STEM RUST SPORES ELICIT RAPID RPG1 PHOSPHORYLATION

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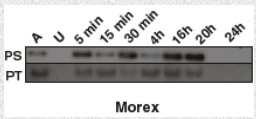
The resurgence of stem rust on wheat and barley, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*), via the highly virulent race TTKSK (aka Ug99) and its variants has brought renewed interest in stem rust resistance genes and their function. The barley *Rpg1* gene has conferred durable resistance against many *Pgt* races for over 60 years (1, 2). The *Rpg1* gene was cloned by a map-based approach (3) and validated by haplotype sequencing and stable transformation of a susceptible cultivar, "Golden Promise", which was rendered completely resistant (4). *Rpg1* is a novel disease resistance gene with a pseudokinase domain (pK1) and an active kinase domain (pK2), both of which are required for disease resistance (5). The RPG1 protein autophosphorylates in vitro, but its in vivo significance was not known. However, failure to autophosphorylate under in vitro conditions correlates with lack of RPG1 protein degradation in vivo (6) and results in disease susceptibility (5, 6). Therefore, to understand how RPG1 perceives the signal from the fungus, we investigated the in vivo phosphorylation status of RPG1 upon inoculation with the rust pathogen. Here we show that RPG1 is rapidly phosphorylated only in response to the avirulent, but not virulent, rust fungus spores and that this phosphorylation is required for disease resistance. The rapidity of the phosphorylation and apparent autophosphorylation suggest that this is the signal to initiate a cascade of biochemical events leading to activation of the defense response.

MATERIALS AND METHODS

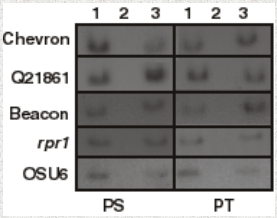
Phospho-immunoprecipitation and western blot analysis: Barley seedlings were collected at different time points post-inoculation with the stem rust fungus as indicated and the total phosphorylated proteins precipitated using phospho-specific antibodies and subjected to SDS-PAGE. The phosphorylated RPG1 was detected by subsequent western blot analysis using RPG1 specific polyclonal antibodies. For the protein kinase inhibitor experiments, plants of the resistant cv. Morex were treated separately with 5 µM concentration of staurosporin, H7, A7, A8, H8, or 2.5 µM concentration of K252a or K252b. The inhibitors were applied to the leaf surface and allowed to dry for 15 min. and then inoculated with the avirulent stem rust race, MCCF. Samples were collected 15 min. post-inoculation and subjected to RPG1 phosphorylation assay or were scored for disease reaction on the 14th day after inoculation.

RPG1 protein is phosphorylated within 5 minutes post-inoculation with the avirulent *Pgt* race MCCF spores in cv. Morex



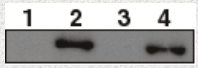
Phosphorylation of RPG1 in cv. Morex, showing its elicitation and stability from 5 minutes to 16h. RPG1 protein is not seen at 20 and 24h time points because it is degraded at that time. A-in vitro phosphorylated RPG1 protein positive control; U-uninoculated control; PS-phosphoserine , PT-phosphothreonine antibody.

In vivo phosphorylation of RPG1 in different barley cultivars producing RPG1 protein in response to inoculation with the avirulent stem rust fungus MCCF



RPG1 phosphorylates within 5 minutes post inoculation with an avirulent rust fungus in all the cultivars producing RPG1 protein even susceptible lines *rpr1* and OSU6 which must act downstream of *Rpg1*. Lane1: in vitro phosphorylated RPG1 protein positive control; lane 2: uninoculated control; lane 3: 5 min. post-inoculation. PS: phosphoserine antibody; PT: phosphothreonine antibody.

Viable but not inviable MCCF spores elicit RPG1 phosphorylation in cv. Morex



RPG1 is phosphorylated only when leaves are inoculated with viable, but not with inviable spores of Pgt race MCCF in cv. Morex Lanes 1 and 2: Total proteins immuno-precipitated with phosphoserine antibodies from the plants inoculated with inviable and viable spores, respectively (30 minutes post-inoculation); lanes 3 and 4: Total proteins immunoprecipitated with phosphothreonine antibodies from the plants inoculated with inviable and viable spores, respectively (30 minutes post-inoculation). Inviable spores are defined as not capable of germination on 2% water agar plates and were generated by storing the spores at room temperature for 3 months.

FUTURE GOALS

***Identify and characterize the elicitor that causes RPG1 to phosphorylate.

***Investigate the mode of action of the elicitor in the RPG1 mediated pathway.

*** Investigate if elicitors can be used to control the stem rust pathogens?

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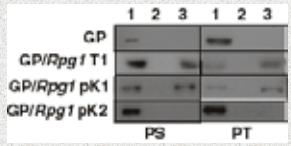
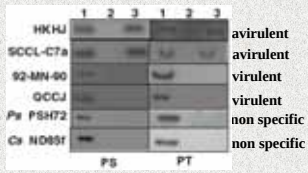
Functional RPG1 pK2 domain is required for in vivo phosphorylation and resistance



RPG1 fails to phosphorylate in response to inoculation with Pgt race MCCF in susceptible transgenic mutant GP/Rpg1 pK2 (KK461/462NQ) whereas the susceptible GP/Rpg1 pK1 (KK152/153NQ) transgenic mutant and the highly resistant GP/Rpg1T1 (wild type Rpg1 transgene) are phosphorylated. Lane1: in vitro phosphorylated RPG1 protein positive control; lane 2: uninoculated negative control; lane 3: 15 min. post-inoculation. PS: phosphoserine antibody; PT: phosphothreonine antibody. GP is a susceptible cultivar and does not have the *Rpg1* gene.

RPG1 phosphorylation is triggered exclusively by the avirulent rust pathotypes



RPG1 is phosphorylated upon inoculation with avirulent but not virulent races of the stem rust fungus or non-specific pathogens in cv. Morex. Avirulent *Pgt* races- HKHJ, SCCL-C7a; Virulent *Pgs* isolate 92-MN-90 and *Pgt* race QCCJ; RPG1 non-specific pathogens barley stripe rust race -*Ps* PSH72, and spot blotch isolate *Cs* ND85F. Lane1: in vitro phosphorylated RPG1 protein positive control; lane 2: uninoculated negative control; lane 3: 15 min. post-inoculation. PS: phosphoserine antibody; PT: phosphothreonine antibody.

Protein kinase inhibitors (PKI) prevent phosphorylation of RPG1 and convert the incompatible disease reaction to compatible in cv. Morex (*Rpg1*), in response to Pgt race MCCF

Seven different protein kinase inhibitors (PKI) prevent phosphorylation of RPG1 and convert the incompatible disease reaction to compatible in cv. Morex (*Rpg1*), in response to *Pgt* race MCCF. Top panel shows autoradiogram depicting the inhibition of in vivo phosphorylation of RPG1, while the bottom panel shows the disease reaction of RPG1 due to the different protein kinase inhibitors. PKI-protein kinase inhibitor; Lanes 1-9 represent Morex no PKI, A8, A7, H8, H7, Staurosporin, K252a, K252b and Steptoe (*rpg1*) no PKI, respectively. Cv. Steptoe does not produce an RPG1 protein.

CONCLUSIONS

**RPG1 is phosphorylated within 5 minutes post-spore landing on the plant surface.

**RPG1 phosphorylation is a highly race specific and takes place only in response to viable spores.

**RPG1 phosphorylation is required for resistance and probably acts as a very early signal in pathogen perception.

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