Supporting Information

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SI Materials and Methods

Plant Materials. Barley lines for the experiments were grown in growth chambers in plastic pots containing potting mix with a day and night temperature of 21 ± 1 °C and 18 ± 1 °C, respectively, and with a 16-h photoperiod provided by cool fluorescent tubes (525 $\mu E \cdot m^2 \cdot s$).

Rust Races. The avirulent wheat stem rust race MCCF was used for all experiments. The urediniospores were increased by infecting the susceptible cv. Steptoe, collected, and used immediately.

Genomic DNA Isolation. Genomic DNA was isolated from sporelings of the various races of the stem rust pathogen using the Omni prep DNA isolation kit (G-Bio sciences).

Transcript Analysis. RNA from spores, sporelings, haustoria, and infected tissues was isolated using the Qiagen RNA isolation kit. PolyA RNA was isolated using the Qiagen Oligotex mRNA isolation kit. RNA was subjected to DNase treatment using the DNase treatment kit from Ambion and was subjected to first-strand cDNA synthesis using the first-strand cDNA synthesis kit from Promega. For real-time PCR, the SYBR Green kit from Qiagen was used. RACE (3′ and 5′) was performed with the Smart 5′ and 3′ RACE kit from Clontech following the manufacturer's recommendations.

The sequences were assembled with Invitrogen Vector NTI software. For annotation, the finished sequences were compared with National Center for Biotechnology Information nonredundant and dbEST database using Blastn, Blastx, and tBlastx algorithms. In addition, FGENESH (Softberry, Inc., Mount Kisco, NY) and GENESCAN (1) were used for gene prediction.

Protein Expression and Purification in *P. pastoris*. *RGD*-binding and *VPS9* genes were amplified as full-length genes using gene-specific primers (pPPICZ NotI RGD forward and reverse for the *RGD*-binding gene and pPICZ NotI VPS9 forward and reverse for the *VPS9* gene, respectively) with the *Not*1 restriction site (Table S2), cloned separately in a pPICZB vector, and expressed as His-tag protein in *P. pastoris*. The proteins were purified by nickel affinity chromatography, using Talon (Clontech) according to the manufacturer's instructions.

Yeast Strains and Growth. *Saccharomyces cerevisiae* strain cdc25H a or α was maintained on yeast peptone dextrose agar supplemented with adenine hemisulfate (YPAD) medium, except when harboring two hybrid plasmids; in such case, strains were handled according to the manufacturer's specifications (Stratagene).

Yeast Two-Hybrid Assay. Genes were cloned in-frame with the pSOS or pMyr vector and tested for the translated protein ability to interact with RPG1 or with each other. The cytotrap-SOS recruitment system (Stratagene) was used for yeast two-hybrid analysis. Cdc25H yeast cells were cotransformed with the bait and the prey plasmids and subsequently incubated at 25 °C on glucose plates lacking leucine and uracil. The colonies growing on Synthetic Drop out/Glucose-Uracil/Leucine (SD/GLU-UL) at 25 °C were picked and spotted on Synthetic Drop out/Galactose-Uracil/Leucine (SD/GAL-UL) at 37 °C and SD/GLU-UL at 37 °C. The colonies growing on SD/GAL-UL at 37 °C but not on SD/GLU-UL at 37 °C were scored as having a positive interaction. The specificity of interaction was tested by reconstruction of the rescued prey construct and repeat of the yeast transformation and screening by directed (bait + rescued prey) analysis. Positive and negative control plasmids were used according to the manufacturer's specifications (Stratagene).

The expression of the proteins from the interacting clones was detected using a commercially available rabbit anti-SOS antibody (Sigma). RPG1 was detected using the RPG1 polyclonal antibody available in our laboratory. Because of the unavailability of a commercial antibody for the myristoylation tag, we used a gene that was cloned in the pMyr vector, for which a protein and an antibody were available. AtSLY1 protein was detected by AtSLY1 polyclonal antibody.

SLY1 Antibody Production. A peptide antibody against the N-terminal region of the AtSLY1 protein corresponding to KRSTTDSDLAGDAHNC (position 2–16 amino acids) was commercially synthesized and raised in rabbits (Sigma) and was validated against expressed AtSLY1 proteins in *Escherichia coli*. The antibody was used to detect the expression of AtSLY1 proteins with a myristoylation tag in the pMyr vector for the two-hybrid assays.

Yeast Protein Isolation and Testing. Total proteins from the interacting yeast two-hybrid clones were isolated using the yeast buster reagent (Novagen) according to the manufacturer's specifications. Total proteins from the interacting clones were run on 10% (wt/vol) SDS/PAGE and subjected to Western blot analysis. The immunoblots were probed with the respective primary antibodies and HRP-conjugated goat anti-rabbit secondary antibodies as described by Nirmala et al. (2).

Phosphorylation and Degradation of RPG1. Experiments were carried out as described by Nirmala et al. (3, 4).

Leaf Infiltration. The purified proteins were mixed equally to a final concentration (0.40 mg/mL) and infiltrated into 10-d-old barley leaves using a syringe without a needle (50 μL per leaf). To test if HR is caused by a nonspecific increase in the infiltration of proteins, we used BSA. BSA was mixed equally with either the RGD-binding or VPS9 protein to a final concentration (0.40 mg/mL) and infiltrated into 10-d-old barley leaves using a syringe (50 μL per leaf). BSA alone as a control and a 1:1:1 combination of BSA/RGD-binding protein/VPS9 protein were also investigated. The plants were scored 2–3 d after infiltration.

Scanning EM. Ten-day-old cv. Steptoe plants were inoculated with either untreated or RGD peptide-treated MCCF rust urediniospores at a rate of 0.05 mg of spores per plant as described by Nirmala et al. (3). Samples were collected at several time points after inoculation as described in the figure and fixed in a fixative [2% (wt/vol) paraformaldehyde, 2% (wt/vol) glutaraldehyde, 0.05 M Pipes buffer (pH 7.2)] to maintain the structural integrity of the cells and were washed twice with water. The samples were freeze-dried overnight and subjected to gold coating. The gold-coated samples were mounted on carbon tapes onto aluminum stubs and examined in field emission scanning electron microscopy (Quanta 200F) at an accelerating voltage of 30 kV, a 10- to 15-mm working distance and high vacuum, or, alternatively, under the same conditions but with a low vacuum when fresh samples were observed.

1. Burge CB, Karlin S (1998) Finding the genes in genomic DNA. *Curr Opin Struct Biol* 8: 346–354.
2. Nirmala J, et al. (2006) Subcellular localization and functions of the barley stem rust resistance receptor-like serine threonine specific protein kinase receptor. *Proc Natl Acad Sci USA* 103:7518–7523.
3. Nirmala J, et al. (2007) Proteolysis of the barley receptor-like protein kinase RPG1 by a proteasome pathway is correlated with *Rpg1*-mediated stem rust resistance. *Proc Natl Acad Sci USA* 104:10276–10281.
4. Nirmala J, Drader T, Chen X, Steffenson BJ, Kleinhofs A (2010) Stem rust spores elicit rapid RPG1 phosphorylation. *Mol Plant Microbe Interact* 23:1635–1642.

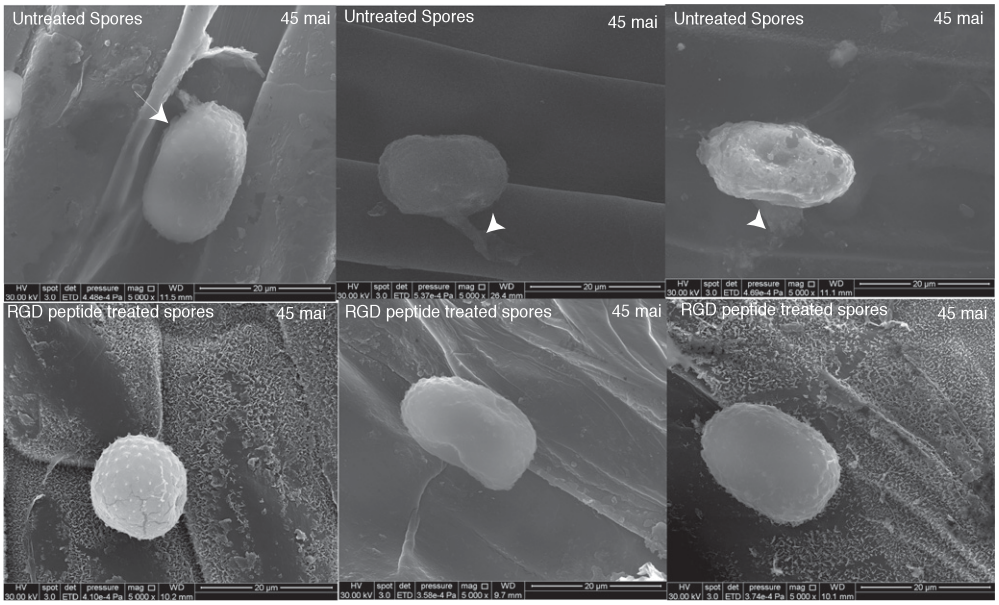

Fig. S1. Photographs showing the formation of adhesion pads only in untreated urediniospores of the *Pgt* stem rust race MCCF on susceptible cv. Steptoe. RGD peptide loop-treated spores do not form any visible adhesion pads. Photographs were taken 45 min after inoculation at 1,000× magnification using scanning EM at low vacuum. mai, minutes after inoculation.

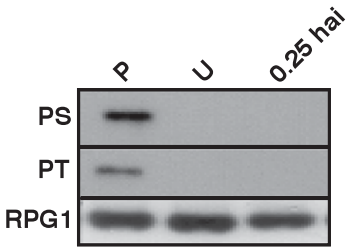

Fig. S2. Stem rust urediniospores of *Pgt* MCCF treated with the RGD peptides failed to induce in vivo phosphorylation of RPG1 in cv. Morex. RPG1 was immunoprecipitated with RPG1-specific antibody, and phosphorylation was detected with either phosphoserine or phosphothreonine antibody or with RPG1 antibody. hai, hours after inoculation with the stem rust urediniospores; P, in vitro phosphorylated RPG1 protein as a positive control; PS, phosphoserine antibody; PT, phosphothreonine antibody; U, uninoculated control. Protein loading is shown in the RPG1-precipitated and RPG1 antibody-visualized row.

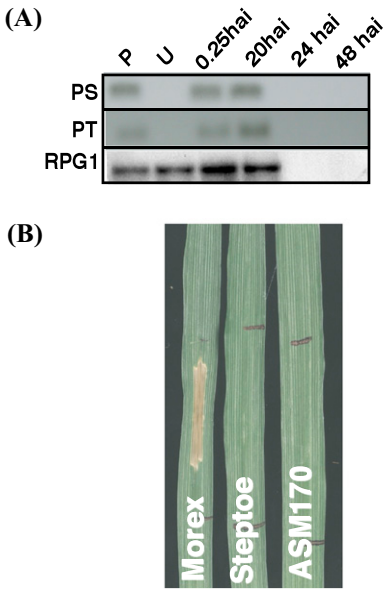

Fig. S3. (*A*) *Pgt* urediniospore extract binding to and eluted from the RGD affinity column induced RPG1 phosphorylation and degradation in barley cv. Morex. The *Pgt* urediniospore extract was applied to the RGD affinity column, washed, and eluted with the RGD peptide. When applied to barley leaf surfaces, the crude eluate elicited RPG1 phosphorylation within 0.25 h and degradation before 24 h, the same as observed when avirulent stem rust urediniospores are applied to the leaf surface (3). RPG1 was immunoprecipitated with RPG1 antibody, and phosphorylation was detected with either phosphoserine or phosphothreonine antibody. hai, hours after inoculation with the stem rust urediniospores; P: in vitro phosphorylated RPG1 protein as a positive control; PS, phosphoserine antibody; PT, phosphothreonine antibody; U, uninoculated control. Protein loading is shown in the RPG1-precipitated and RPG1 antibody-visualized row. (*B*) *Pgt* urediniospore extract binding to and eluted from the RGD affinity column induced HR in barley harboring a functional *Rpg1* gene. The *Pgt* urediniospore extract was applied to the RGD affinity column, washed, and eluted with the RGD peptide. When applied to barley leaf surfaces, the crude eluate elicited RPG1 phosphorylation, and HR, in the resistant cv. Morex but not in susceptible cv. Steptoe and susceptible line ASM170, the latter of which has a recombination within *Rpg1* rendering it nonfunctional.

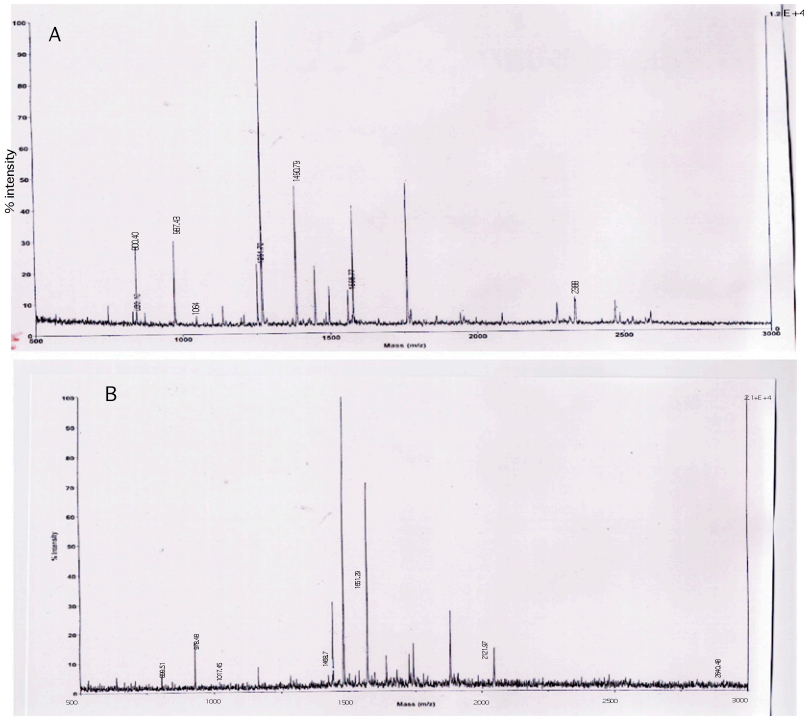

Fig. S4. MALDI TOF-mass spectra showing the identification of RGD-binding and VPS9 peptides from the spore extracts of *Pgt*. RGD-binding protein (*A*) and VPS9 protein (*B*).

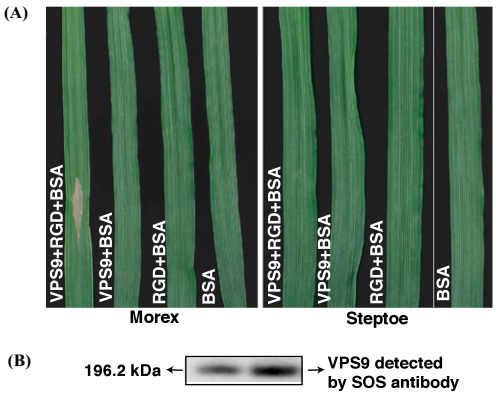

Fig. S5. (*A*) RPG1-specific HR attributable to infiltration of RGD-binding and VPS9 proteins is not caused or altered by increased nonspecific protein concentration. To determine if HR was attributable to increased protein concentration in the infiltrated sample, BSA was added to all samples. Infiltration of BSA alone or in a 1:1 combination with either the RGD-binding protein or VPS9 protein did not cause HR on the resistant cv. Morex or susceptible cv. Steptoe. Infiltration of a 1:1:1 mixture of BSA/RGD-binding protein/VPS9 protein did not inhibit the specific HR or cause nonspecific HR. (*B*) Western blot showing that the SOS-VPS9 fusion protein is expressed in yeast. Total yeast proteins from the SOS-VPS9 + Myr RGD yeast clones exhibiting a positive interaction were subjected to SDS/PAGE and Western blotting. The Western blot probed with an SOS primary antibody confirmed expression of the VPS9 protein.

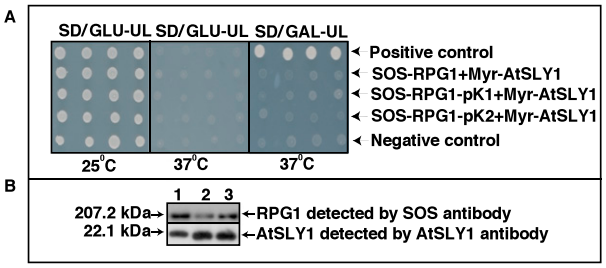

Fig. S6. RPG1 or its mutant derivatives do not interact with AtSLY1. (*A*) RPG1 and its mutant derivatives RPG1-pK1 and RPG1-pK2 were cloned as the bait in pSOS vector, and AtSLY1 was cloned in pMyr vector as the prey. The prey and bait constructs were cotransformed into the cdc25Ha yeast strain as indicated in the figure. The cotransformants were plated on SD/Glu-UL medium and allowed to grow at 25 °C. The colonies growing at 25 °C were checked for interaction on SD/Glu-UL and SD/GAL-UL medium at 37 °C. The clones growing on SD/GAL-UL medium at 37 °C and not growing on SD/Glu-UL medium at 37 °C were scored as positive, exhibiting protein-protein interaction. RPG1, RPG1-pK1, and RPG1-pK2 failed to interact with AtSLY1. (*B*) Western blot showing expression of SOS-RPG1, SOS-RPG1-pK1, SOS-RPG1-pK2, and the Myr-AtSLY1 fusion proteins in the yeast clones. RPG1 was detected with RPG1 antibody, and AtSLY1 was detected with SLY1 antibody. SOS-MAFB + Myr-MAFB served as a positive control, and SOS-MAFB + Myr Lamin C served as a negative control. The positive and negative controls were provided by the suppliers of the Cytotrap kit (Stratagene): 1, SOS-RPG1 + Myr-AtSLY1; 2, SOS-RPG1-pK1 + AtSLY1; 3, SOS-RPG1-pK2 + Myr-AtSLY1. MAFB, V-maf masculoaboneurotic fibrosarcoma oncogene homolog B.

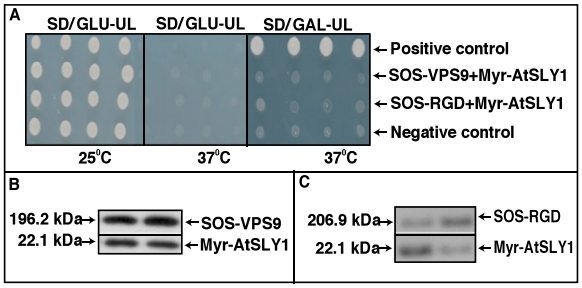

Fig. S7. AtSLY1 does not interact with the RGD-binding or VPS9 protein in yeast. (*A*) RGD-binding or VPS9 gene was cloned as the bait in pSOS vector, and AtSLY1 was cloned in pMyr vector as the prey. The bait and the prey constructs were cotransformed into the cdc25Ha yeast strain as indicated in the figure. The cotransformants were plated on SD/GLU-UL medium and allowed to grow at 25 °C. The colonies growing at 25 °C were checked for interaction on SD/GLU-UL and SD/GAL-UL medium at 37 °C. The clones growing on SD/GAL-UL medium at 37 °C and not growing on SD/GLU-UL medium at 37 °C were scored as positive, exhibiting protein-protein interaction. RGD-binding protein and the VPS9 protein failed to interact with ATSLY1. (*B*) Western blot showing that the VPS9, RGD-binding, and AtSLY1 proteins are expressed in yeast. RGD-binding and the VPS9 proteins were detected with SOS antibody, and AtSLY1 protein was detected with SLY1 antibody. SOS-MAFB + Myr-MAFB served as a positive control, and SOS-MAFB + Myr Lamin C served as a negative control. The positive and negative controls were provided by the suppliers of the Cytotrap kit (Stratagene). MAFB, V-maf masculoaboneurotic fibrosarcoma oncogene homolog B.

Table S1. Effect of RGD peptide loop on the formation of adhesion pads and appresoria with *Pgt* race MCCF urediniospores on the susceptible cv. Steptoe

Treatment	Adhesion pad formation by 1 hai	Appresoria formation by 16 hai	Appresoria formation by 24 hai
Untreated uredinispores	2,412	3,018	Hard to count or differentiate appresoria
RGD peptide loop-treated spores	0	0	3

A total of 20 leaves per time point were visualized by scanning EM directly under low vacuum without fixing at a magnification of 1,000×–2,000×, and the number of adhesion pads or appresoria encountered was counted and recorded. Sample photographs are shown in Fig. 1 and Fig. S1. hai, hours after inoculation.

Table S2. List of primers used in this study

Primer name	5′-3′ sequence
JNF1	AACGGTCAACGTCTTGGAAC
JnR1	GAAATCGCAAGGCTTACAGG
JNF2	GCCCCAACTTTACAGGACAA
JNR2	GCATCGAAAGTGGCAAGTCT
JNF3	TGATGAATCAGCCTGTGGAA
JNR3	CACAACCAGGAGGCAGAAGT
JNF4	AACGGTCAACGTCTTGGAAC
JNR4	ACGATCACCCCAAAACAGAC
JNF5	CCTGTAAGCCTTGCGATTTC
JNR5	ATTGGAGTCTGAGCCAATCG
JNF6	ACTCGGAGAAAACGCTGAA
JNR6	ACGTGATGAGATCGGTAGGC
JNF8	ATGACCGGATCTTTTGGTG
JNR8	TCAATCGGCAAGATGATTAGGA
pSOS NotI RGD F	GAGCGGCCGCTATGACCGGATCTTTTGGTGGA
PSOS NotI RGD R	GCGCGGCCGCTTCAATCGGCAAGATGATTAGG
pPICZ NotI RGD F	GAGCGGCCGCTATGACCGGATCTTTTGGTGGA
pPICZ NotI RGD R	GCGCGGCCGCTATCGGCAAGATGATTAGG
pMyr VPS9 EcoRI F	GCGAATTCATGTCAACAGCAACCATCCAA
pMyr VPS9 XhoI R	GACTCGAGTCAAGACATTTCCAATAAAGA
pPICZ VPS9 NotI F	GCGCGGCCGCTATGTCAACAGCAACCATCCAA
ppicz VPS9 NotI R	GAGCGGCCGCTAGACATTTCCAATAAAGA
pMyr SalI RGD F	GCGTCGACATGACCGGATCTTTTGGTGGA
pMyr SalI RGD R	GAGTCGACTCAATCGGCAAGATGATTAGG
VPS9 seq1	GCAAGTAGAAATT
VPS9 seq2	GCATCTCGACCTCTCT
VPS9 seq3	GCTCTCATCCTCATCCTC
VPS9 seq4	GGTCGTCCTACGCGC
VPS9 seq5	GCATGAGCATGTCTCCTAATC

F, forward; R, reverse.