 2012), leaf and root development (Guo and Chen, 2008; Guo et al., 2009b), drought and salt stress responses (Ullah et al., 2008; Guo et al., 2009a), flooding stress (Komatsu et al., 2014), nodulation (Islas-Flores et al., 2011, 2012), seed germination (Komatsu et al., 2005; Islas-Flores et al., 2009; Zhang et al., 2014), hydrogen peroxide production (Zhang et al., 2014), innate immunity (Nakashima et al., 2008), plant response to fungal pathogens (Wang et al., 2014), association with ribosomes (Chang et al., 2005; Gialvalisco

¹ This work was supported by the National Institute of General Medical Sciences (grant no. R01GM065989), the U.S. Department of Energy (grant no. DE-FG02-05er15671), the National Science Foundation (grant nos. MCB-0723515 and MCB-0718202 to A.M.J.), the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory (to J.-G.C.), the Oak Ridge National Laboratory (managed by UT-Battelle, LLC for the U.S. Department of Energy under contract no. DE-AC05-00OR22725), the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences of the U.S. Department of Energy (technical support through grant no. DE-FG02-05er15671 to A.M.J.), and the China Scholarship Council (scholarship to X.W.).

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D.U., A.M.J., and J.-G.C. conceived and designed the experiments; D.U., O.C., and X.W. performed the experiments; D.U., O.C., X.W., A.M.J., and J.-G.C. analyzed the data; D.U., O.C., X.W., A.M.J., and J.-G.C. wrote the article.

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www.plantphysiol.org/cgi/doi/10.1104/pp.114.247460

et al., 2005), protein translation (Guo et al., 2011), and microRNA abundance (Speth et al., 2013). However, little is known about the molecular mechanism of action of RACK1.

RACK1 contains a seven-Trp-Asp repeat domain (WD40) similar to the heterotrimeric GTP-binding protein β -subunit (Ullah et al., 2008). We previously screened for *Arabidopsis thaliana* RACK1

interacting partners (Klopffleisch et al., 2011) and found that RACK1B and RACK1C interact with WITH NO LYS KINASE8 (WNK8), a member of 10 *Arabidopsis* WNK family kinases (Nakamichi et al., 2002; Wang et al., 2008). WNKs are composed of an N-terminal kinase domain and a C-terminal regulatory domain. WNKs have an atypical displacement of a catalytic Lys residue within the kinase subdomain II (Xu et al., 2000;

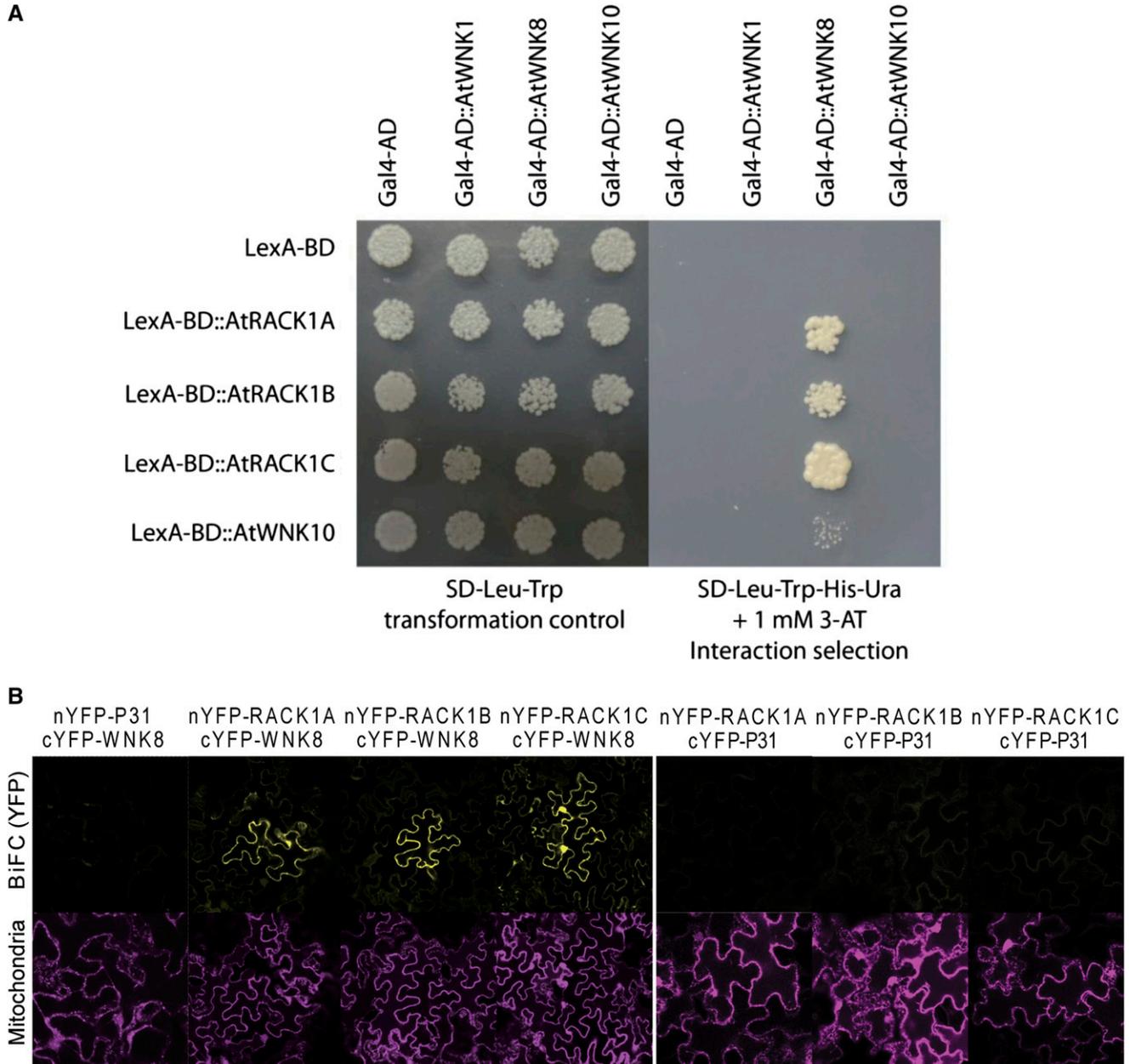


Figure 1. WNK8 interacts with RACK1. A, Y2H assays. Yeasts expressing Gal4-AD and LexA-BD fusion proteins were grown on Synthetic Defined (SD)-Leu-Trp medium to validate efficient transformation or SD-Leu-Trp-His-Ura + 3-amino-1,2,4-triazole (3-AT) selection medium to test interactions of the indicated protein pairs. B, BiFC assay. Carboxyl terminal domain of yellow fluorescent protein (cYFP)-tagged WNK8 was expressed with amino-terminal domain of YFP (nYFP)-tagged RACK1A, RACK1B, or RACK1C in tobacco leaf epidermal cells. mCherry-mitochondrial marker was coinfecting as the expression control. Complementation of split YFP and fluorescence of mCherry are shown. cYFP-tagged WNK8 expressed with nYFP-tagged P31 and nYFP-tagged RACK1A, RACK1B, and RACK1C expressed with cYFP-tagged P31 were used as negative controls.

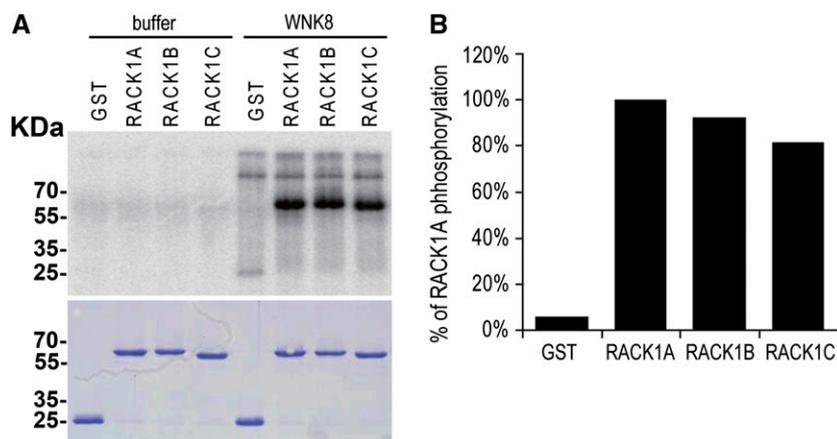


Figure 2. WNK8 phosphorylates RACK1. A, Recombinant GST or GST-tagged RACK1A, RACK1B, or RACK1C protein was incubated with [γ - 32 P] ATP and GST-WNK8 for 6 h at room temperature. Phosphorylated proteins were then separated with SDS-PAGE and detected with a phosphoimage analyzer. Bottom shows Coomassie Blue staining of the gel. B, Quantification of phosphorylation level of RACK1 proteins.

Huang et al., 2007). This Lys residue is conserved among all other kinases and essential for the coordination of ATP in the active center (Xu et al., 2000; Huang et al., 2007).

In this study, we report that RACK1 is a substrate of WNK8 and that phosphorylation negatively affects RACK1 function. Recombinant WNK8 physically

bound and phosphorylated three Arabidopsis RACK1 proteins: RACK1A, RACK1B, and RACK1C. The phosphomimetic mutations on RACK1A abolished its expression at the protein level but not at the transcript level. These results reveal a regulatory system in which the action of RACK1 is controlled by phosphorylation and subsequent protein degradation.

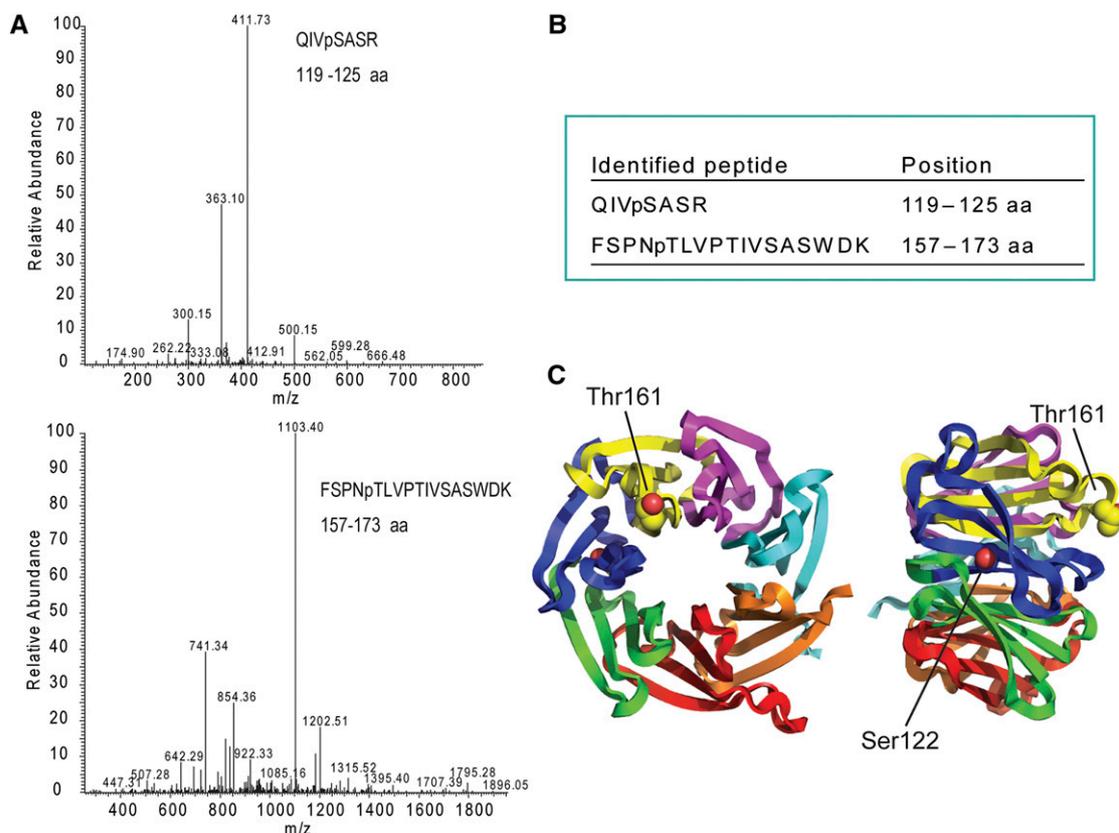


Figure 3. RACK1 phosphorylation sites. A, Liquid chromatography-tandem mass spectrometry (LC-MS/MS) spectra of phosphorylated peptides of RACK1C. GST-RACK1C protein was phosphorylated by WNK8 and digested with trypsin. The digested sample was supplied to LC-MS/MS analysis. Peaks corresponding to phosphorylated RACK1 peptide were obtained from MS/MS analysis. B, Phosphorylated peptides identified by MS. RACK1C phosphorylated by WNK8 was used for MS analysis. Amino acid sequences and positions of two phosphorylated peptides are shown. pS, Phosphorylated Ser; pT, phosphorylated Thr. C, Phosphorylation sites on Arabidopsis RACK1A structure (Protein Data Bank ID code 3DM0; Ullah et al., 2008). Each WD40 repeat is drawn with different colors. Two phosphorylation sites identified by MS are drawn with space fill model. Note that Thr-162 of RACK1A corresponds to Thr-161 of RACK1C.

RESULTS

WNK8 Interacts with RACK1

We previously reported interaction between WNK8 and RACK1 proteins (Kloppfleisch et al., 2011). To analyze subclass specificity of the interaction, we tested complementation by yeast two-hybrid (Y2H) assay of the full-length open reading frame (ORF) of Arabidopsis WNK8 against each of three Arabidopsis RACK1 proteins: RACK1A, RACK1B, and RACK1C. We found that all three RACK1 proteins interacted with WNK8 (Fig. 1A). We also tested two other WNK family kinases, WNK1 and WNK10. WNK1 is the most divergent from WNK8, whereas WNK10 is the most similar to WNK8 at the amino acid sequence level (Urano et al., 2012). Neither WNK1 nor WNK10 interacted with RACK1 proteins in the Y2H assays (Fig. 1A), suggesting that, among WNK family kinases, the interaction between WNK8 and RACK1 proteins is specific.

To validate the interactions between WNK8 and RACK1 proteins in vivo, we used bimolecular fluorescence complementation (BiFC) by expressing YFP fragment-tagged test proteins in tobacco leaf epidermal cells (Fig. 1B). Consistent with the results of Y2H assays, WNK8 interacted with all three RACK1 proteins, whereas a soluble protein, P31, used as a control did not interact with WNK8 or RACK1 proteins.

WNK8 Phosphorylates RACK1

As shown above (Fig. 1), RACK1A, RACK1B, and RACK1C interacted with WNK8 in yeast (*Saccharomyces cerevisiae*) and in planta. WNK8 has been reported as a functional kinase through molecular and genetic studies (Hong-Hermesdorf et al., 2006; Tsuchiya and Eulgem, 2010; Urano et al.; 2012); therefore, we tested if WNK8 phosphorylates RACK1 proteins in vitro using recombinant proteins and radio-labeled [γ ³²P]ATP. WNK8 phosphorylated all three RACK1 proteins (Fig. 2). No substrate specificity was observed between the three RACK1 proteins. Phosphorylation sites were determined by MS (Fig. 3). Recombinant RACK1C protein phosphorylated by WNK8 contained two phosphorylated sites: Ser-122 and Thr-161 (Fig. 3, A and B). The Ser-122 on the side of blade 3 of RACK1 (Fig. 3C) is conserved in all three Arabidopsis RACK1 proteins (RACK1A, RACK1B, and RACK1C), both rice (*Oryza sativa*) RACK1 proteins (OsRACK1A and OsRACK1B), and RACK1 proteins from human, fruit fly, and yeast (Supplemental Fig. S1). Thr-161 of RACK1C (Thr-162 of RACK1A and Thr-161 of RACK1B) is present in all three Arabidopsis RACK1 proteins and one of two rice RACK1 proteins (OsRACK1A) but not conserved in nonplant species, indicating that the Thr-161 phosphorylation site is plant specific.

Ser-122 Is the Dominant Phosphorylation Site of RACK1 by WNK8

MS covered only 81% of the amino acids of RACK1 protein (Supplemental Fig. S2); therefore, we asked whether Ser-122 and Thr-161 represent major phosphorylation sites by using RACK1A mutant proteins (Fig. 4). We substituted Ser-122 and Thr-162 of RACK1A with Asp and Glu, respectively, to create two RACK1 phosphomimetic mutant proteins RACK1A^{S122D} and RACK1A^{T162E}, respectively, and tested the phosphorylation of the mutant proteins by WNK8. These two amino acid substitutions are commonly used as phosphomimetics that mimic the phosphorylated isoform. As shown in Figure 4, mutation of S122D abolished the phosphorylation of RACK1A protein, whereas the mutation of T162E did not affect the phosphorylation. To further test this, we created a mutant protein containing mutations at both Ser-122 and Thr-162 (RACK1A^{S122D/T162E}). Similar to the RACK1A^{S122D} single-mutant protein, the phosphorylation of the RACK1A^{S122D/T162E} double mutant by WNK8 was abolished (Fig. 4). These results indicate that Ser-122 is the single major phosphorylation site of RACK1A by WNK8.

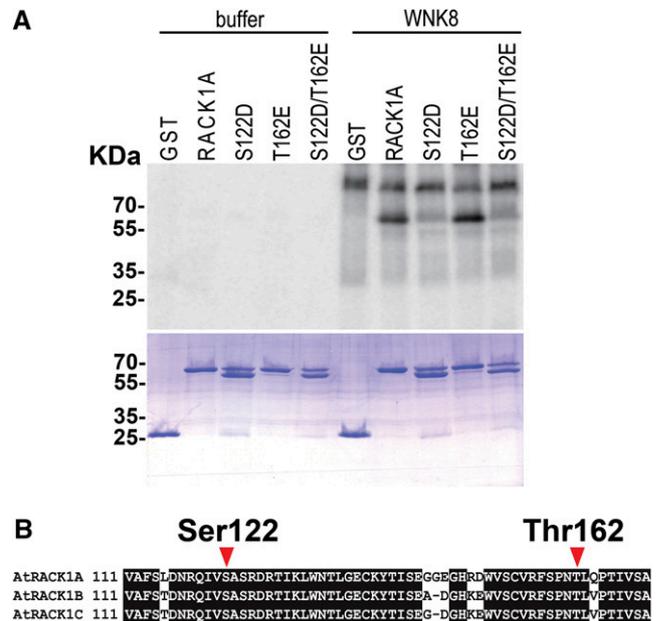


Figure 4. Ser-122 is required and sufficient for the phosphorylation of RACK1. A, Recombinant GST-tagged RACK1A and the point mutants were incubated with [γ -³²P]ATP and GST-WNK8 for 6 h at room temperature. Proteins were then separated by SDS-PAGE: S122D, RACK1A^{S122D}, T162E, RACK1A^{T162E}, and S122D/T162E, RACK1A^{S122D/T162E}. Note that the Asp substitution at Ser-122 induced a partial cleavage of RACK1A protein, which resulted in a cleaved protein that was approximately 5 kD smaller than the native RACK1A protein. Neither the intact protein nor the cleaved protein was phosphorylated by WNK8. B, Partial amino acid sequence indicating the positions of Ser-122 and Thr-162. Thr-161 of RACK1C corresponds to Thr-161 of RACK1B and Thr-162 of RACK1A.

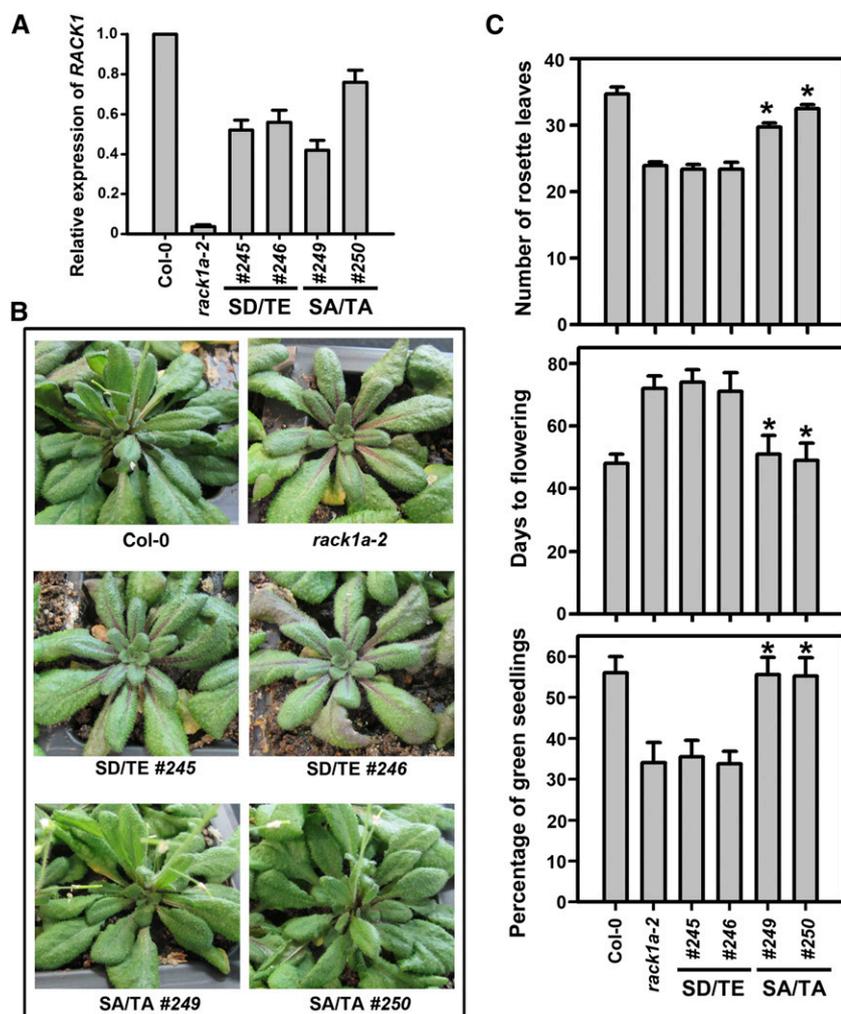


Figure 5. Genetic complementation of *rack1a* mutants. **A**, qRT-PCR analysis of *RACK1A* transcripts. Total RNAs were isolated from rosette leaves of 4-week-old plants. Amplification of *ACTIN8* was used as a control. Shown are means of three replicates \pm SE. SA/TA, *RACK1A*^{S122A/T162A}; SD/TE, *RACK1A*^{S122D/T162E}. **B**, Seven-week-old transgenic plants. Plants were grown under a 10-h-light/14-h-dark photoperiod. **C**, Rosette leaf production, days to flowering, and Glc sensitivity. Shown are means of a minimum 10 plants \pm SE for rosette leaf production and flowering assays. The percentages of green seedlings were recorded 10 d after seeds had been transferred to germination conditions with 6% Glc. At 1% Glc, the percentages of green seedlings for all genotypes were 100%. Shown are means of three biological replicates \pm SE. *, Significant difference from *rack1a-2* mutant, $P < 0.05$.

Phosphorylation Affects RACK1 Function

Having shown that RACK1 proteins are phosphorylated by WNK8 at Ser-122 and Thr-162, we investigated the biological consequence of RACK1 phosphorylation. We tested whether expression of the double phosphomimetic form, *RACK1A*^{S122D/T162E}, rescues the *rack1a* mutant phenotypes. In addition, we created a double phosphorylation-dead isoform of RACK1A by substituting Ser-122 and Thr-162 with Ala (*RACK1A*^{S122A/T162A}). The expression of the phosphomimetic form (*RACK1A*^{S122D/T162E}) and the phosphorylation-dead form (*RACK1A*^{S122A/T162A}) was driven by *RACK1A*'s native promoter (Guo et al., 2009a). $P_{RACK1A}::RACK1A^{S122D/T162E}$ or $P_{RACK1A}::RACK1A^{S122A/T162A}$ was transformed into the *rack1-2* null mutant background. Quantitative reverse transcription (qRT)-PCR analysis indicated that both *RACK1A*^{S122D/T162E} (lines 245 and 246) and *RACK1A*^{S122A/T162A} (lines 249 and 250) were expressed in the transgenic lines (Fig. 5A). One of the characteristic phenotypes of the *rack1a* mutant, rosette leaf production (Chen et al., 2006a, 2006b), was used to determine genetic complementation. Under a 10-h-light/14-h-dark photoperiod (23°C) in the

vegetative growth stage, the Columbia-0 (Col-0) wild type produced approximately 35 rosette leaves within 40 d, whereas the *rack1a-2* mutant produced approximately 24 rosette leaves (Fig. 5, B and C). Expression of the phosphomimetic form, *RACK1A*^{S122D/T162E}, had no effect on rosette leaf production in the *rack1a-2* mutant background, indicating that *RACK1A*^{S122D/T162E} is not able to rescue *rack1-2* mutants. In contrast, expression of the phosphorylation-dead form, *RACK1A*^{S122A/T162A}, resulted in near-full rescue of rosette leaf production phenotype of *rack1a-2* mutants (Fig. 5C). Similarly, the late flowering and Glc hypersensitivity phenotypes of *rack1a* mutant were rescued by the expression of *RACK1A*^{S122A/T162A} but not *RACK1A*^{S122D/T162E} (Fig. 5C). These results imply that phosphorylation negatively affects RACK1A function and that the nonphosphorylated isoform of RACK1 is required for its function.

To further explore the possible mechanism of RACK1 phosphorylation, we examined whether phosphorylation affects the stability of RACK1 protein in plants. We used an anti-RACK1A peptide antibody to detect RACK1 protein in transgenic lines. Interestingly, we

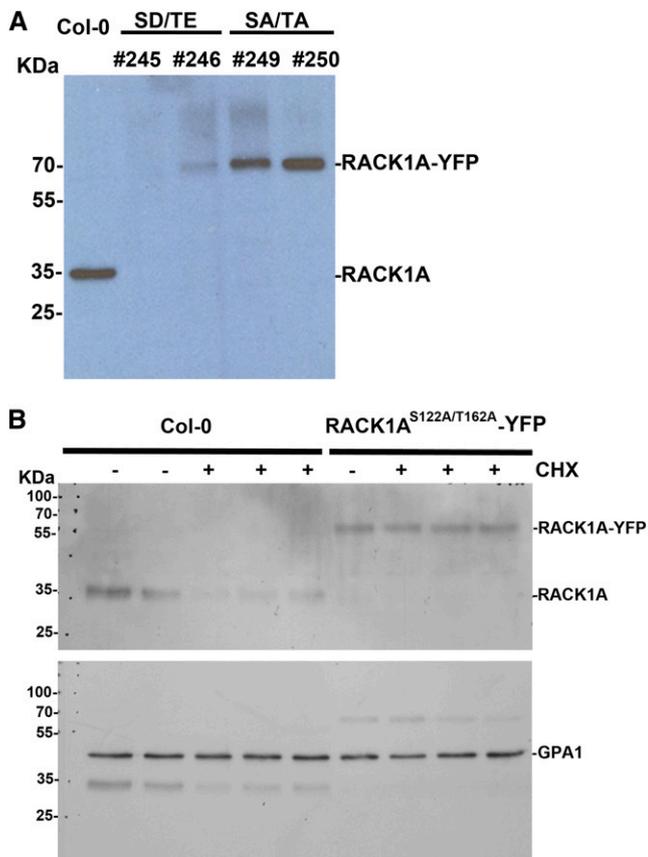


Figure 6. Immunoblot analysis of RACK1A protein. **A**, RACK1A protein in wild-type (Col-0) and transgenic plants. Total proteins were extracted from leaves of 6-week-old plants and loaded to an SDS-PAGE gel. Anti-RACK1A peptide antibodies were used for immunoblot analysis. SA/TA, RACK1A^{S122A/T162A}; SD/TE, RACK1A^{S122D/T162E}. **B**, The stability of RACK1A protein. Total proteins were extracted from 1-week-old Arabidopsis seedlings of Col-0 and *P*_{RACK1A}::RACK1A^{S122A/T162A}-YFP transgenic plants (in *rack1a-2* mutant background) grown in one-half-strength Murashige and Skoog liquid medium. Anti-RACK1A peptide antibodies were used for immunoblot analysis. The same membrane was blotted with anti-Arabidopsis GTP-binding protein α subunit1 (GPA1) antibodies as a loading control. Lanes 1 and 2, Col-0 without cycloheximide (CHX) treatment; lanes 3 to 5, Col-0 treated with 70 μ M CHX for 6 h; lane 6, *P*_{RACK1A}::RACK1A^{S122A/T162A}-YFP without CHX treatment; and lanes 7 to 9, *P*_{RACK1A}::RACK1A^{S122A/T162A}-YFP treated with CHX for 6 h.

found that, although RACK1A^{S122A/T162A} was detected at a similar level as wild-type RACK1A in Col-0, RACK1A^{S122D/T162E} proteins were nearly undetectable (Fig. 6A). These results suggest that phosphorylation affects the stability of RACK1A proteins. Consistent with this view, we found that the phosphorylation-dead form, RACK1A^{S122A/T162A}, is more stable than the wild-type RACK1A protein (Fig. 6B).

RACK1 Acts Downstream of WNK8

We next tested genetic interaction between RACK1A and WNK8. *rack1a-2* (Chen et al., 2006b) and *wnk8-1* (Wang et al., 2008), loss-of-function mutations in

the corresponding genes (Chen et al., 2006b; Wang et al., 2008), were combined for epistasis analysis. *rack1a* mutants displayed pleiotropic phenotypes, such as impairment in plant hormone signaling, stress responses, and developmental processes (Chen et al., 2006b; Guo and Chen, 2008; Guo et al., 2009a, 2009b; Guo et al., 2011). In contrast, the adult *wnk8* plants have wild-type morphology (Wang et al., 2008). Morphologically, the *rack1a wnk8* double mutant phenocopied the *rack1a* single mutant (Fig. 7A), suggesting that the *rack1a-2* null allele is epistatic to the *wnk8-1* null allele. To further define the genetic relationship between RACK1A and WNK8, we analyzed one opposing developmental phenotype (flowering time) and one opposing conditional phenotype (Glc sensitivity) reported for *rack1a-2* and *wnk8-1* single mutants. The *rack1a-2* mutant flowered later than the wild type (Chen et al., 2006b), whereas the *wnk8-1* mutant flowered earlier (Wang et al., 2008; Park et al., 2011). The *rack1a-2* mutant was hypersensitive to Glc (Fig. 7B), whereas *wnk8-1* mutant was shown previously to be hyposensitive to Glc (Urano et al., 2012; Fu et al., 2014). These two opposing phenotypes of *rack1a-2* and *wnk8-1* single mutants provided an efficient way to determine the genetic relationship between *rack1a-2* and *wnk8-1*. As shown in Figure 7, *rack1a-2 wnk8-1* double mutant phenocopied *rack1a-2* single mutant's Glc hypersensitivity and late flowering phenotypes. Consistent with the flowering phenotypes, the transcripts of flowering marker genes *CONSTANS* (CO) and *FLOWERING LOCUS T* (FT; Putterill et al., 1995; Kardailsky et al., 1999; Kobayashi et al., 1999) were up-regulated in *wnk8-1* single mutant but down-regulated in *rack1a-2* single mutant and *rack1a-2 wnk8-1* double mutant (Supplemental Fig. S3). These results indicate that the *rack1a-2* null allele is epistatic to the *wnk8-1* null allele and suggest that RACK1 and WNK8 work in the same pathway to regulate flowering and Glc responsiveness.

DISCUSSION

We showed that RACK1 proteins are phosphorylated by WNK8 and that the phosphorylation negatively affects RACK1 function. MS analysis revealed that Ser-122 and Thr-162 are phosphorylated sites and that these two sites are conserved in all three Arabidopsis RACK1 proteins (Figs. 3 and 4; Supplemental Fig. S1). Mutational analysis suggested that Ser-122 is necessary and sufficient for the phosphorylation of RACK1 proteins (Fig. 4). Ser-122 is conserved in RACK1 proteins from all species analyzed, including human, yeast, fruit fly, and plant, whereas Thr-162 is plant specific (Supplemental Fig. S1). Because a mutation at Thr-162 has a minimal effect on the phosphorylation level of RACK1A protein (Fig. 4), the impact of phosphorylation at Thr-162 is unknown. Our results also do not rule out the possibility that other phosphorylation sites may exist. For example, mammalian RACK1 is phosphorylated by protein Tyr

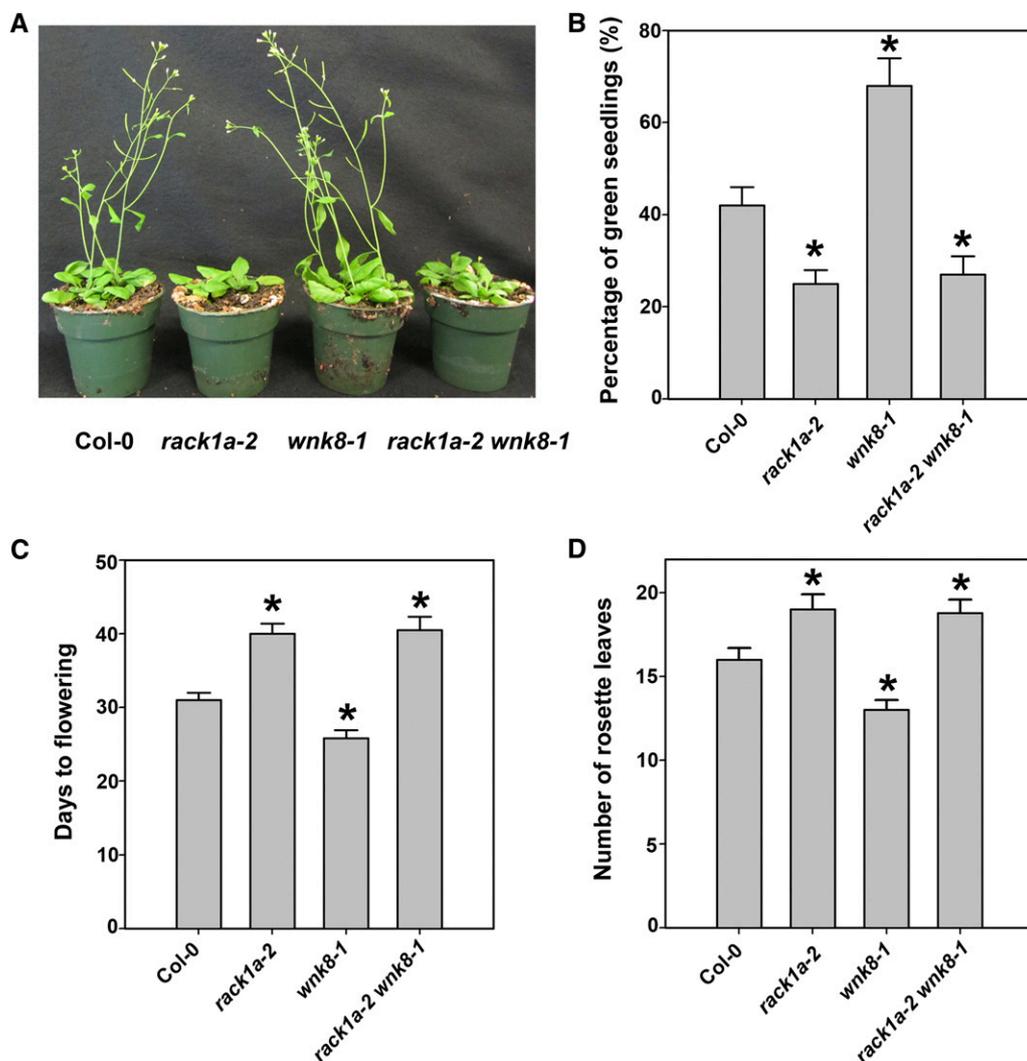


Figure 7. *rack1a wnk8* double mutants. A, Five-week-old *rack1a wnk8* double mutants. Plants were grown under a 14-h-light/10-h-dark photoperiod. B, Glc sensitivity phenotype determined by the percentage of green seedlings. Seeds from the Col-0 wild type and mutants were germinated on one-half-strength Murashige and Skoog medium with 1% or 6% Glc. The percentages of green seedlings were recorded 10 d after seeds had been transferred to germination conditions with 6% Glc. At 1% Glc, the percentages of green seedlings for all genotypes were 100%. Shown are means of three biological replicates \pm SE. C, Flowering phenotype determined by the number of days to flowering. Shown are means of a minimum of 10 plants \pm SE. D, Flowering phenotype determined by the number of rosette leaves upon bolting. Shown are means of a minimum of 10 plants \pm SE. *, Significant difference from Col-0, $P < 0.05$.

kinases at Tyr-52, Tyr-228, and Tyr-246 (Chang et al., 2002; Kiely et al., 2009), and these sites are conserved in all plant RACK1 proteins examined (Supplemental Fig. S1).

RACK1 and WNK8 coordinate flowering and Glc responsiveness (Fig. 7). In addition to interacting with and phosphorylating RACK1 proteins, WNK8 interacts with and phosphorylates other proteins, such as subunit C of vacuolar H^+ -ATPase (Hong-Hermesdorf et al., 2006) and regulator of G-protein signaling1 (Urano et al., 2012).

Transgenic lines expressing the nonphosphorylatable form (RACK1A^{S122A/T162A}) of RACK1A but not the

phosphomimetic form (RACK1A^{S122D/T162E}) rescued *rack1a* mutant (Fig. 5), suggesting that the phosphorylated RACK1A was functionally inactive. Furthermore, phosphorylation of RACK1A negatively affects its function, possibly by decreasing RACK1A protein stability (Fig. 6). However, we did not detect a difference in RACK1A protein abundance between *wnk8* mutant and the wild type (Supplemental Fig. S4), implying that endogenous RACK1A protein is also subject to other forms of posttranslational modifications that counteract the impact of phosphorylation by WNK8. Nonetheless, we found that the phosphorylation-dead form, RACK1A^{S122A/T162A}, is

more stable than the wild-type RACK1A (Fig. 6B), supporting that phosphorylation affects the stability of RACK1A protein.

In summary, we provide biochemical evidence that RACK1 is a substrate of WNK8. Genetic analysis suggests that RACK1 acts downstream of WNK8 in the same pathway to regulate flowering and Glc responsiveness. Furthermore, phosphorylation negatively regulates the function of RACK1 protein by rendering the protein unstable. These findings provide unique insights into the molecular mechanism of action of RACK1 and open a unique window to dissect RACK1 signaling pathways.

MATERIALS AND METHODS

Plant Materials

The *rack1a-2* (Chen et al., 2006b) and *wnk8-1* (Wang et al., 2008) mutants were described previously. The *rack1a-2 wnk8-1* double mutant was generated by crossing *wnk8-1* mutant with *rack1a-2* mutant, and double mutants homozygous for the *rack1a-2* and *wnk8-1* loci were isolated by PCR genotyping.

Y2H Assays

Full-length ORFs of RACK1A, RACK1B, and RACK1C were cloned into LexA-BD vector pBTM117c (Wanker et al., 1997). Full-length ORFs of WNK1, WNK8, and WNK10 were cloned into Gal4-AD vector pGAD10 (GenBank accession no. U13188). Growth of yeast (*Saccharomyces cerevisiae*) on SD-Leu-Trp medium was used as a control of successful transformation, and growth of yeasts on selection medium SD-Leu-Trp-His-Ura plus 1 mM 3-amino-1,2,4-triazole was used to determine positive interactions between bait and prey proteins.

BiFC

The full-length ORFs of RACK1 proteins were cloned into pCL112 (N-terminal split-nYFP tag). Approximately 4- to 5-week-old tobacco (*Nicotiana tabacum*) leaves were infected with *Agrobacterium tumefaciens* harboring pCL113-WNK1, -WNK8, -WNK10, or -P31 (At3G01290, a negative control) and pCL112-RACK1A, -RACK1B, or -RACK1C to express split nYFP- and cYFP-tagged proteins. BiFC was performed as described previously (Klopffleisch et al., 2011) with a few modifications. An internal transformation control (mitochondrial marker; mCherry-mt-rk) was used to confirm infiltration of multiple plasmids (p19 gene silencing suppressor, mt-rk, and two BiFC halves). Leaf epidermis was imaged using a confocal laser-scanning microscope (LSM710; Zeiss) with an Apochromat 40 \times water-emersion objective (numerical aperture = 1.2). YFP and mCherry were excited by a 514-nm argon laser and a 560-nm diode laser, and their fluorescence was detected at 526 to 569 and 565 to 621 nm, respectively.

Recombinant Proteins

The full-length ORFs of RACK1 proteins were subcloned to pDEST15 (N-terminal glutathione S-transferase [GST] tag). Recombinant GST-tagged RACK1 proteins were expressed in ArcticExpress RP (Agilent Technologies) with 0.5 mM isopropylthio- β -galactoside at 12 $^{\circ}$ C and solubilized in buffer A (90 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 5 mM 2-Mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μ g mL $^{-1}$ leupeptin, 0.25 mg mL $^{-1}$ lysozyme, and 0.2% [w/v] nonyl phenoxypolyethoxyethanol). The tagged proteins were captured from the soluble fraction with glutathione-Sepharose 4B (GE Healthcare), washed with buffer A containing 500 mM NaCl and 0.1% sodium cholate, and eluted with buffer B (100 mM Tris-HCl [pH 8.8], 200 mM NaCl, 5 mM dithiothreitol [DTT], 20 mM glutathione, 1 μ g mL $^{-1}$ leupeptin, and 1 mM PMSF). The purified proteins were dialyzed with buffer C (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM MgCl $_2$, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF)

and stored at -80° C. The expression and purification of WNK8 kinase have been described previously (Urano et al., 2012).

Phosphorylation mutations of RACK1A (S122D, T162E, S122D/T162E, and S122A/T162A) were generated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Invitrogen). Mutant proteins (S122D, T162E, and S122D/T162E) were purified in the same manner as the wild-type RACK1 proteins described above.

In Vitro Phosphorylation Assay

Seventy-five picomoles of GST, GST-RACK1A, GST-RACK1B, or GST-RACK1C was incubated with 0.05 pmol of GST or GST-WNK8 in 15 μ L of buffer D (50 mM Tris-HCl [pH 7.0], 10 mM MgCl $_2$, 5 mM MnCl $_2$, 2 mM DTT, 1 mM PMSF, and 1 μ g mL $^{-1}$ leupeptin) containing 0.2 mM [γ - 32 P]ATP for 6 h at room temperature. The reactions were terminated by adding Laemmli sample buffer, and proteins were separated by SDS-PAGE. Incorporation of 32 P into proteins was visualized and quantified with a phosphorimager.

MS

GST-RACK1C protein was phosphorylated by WNK8 and analyzed by SDS-PAGE as described above. The gel band of GST-RACK1C was excised from the polyacrylamide gel, and the proteins in the band were digested using trypsin. The peptides were extracted, and the solution was lyophilized. A fraction of the sample was analyzed by matrix-assisted laser-desorption ionization time of flight/time of flight (MALDI-TOF/TOF), and the remaining was used for analysis by LC-electrospray ionization-MS/MS. A 90-min gradient was used, and MS analysis was performed with an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific). MS spectra were searched using the MASCOT algorithm (Perkins et al., 1999) against the non-redundant National Center for Biotechnology Information database and the RACK1C-specific database with possible phosphorylation of Ser, Thr, and Tyr. The phosphorylated peptides were identified based on MASCOT results, and the phosphorylation sites were defined by manual inspection of the spectra.

Flowering Assay

The flowering phenotypes were determined by counting the number of rosette leaves upon bolting (florescence stem > 0.5 cm) and recording the number of days to bolting. Plants of Col-0, *rack1-2*, *wnk8-2*, and *rack1-2 wnk8-1* were grown side by side under identical conditions of 23 $^{\circ}$ C \pm 1 $^{\circ}$ C with a 14-h-light/10-h-dark photoperiod (approximately 125 μ mol photons m $^{-2}$ s $^{-1}$). A minimum of 10 plants each genotype was used for the assay.

For RACK1 phosphorylation mutants, a 10-h-light/14-h-dark photoperiod (approximately 125 μ mol photons m $^{-2}$ s $^{-1}$) was used for the flowering assay.

Glc Sensitivity Assay

Seeds from Col-0, *rack1a-2*, *wnk8-1*, *rack1-2 wnk8-1*, and RACK1 phosphorylation mutants were sown on one-half-strength Murashige and Skoog medium with 0.7% phytoagar plus 1% (w/v) or 6% (w/v) Glc, placed at 4 $^{\circ}$ C for stratification for 2 d, and then transferred to germination conditions at 23 $^{\circ}$ C \pm 1 $^{\circ}$ C with continuous light (approximately 100 μ mol photons m $^{-2}$ s $^{-1}$). The percentages of green seedlings were scored 10 d after seeds had been transferred to germination conditions. Approximately 50 seeds were used for each genotype in each petri dish with three biological replicates.

Genetic Complementation

The RACK1A^{S122D/T162E} and RACK1A^{S122A/T162A} mutant lines were generated as follows. The genomic DNA containing RACK1A promoter and RACK1A genomic sequence (Guo et al., 2009a) was cloned into the pENTR-D-TOPO vector (Invitrogen) and then transferred into the pGWB40 plant destination vector (Nakagawa et al., 2009), which fuses YFP at the carboxyl terminus of RACK1A protein. The QuikChange Lightning Site-Directed Mutagenesis Kit was used to introduce mutations at Ser-122 and Thr-162 of RACK1A protein. pGWB40 vectors containing P_{RACK1A}::RACK1A^{S122D/T162E} or P_{RACK1A}::RACK1A^{S122A/T162A} were transformed into *rack1a-2* mutant background through *Agrobacterium tumefaciens*-mediated transformation. The expression of RACK1A mutant genes was analyzed by qRT-PCR and immunoblot.

Genetic complementation of *rack1* mutants was determined by counting the number of rosette leaves in a 40-d period. Plants of Col-0, *rack1a-2*, RACK1A^{S122D/T162E}, and RACK1A^{S122A/T162A} were grown side by side under identical conditions (23°C ± 1°C, 10-h-light/14-h-dark photoperiod, approximately 125 μmol photons m⁻² s⁻¹). A minimum of 10 plants of each genotype were used for the assay.

qRT-PCR

For qRT analysis of the expression of RACK1A^{S122D/T162E} and RACK1A^{S122A/T162A} transgene in *rack1a-2* mutant background, total RNA was extracted from rosette leaves of 4-week-old plants using an Invisorb Spin Plant Mini Kit (Stratag Molecular). Two micrograms of total RNA was reversely transcribed to complementary DNA using Fermentas RevertAid Reverse Transcriptase (Thermo Scientific). The expression of *ACTIN8* was used as a control.

For qRT analysis of the transcript levels of flowering marker genes *FT* and *CO* in *rack1a-2* and *wnk8-1* single and double mutants compared with those in Col-0, RNA was isolated from fully expanded rosette leaves of plants that had been grown for 51 d under an 8-h-light/16-h-dark photoperiod. qRT was performed by using StepOnePlus (Applied Biosystems), Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific), and complementary DNA corresponding to 80 ng of RNA in a total volume of 25 μL. The following cycling conditions were used for PCR: 10 min at 95°C and 35 cycles of 15 s at 95°C and 60 s at 60°C. The transcript level of *FT* and *CO* was normalized against that of *ACTIN2*. The sequences of *FT* and *CO* gene-specific primers used for qRT were the same as the ones reported by Park et al. (2011).

Immunoblot Analyses

The total protein lysates were prepared from rosette leaves of 6-week-old Arabidopsis (*Arabidopsis thaliana*) plants or 1-week-old seedlings in buffer E (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 5 mM EGTA, 2 mM DTT, 1% Triton-X100, and a protease inhibitor cocktail; P9599; Sigma). One milliliter of buffer E was added per 0.1 g of plant tissue. Twenty microliters of centrifuged protein extracts was subjected to SDS-PAGE followed by western blotting with anti-RACK1A antibody raised against the sequence K₂₇₃VDLKAEAKADNSGPAAT₂₉₁ in Arabidopsis RACK1A (Chang et al., 2005). Note that the epitope region is highly divergent among three Arabidopsis RACK1 proteins. In the protein stability assays, the same membrane was blotted with anti-GPA1 peptide antibodies (Chen et al., 2006a) as a loading control.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g18080 (RACK1A), At1g48630 (RACK1B), At3g18130 (RACK1C), At3g04910 (WNK1), At5g41990 (WNK8), and At1g64630 (WNK10).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Alignment of RACK1 amino acid sequences.

Supplemental Figure S2. Peptides detected in MS analysis.

Supplemental Figure S3. qRT-PCR analysis of the transcript level of flowering marker genes *CO* and *FT*.

Supplemental Figure S4. Immunoblot analysis of RACK1A protein in *wnk8* mutant and wild-type background.

ACKNOWLEDGMENTS

We thank Dr. David Smalley for determining phosphorylation sites of RACK1, Jing Yang and Ariko Urano for assistance in gene cloning and plant transformation, and Dr. Julia Bailey-Serres for providing anti-RACK1A antiserum.

Received July 28, 2014; accepted December 5, 2014; published December 8, 2014.

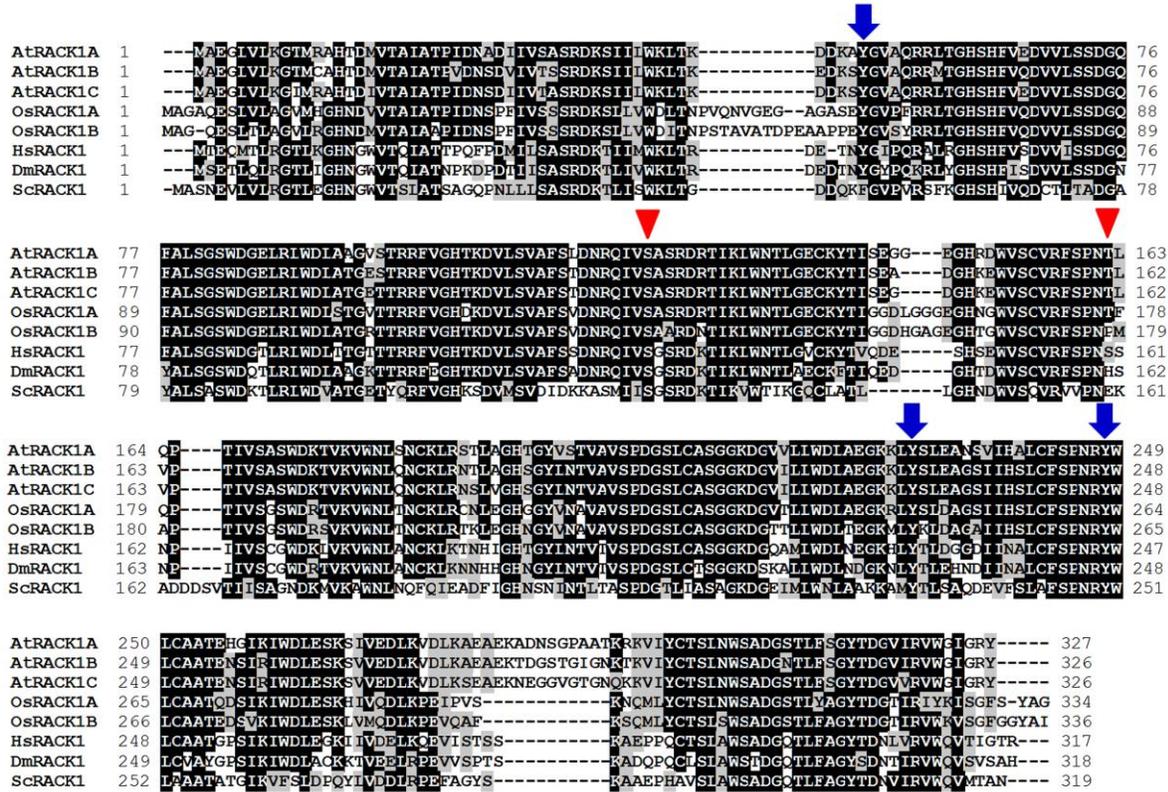
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SUPPLEMENTAL DATA

Figure S1



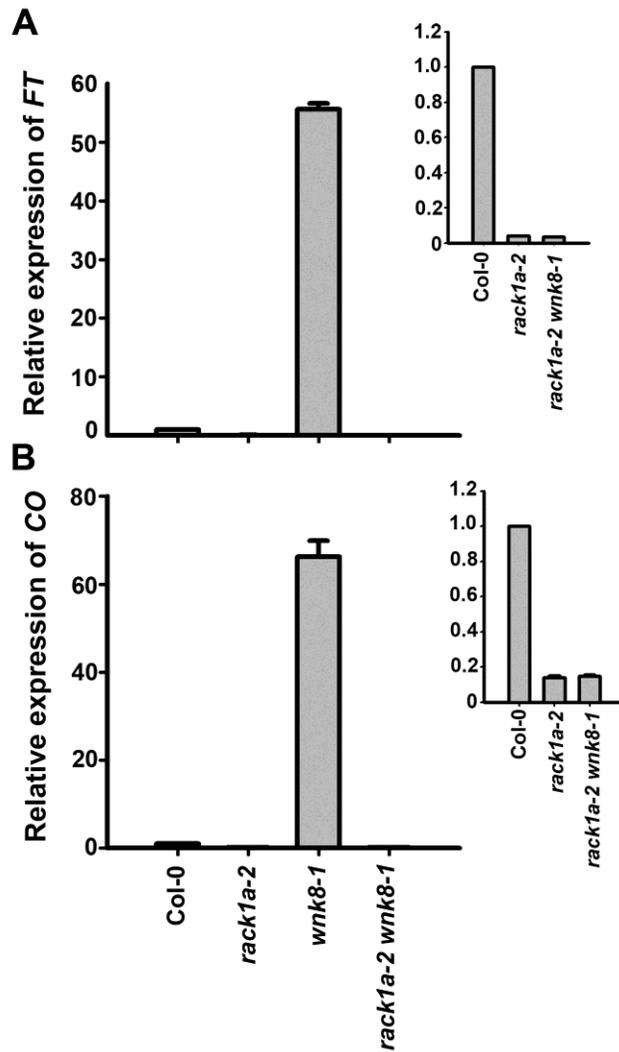
Supplemental Figure S1. Alignment of RACK1 amino acid sequences. The amino acid sequences were aligned by CLUSTALW multiple alignment using BioEdit (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Amino acids that are identical or similar are shaded with black or gray, respectively. Gaps are shown as dashed lines. The proteins aligned are (name of species and accession number in parentheses): AtRACK1A (*Arabidopsis thaliana*, NP_173248), AtRACK1B (*Arabidopsis thaliana*, NP_175296), AtRACK1C (*Arabidopsis thaliana*, NP_188441), OsRACK1A (*Oryza sativa*, NP_001043910), OsRACK1B (*Oryza sativa*, NP_001056254), HsRACK1_Hs (*Homo sapiens*, NP_006089), DmRACK1 (*Drosophila melanogaster*, NP_477269), ScRACK1 (*Saccharomyces cerevisiae*, NP_013834). Arabidopsis RACK1 phosphorylation sites (Ser122; Thr 162 of AtRACK1A and Thr161 of AtRACK1B and AtRACK1C) identified in this study are indicated by red arrow heads on the top of amino acids. Human RACK1 phosphorylation sites (Tyr52, Tyr228 and Tyr246 of HsRACK1) are indicated by blue arrows on the top of amino acids.

Figure S2

MAEGLVLKGI**MR**AHTDIVTAIATPIDNSDIIVTASRDKSIILWKLTKDDKSYGVA**QRRL**TGHSHFVEDVV
LSSDGQFALSGSWDGELRLWDLATGETTRRFVGH**TKD**VLSVAFSTDNR**QIVSASR**DRTIKLWNTLGE**CKY**
TISEGDGHKEWV**SCVRF**SPNTLVPTIVSAS**WDR**TVKVVWNLQ**NCKLR**NSLVGHSGYLNTVAVSPDGSLCAS
GGKDGVILLWDLAEG**KK**LYSLEAGSIIH**SL**CFSPNRYWLCAATENSIR**I**WDLESKSVVEDLKVDLKSEAE
KNEGGVGTGN**Q**KKVIYCTSLN**WSADG**STLFSGYTDGVV**RV**WGIGRY

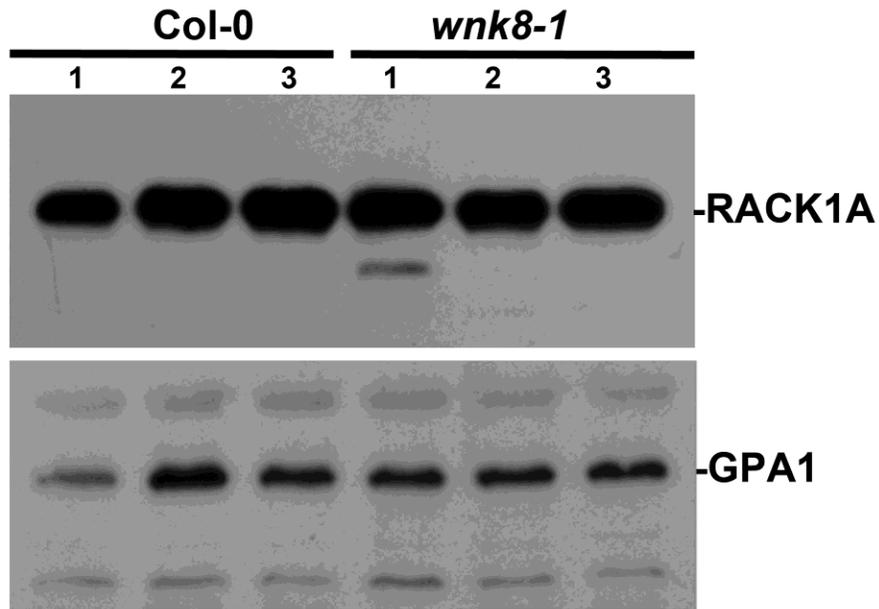
Supplemental Figure S2. Peptides detected in mass spectrometry analysis. The amino acid sequence of RACK1C is shown. Non-phosphorylated peptides detected are marked in yellow. Peptides containing phosphorylation sites are marked in blue.

Figure S3



Supplemental Figure S3. Quantitative RT-PCR analysis of the transcript level of flowering marker genes *FT* (A) and *CO* (B) and. RNA was isolated from fully-expanded rosette leaves of plants that had been grown for 51 days under 8/16 photoperiod. Insets illustrate the difference in transcript levels between Col-0, *rack1a-2* single mutant and *rack1a-2 wnk8-1* double mutant. Shown are means of three replicates \pm s.e.

Figure S4



Supplemental Figure S4. Immunoblot analysis of RACK1A protein in *wnk8* mutant and Col-0 wild-type background. Total proteins were extracted from one-week old Arabidopsis seedlings grown in ½ MS liquid media. Anti-RACK1A peptide antibodies were used for immunoblot analysis. The same membrane was blotted with anti-GPA1 peptide antibodies as a loading control. Three biological replicates were used for each genotype.