

Stem Rust Spores Elicit Rapid RPG1 Phosphorylation

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Stem rust threatens cereal production worldwide. Understanding the mechanism by which durable resistance genes, such as *Rpg1*, function is critical. We show that the RPG1 protein is phosphorylated within 5 min by exposure to spores from avirulent but not virulent races of stem rust. Transgenic mutants encoding an RPG1 protein with an in vitro inactive kinase domain fail to phosphorylate RPG1 in vivo and are susceptible to stem rust, demonstrating that phosphorylation is a prerequisite for disease resistance. Protein kinase inhibitors prevent RPG1 phosphorylation and result in susceptibility to stem rust, providing further evidence for the importance of phosphorylation in disease resistance. We conclude that phosphorylation of the RPG1 protein by the kinase activity of the pK2 domain induced by the interaction with an unknown pathogen spore product is required for resistance to the avirulent stem rust races. The pseudokinase pK1 domain is required for disease resistance but not phosphorylation. The very rapid phosphorylation of RPG1 suggests that an effector is already present in or on the stem rust urediniospores when they are placed on the leaf surface. However, spores must be alive, as determined by their ability to germinate, in order to elicit RPG1 phosphorylation.

The ability of plants to successfully defend themselves against invading pathogens depends on early perception of the pathogen and ability to initiate the appropriate signaling process to recruit and execute multicomponent defense responses. Extensive research in the last decade has revealed a sequence of biochemical events that appear to participate in the activation of plant disease resistance. The activation of defense responses in plants is initiated by host recognition of pathogen-associated molecular patterns (PAMPs). These are molecules that are conserved by specific classes of microbes and are often referred to as “general elicitors.” They are recognized by specific pattern recognition receptors encoded by the host and mediate innate immunity. In case the basal defense system is overcome, plants have evolved a recognition system based on specific resistance (R) proteins that recognize specific pathogen effectors, which leads to a rapid defense response termed effector-triggered immunity (ETI) (Jones and Dangl 2006; Gohre and Robatzek 2008; de Wit et al. 2009; Stergiopoulos and de Wit 2009). Resistance or susceptibility of host plants to different races of a pathogen is determined, in many cases, by dominant R genes in the plant and corresponding dominant avirulence (*Avr*) genes in the pathogen (Flor 1971). The *Avr* genes and their products have been characterized in a few well-studied pathogen–plant systems and have been shown to be elicitor mole-

cules that interact directly or indirectly with R gene products (Mansfield 2009; Stergiopoulos and de Wit 2009). The R genes, on the other hand, encode receptor molecules that detect the elicitor molecules either directly or indirectly via molecules they “guard” (Jones and Dangl 2006) or which act as “decoys” (van der Hooft and Kamoun 2008). The decoy model proposes that the decoy protein acts solely to detect the pathogen effector and does not have a virulence function. Most R genes cloned to date are of the nucleotide-binding site leucine-rich repeat (NBS-LRR) type but little is known about how the interaction of pathogen elicitors with host receptors activates signal transduction cascades that may involve protein phosphorylation, ion fluxes, reactive oxygen species (ROS), and other signaling events, leading to disease resistance in the plant host (Piedras et al. 1998; Blatt et al. 1999).

Receptors for fungal elicitors appear to reside either on the plasma membrane or in the cytoplasm of the plant cell (Yang et al. 1997; Hoefle and Huckelhoven 2008). Rapid changes at the cell surface that may be involved in the activation of inducible defense responses have been reported (Vera-Estrella et al. 1992). Plants activate their defenses against a variety of microorganisms by using a combination of signaling molecules, such as K⁺ and Cl⁻ efflux, as well as the production of O₂⁻, H₂O₂, and nitric oxide (Richberg et al. 1998), which induce significant changes on the plant cell surface. A crucial role for protein phosphorylation was suggested by the isolation of the *Pto* gene from tomato that encodes an S/T kinase (Martin et al. 1993). Subsequent cloning of the *Xa21* gene from rice, a gene that encodes an LRR transmembrane receptor-like kinase (Song et al. 1995); *Pbs1* from *Arabidopsis*, encoding an S/T kinase (Swiderski and Innes 2001); *Rpg1* and *Rpg5* from barley, which encode dual kinase domains and an NBS-LRR-S/T kinase, respectively (Brueggeman et al. 2002, 2008); and the *Yr36* gene from wheat, which codes for a kinase START domain (Fu et al. 2009) strengthened this idea. In addition to the cloned genes, in vivo studies with phosphatase and protein kinase inhibitors have demonstrated that protein kinases and phosphatases play a critical role in the activation of early defense responses (Yang et al. 1997; Scheel 1998). Some downstream mitogen-activated protein kinases (MAPK) (Ligterink et al. 1997; Zhang and Klessig 1998b; Zhang et al. 1998; Romeis et al. 1999), calmodulin protein kinases (Romeis et al. 2000), syntaxin like proteins (Heese et al. 2005), and kinases involved in brassinosteroid signaling (Wang et al. 2005) are rapidly phosphorylated in response to specific or nonspecific elicitation. More recently, large-scale phosphoproteomics analyses have identified many proteins, which become rapidly phosphorylated upon exposure of the cells to the PAMP elicitor flagellin (Benschop et al. 2007; Nuhse et al. 2007). Proteins identified include kinases and other proteins potentially involved in disease resistance. Direct identification of phosphorylation

sites in probable disease *R* gene products such as the NBS-LRR proteins was recently reported (Nakagami et al. 2010). Here, we report direct evidence for *in vivo* phosphorylation of a race-specific *R* protein, RPG1, and suggest that the phosphorylation results in activation of the stem rust disease resistance response.

The resurgence of stem rust on wheat and barley, caused by *Puccinia graminis* f. sp. *tritici*, via the highly virulent race TTKSK (also known as Ug99) and its variants has brought renewed interest in stem rust *R* genes and their function. The barley *Rpg1* gene has conferred durable resistance against many *P. graminis* f. sp. *tritici* races for over 60 years (Steffenson 1992; Kleinhofs et al. 2009). The *Rpg1* gene was cloned by a map-based approach (Brueggeman et al. 2002) and validated by haplotype sequencing and stable transformation of a susceptible cultivar, Golden Promise, which was rendered completely resistant (Horvath et al. 2003). *Rpg1* is a novel disease *R* gene encoding a kinase with a pseudokinase domain (pK1) and an active kinase domain (pK2), both of which are required for disease resistance (Nirmala et al. 2006). The RPG1 protein autophosphorylates *in vitro* but its *in vivo* significance is not known. However, failure to autophosphorylate under *in vitro* conditions correlated with lack of RPG1 protein degradation *in vivo* (Nirmala et al. 2007) and disease susceptibility (Nirmala et al. 2006, 2007).

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 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 an early signal needed to initiate a cascade of biochemical events leading to activation of the defense response and disease resistance.

RESULTS

RPG1 protein is phosphorylated within 5 min postinoculation with stem rust race MCCF spores.

To understand how RPG1 perceives the signal from the stem rust fungus, we analyzed the *in vivo* phosphorylation status of RPG1 upon inoculation with the pathogen. Total phosphorylated proteins were precipitated with phosphoserine or phosphothreonine-specific antibodies and tested with RPG1-specific antibodies in a Western blot. To eliminate the possibility that unphosphorylated RPG1 was co-precipitated with phosphorylated proteins, the total proteins were also first immunoprecipitated with the RPG1 antibody and then tested with phosphoserine or phosphothreonine-specific antibodies. In both cases, the results showed that RPG1 was not phosphorylated in the absence of the fungus but became rapidly phosphorylated within only 5 min after the avirulent *P. graminis* f. sp. *tritici* race MCCF spores were delivered on the leaf surface of the resistant cv. Morex (Fig. 1). To eliminate the possibility that lack of the RPG1 protein phosphorylation prior to exposure of the leaf to avirulent rust spores was due to *in vitro* dephosphorylation during extraction, we included phosphatase inhibitors in the protein extraction buffer. The RPG1 phosphorylated state was maintained past 20 h postinoculation (Fig. 1), after which it was degraded in cv. Morex as previously reported (Nirmala et al. 2007). RPG1 was also phosphorylated in the moderately resistant cv. Beacon, highly resistant cvs. Chevron and Q21861 (Fig. 2), and the highly resistant *Rpg1* transgene line (GP/*Rpg1T1*) (Fig. 3). RPG1 was also phosphorylated in two lines that produce the RPG1 protein but are susceptible to stem rust, demonstrating that, although phosphorylation is important for disease resistance, it is not sufficient by itself. The two lines are the *rpr1* mutant (Zhang et al. 2006), which has an intact *Rpg1*, and OSU6 (PBI004-7-0-015 accession no. 8321), a wild barley accession with a GTT insertion in the *Rpg1* gene resulting in an S to R amino acid substitution plus an F insertion (Fig. 2) (Brueggeman et al. 2002; Mirlohi et al. 2008). RPG1 was not phosphorylated when mock inoculated with talc only.

RPG1 is phosphorylated only when plants are inoculated with viable but not with unviable race MCCF urediniospores.

To determine whether the avirulent stem rust urediniospores need to be viable, we tested the ability of both viable and unviable spores of *P. graminis* f. sp. *tritici* race MCCF to elicit RPG1 phosphorylation. The results revealed that RPG1 is specifically phosphorylated only when inoculated with viable spores, determined to be capable of germinating on 2% water agar plates, but not by unviable spores, which failed to germinate on 2% water agar after exposure to room temperature for 3 months. (Fig. 4).

RPG1 is phosphorylated when plants are inoculated with avirulent but not virulent races of the stem rust fungus or nonspecific pathogens.

Urediniospores from avirulent and virulent *P. graminis* f. sp. *tritici* races were used to inoculate leaves of cv. Morex in order to determine whether or not their ability to phosphorylate RPG1 was race specific. RPG1 was rapidly phosphorylated when plants were inoculated with the avirulent races MCCF

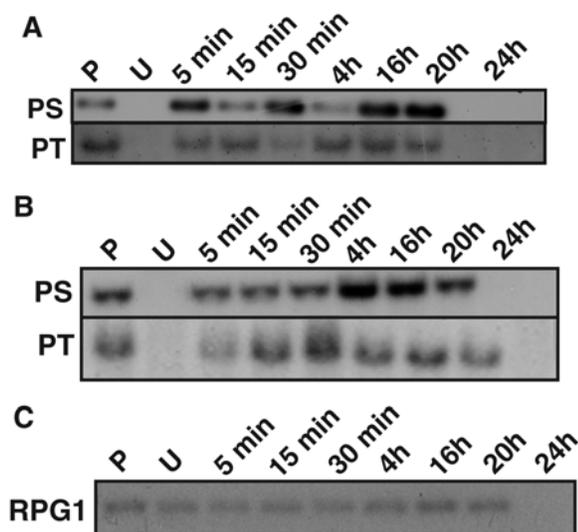


Fig. 1. Western blots showing that the RPG1 protein is phosphorylated within 5 min postinoculation with the avirulent *Puccinia graminis* f. sp. *tritici* race MCCF spores. The RPG1 protein is retained in the phosphorylated state for up to 20 h after inoculation, at which time it is degraded, as we have previously demonstrated (Nirmala et al. 2007). The RPG1 protein is not phosphorylated in the uninoculated sample (U). **A**, Total seedling leaf proteins were precipitated with either phosphoserine or phosphothreonine antibodies and were separated on polyacrylamide gels, blotted, and visualized with an RPG1-specific antibody. **B**, Total seedling leaf proteins were precipitated with an RPG1-specific antibody, processed as above, and visualized with phosphoserine or phosphothreonine antibodies separately. **C**, Total seedling leaf proteins were precipitated with an RPG1-specific antibody, processed as above, and visualized with RPG1-specific antibodies to demonstrate the presence of the RPG1 protein in the extracts. P = *in vitro* phosphorylated RPG1 protein positive control; U = uninoculated control; PS = phosphoserine antibody; PT = phosphothreonine antibody.

(Fig. 1), SCCL-C7a, and HKHJ but not with the virulent *P. graminis* f. sp. *tritici* race QCCJ or a virulent *P. graminis* f. sp. *secalis* (rye stem rust) isolate 92-MN-90 (Fig. 5). Further tests for specificity were conducted with the virulent but not RPG1-specific pathogens *P. striiformis* f. sp. *hordei* (barley stripe rust) and *Cochliobolus sativus*, the spot blotch pathogen. Barley cv. Morex is susceptible to stripe rust but moderately resistant to spot blotch. These pathogens did not elicit phosphorylation of RPG1 (Fig. 5). These results demonstrate that the in vivo phosphorylation of RPG1 protein is a highly specific response initiated between the *Rpg1* gene product and a thus-far-unknown fungal effector or an *AvrRpg1* gene product and not with other *Avr* gene products carried by other stem rust isolates or nonspecific pathogens.

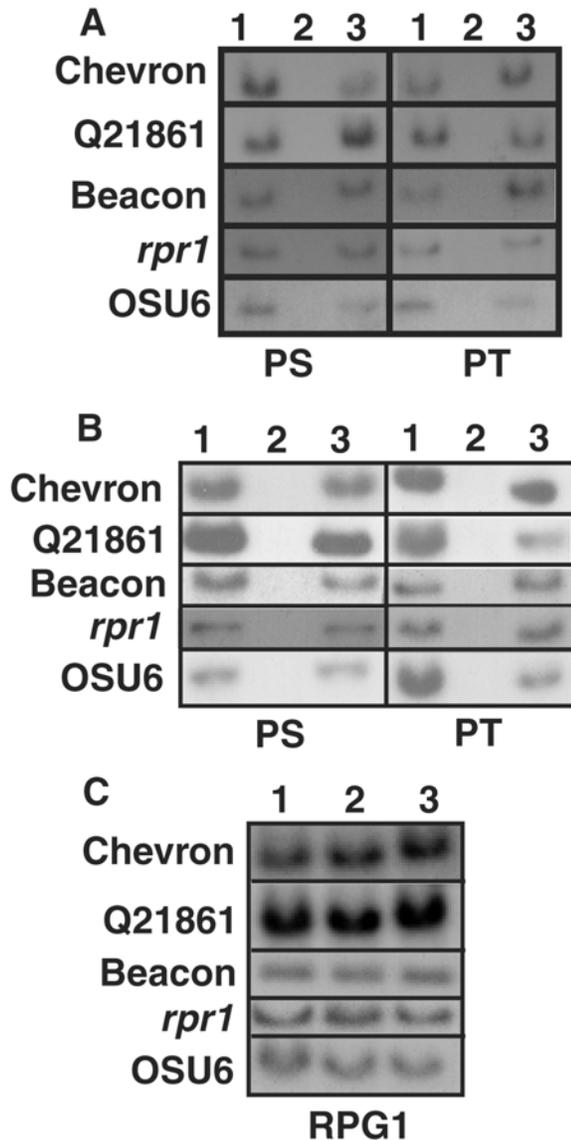


Fig. 2. Western blots showing that the RPG1 protein is phosphorylated postinoculation with the avirulent *Puccinia graminis* f. sp. *tritici* race MCCF spores in *Rpg1*-gene-containing barley cultivars. Total seedling leaf proteins were precipitated with either **A**, phosphoserine or phosphothreonine antibodies or **B** and **C**, RPG1-specific antibodies and were separated on polyacrylamide gels, blotted, and visualized with an RPG1-specific antibody. Bands were visualized with either **A** and **C**, RPG1-specific antibody or **B**, phosphoserine or phosphothreonine antibodies separately. Lane 1, in vitro phosphorylated RPG1 protein positive control; lane 2, uninoculated control; lane 3, 5 min postinoculation. PS = phosphoserine antibody; PT = phosphothreonine antibody.

Phosphorylation of RPG1 is correlated with disease resistance.

To determine whether the in vivo phosphorylation of RPG1 is required for disease resistance, we tested the ability of two different transgenic loss-of-function mutants, *GP/Rpg1*-pK1 (KK152, 153NQ) and *GP/Rpg1*-pK2 (KK461, 462NQ), for their response to inoculation with the avirulent *P. graminis* f. sp. *tritici* race MCCF. The catalytically inactive *GP/Rpg1*-pK2 domain mutant (KK461, 462NQ), incapable of autophosphorylating RPG1 in vitro, also failed to phosphorylate in vivo upon inoculation with rust spores, suggesting that RPG1 autophosphorylation in vivo is required for disease resistance (Fig. 3). The *GP/Rpg1*-pK2 and *GP/Rpg1*-pK1 mutants were previously shown to be susceptible to stem rust and to express the RPG1 protein (Nirmala et al. 2006, 2007). The RPG1 pK1 domain mutant (KK152/153NQ), capable of being phosphorylated in vitro, is phosphorylated in vivo (Fig. 3) but is susceptible to rust, suggesting that this domain functions downstream in the resistance-signaling pathway. To further confirm the role of RPG1 phosphorylation in disease resistance, we tested seven different serine-threonine protein kinase inhibitors for their ability to prevent

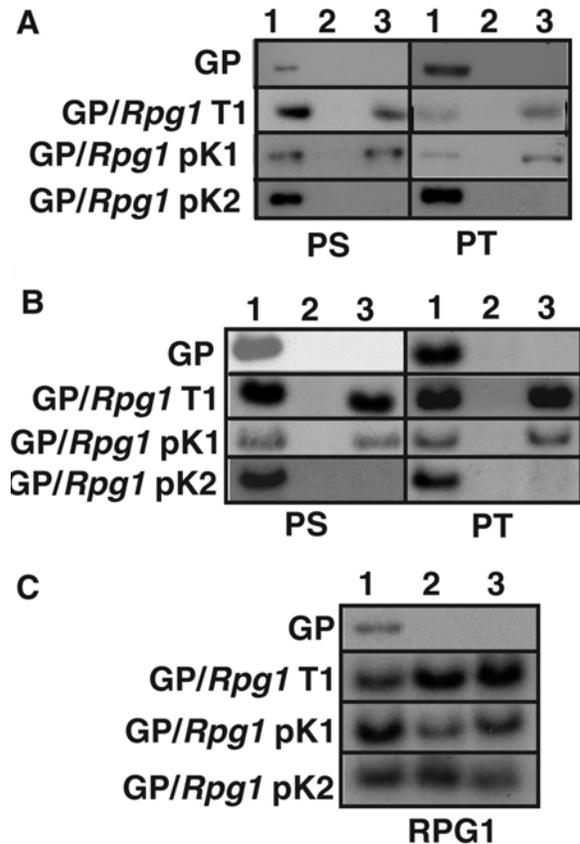


Fig. 3. Western blots showing that RPG1 phosphorylation is dependent on a functional protein kinase 2 domain. The RPG1 protein fails to become phosphorylated in response to inoculation with *Puccinia graminis* f. sp. *tritici* race MCCF in the susceptible transgenic mutant *GP/Rpg1* pK2 (KK461/462NQ) whereas the RPG1 protein produced by the susceptible transgenic mutant *GP/Rpg1* pK1 (KK152/153NQ) and the highly resistant wild-type *Rpg1* transgene *GP/Rpg1*/T1 are phosphorylated. Total seedling leaf proteins either **A**, were precipitated with phosphoserine or phosphothreonine antibodies and the bands visualized with RPG1-specific antibody or **B**, were precipitated with RPG1-specific antibody and the bands visualized with phosphoserine or phosphothreonine antibodies separately or **C**, with RPG1-specific antibodies. Lane 1, in vitro phosphorylated RPG1 protein positive control; lane 2, uninoculated control; lane 3, 15 min postinoculation. Golden Promise (GP) does not have an RPG1 protein and a protein cross-reacting with the RPG1 antibody is not detected. PS = phosphoserine antibody; PT = phosphothreonine antibody.

in vivo phosphorylation of RPG1 and their effect on disease reaction to *P. graminis* f. sp. *tritici* race MCCF on cv. Morex. All seven kinase inhibitors (identified in Figure 6) inhibited phosphorylation of RPG1 and converted the incompatible reaction to a compatible reaction in the resistant cv. Morex (Fig. 6).

DISCUSSION

The barley stem rust resistance protein RPG1 is phosphorylated within 5 min after inoculating the leaves with hydrated spores from avirulent but not virulent stem rust races or non-specific pathogens. The RPG1 phosphorylated state is maintained for 20 h postinoculation, after which it is degraded as previously demonstrated (Nirmala et al. 2007). The speed at which RPG1 is phosphorylated is remarkable because obvious spore germination does not take place until approximately 30 min after they are placed on the leaf surface and provided with free moisture. This very rapid pathogen recognition suggests that it is a primary event that sets the host disease resistance signaling pathway in action. Because the 5-min time frame is well before the rust spores have time to germinate and infect the host cells, we postulate that an unknown effector may already be present on or in the spore, waiting to sense the presence of a suitable host surface. However, metabolic activity seems to be required because unviable spores failed to elicit the phosphorylation response. Research with the flax rust fungus has shown that AVR proteins are synthesized within haustoria and transported into the host cytoplasm, where they interact with the R proteins and activate the downstream signaling cascades (Dodds et al. 2004; Catanzariti et al. 2006). Stem rust appressoria formation and penetration of the stomata takes place approximately 12 to 24 h postinoculation and obvious differences between resistant (*Rpg1*) and susceptible (*rpg1*) cultivars were not observed during this time (Sellam and Wilcoxon 1976) (B. Steffenson, *unpublished*). Thus, *Rpg1*-mediated resistance to stem rust does not become visibly (via microscope) functional until some time after 12 to 24 h postinoculation and may correlate with haustoria formation. Our observations demonstrate that the RPG1 protein recognizes the avirulent spores long before haustoria formation.

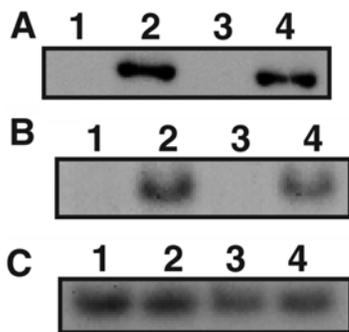


Fig. 4. Western blots showing that RPG1 is phosphorylated only when leaves are inoculated with viable but not with unviable spores of *Puccinia graminis* f. sp. *tritici* race MCCF in cv. Morex. Total seedling leaf proteins were precipitated and were separated on polyacrylamide gels, blotted, and visualized with an RPG1-specific antibody. **A**, Precipitated with either phosphoserine or phosphothreonine antibodies and the bands visualized with RPG1-specific antibody. **B** and **C**, Precipitated with RPG1-specific antibody and the bands visualized with **B**, phosphoserine or phosphothreonine antibodies separately or **C**, RPG1-specific antibodies. Lanes 1 and 3, total proteins immunoprecipitated from the plants inoculated with unviable spores, respectively; lanes 2 and 4: total proteins immunoprecipitated from plants inoculated with viable spores, respectively (all at 30 min postinoculation). Unviable 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.

This recognition leads to RPG1 phosphorylation and disease resistance, as demonstrated by mutant and inhibitor studies described herein. It is possible that a mechanism of resistance different from AVR protein recognition exists or that the specific AVR protein may already be present in the spores and is released into the plant cells and detected by the RPG1 R protein. In the flax rust, *Avr* genes were primarily expressed at the RNA level only in infected tissues, probably in haustoria and the mycelium, except for *AvrM*, where they were also expressed in in-vitro-germinated spores. Among the *Avr* genes analyzed, expression was not detected in ungerminated spores (Dodds et

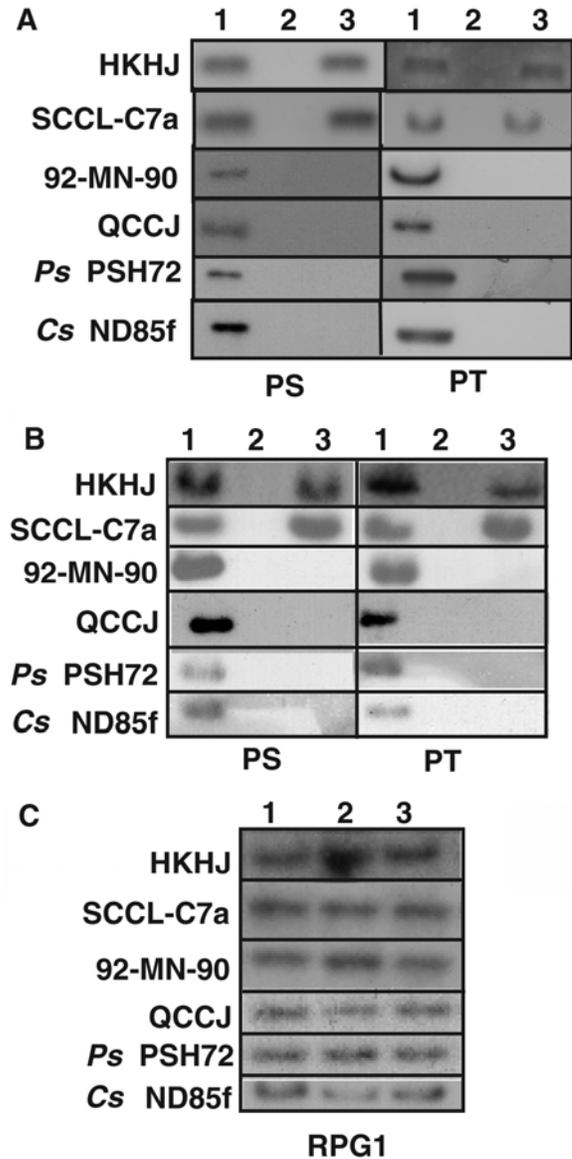


Fig. 5. Western blots showing that RPG1 is phosphorylated upon inoculation with avirulent but not virulent races of the stem rust fungus or nonspecific pathogens in cv. Morex. **A**, Total seedling leaf proteins were precipitated with either phosphoserine or phosphothreonine antibodies and the bands visualized with RPG1-specific antibody or **B**, protein was precipitated with RPG1-specific antibody and the bands visualized with phosphoserine or phosphothreonine antibodies separately or **C**, with RPG1-specific antibodies. Avirulent *Puccinia graminis* f. sp. *tritici* races HKHJ and SCCL-C7a, virulent *P. graminis* f. sp. *secalis* isolate 92-MN-90 and *P. graminis* f. sp. *tritici* race QCCJ, RPG1 nonspecific pathogens barley stripe rust race -*Ps* PSH72, and spot blotch isolate *Cs* ND85f. Lane 1, in vitro phosphorylated RPG1 protein positive control; lane 2, uninoculated control; lane 3, 15 min postinoculation. PS = phosphoserine antibody; PT = phosphothreonine antibody.

al. 2004; Catanzariti et al. 2006). These experiments suggest that there is variation in the expression pattern among the different *Avr* genes and they do not exclude the possibility that stored AVR protein may be present in ungerminated spores.

Rpg1 mRNA and protein are present in the barley leaf epidermal cells at levels considerably higher than those found elsewhere in the leaf (Rostoks et al. 2004) (J. Nirmala, *unpublished*). A small but significant amount of RPG1 protein is also present in the plasma membrane (Nirmala et al. 2006). The plant plasma membrane is expected to be the initial site where elicitor–receptor interaction is most likely to take place; therefore, it is possible that rust elicitors interact or mediate via the plasma-membrane-associated RPG1. The RPG1 protein structure and some functional aspects are strikingly similar to the animal Janus kinases (JAK) (O’Shea and Leonard 1998). JAK family members and their functions are diverse. Briefly, they function via a pathway whereby transmembrane receptors are activated by external effectors which, in turn, activate the JAK. JAK proteins activate transcription factors which migrate to the nucleus and activate the appropriate response genes (Aaronson and Horvath 2002). It would be naive to expect that RPG1 will function in a very similar manner but the JAK pathway does provide a valuable model for testing ideas.

The role of RPG1 phosphorylation in disease resistance is underscored by the observation that the highly susceptible *Rpg1* transgenic pK2 mutant, which encodes an RPG1 protein that

fails to phosphorylate in vitro, also fails to phosphorylate in vivo upon inoculation with the avirulent *P. graminis* f. sp. *tritici* race MCCF. This demonstrates that RPG1 is a very early or initial recipient of the fungal effector, and the observation that the inactive kinase mutant pK2 is not phosphorylated in vivo and does not confer disease resistance strongly suggests that RPG1 auto-phosphorylates and this reaction is required for disease resistance. Additional evidence for the role of phosphorylation in the stem rust resistance-signaling pathway comes from the use of kinase inhibitors (Fig. 6), which most likely inhibit other host kinases besides RPG1 but also prevented the phosphorylation of RPG1 and converted the incompatible reaction to a compatible reaction in the resistant cv. Morex.

The pK2 domain of RPG1 is involved in phosphorylation and disease resistance. However, the specific role of the pK1 domain is not clear, despite its requirement for disease resistance. Two susceptible *Rpg1* pK1 mutants with KK152/153NQ substitutions and a GTT insertion encode RPG1 proteins that are phosphorylated. The *Rpg1* gene with a GTT insertion, encoding a protein with S to R amino acid substitution plus an F insertion (Brueggeman et al. 2002; Mirlohi et al. 2008), is also degraded just like wild-type RPG1 (J. Nirmala, *unpublished*). Thus, although RPG1 phosphorylation and degradation are essential for RPG1-mediated stem rust resistance, it is not sufficient. We speculate that the pK1 domain acts downstream of these events and is perhaps involved in protein–protein interac-

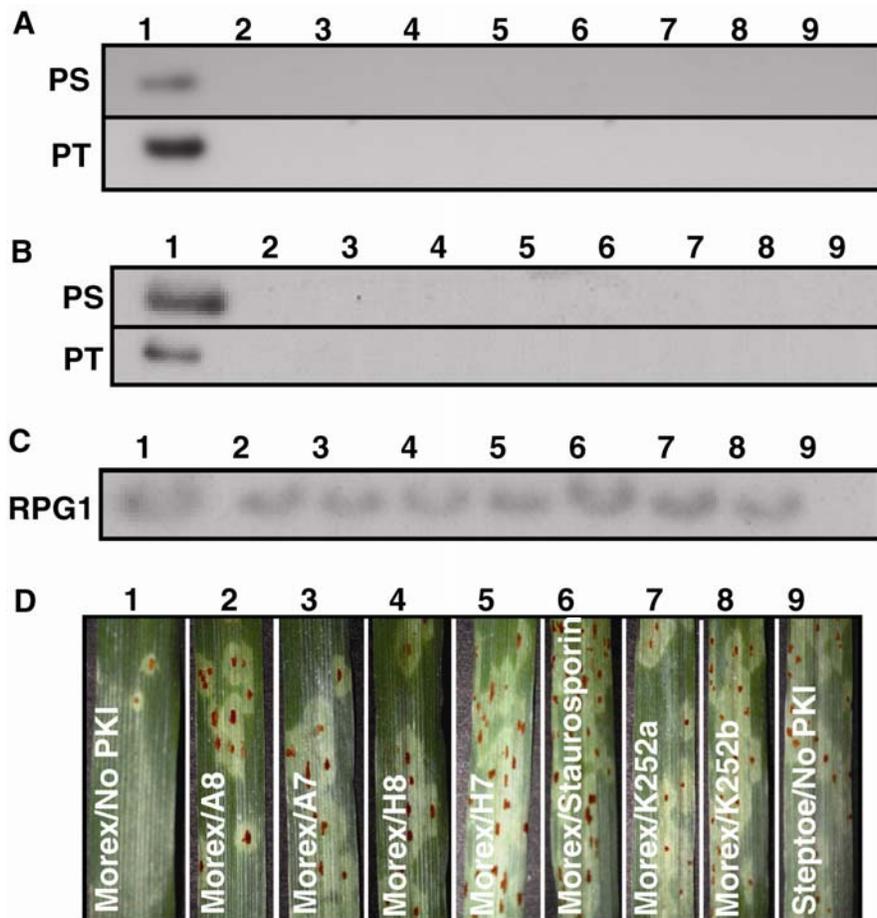


Fig. 6. Seven different protein kinase inhibitors (PKI) prevent phosphorylation of RPG1 and convert the incompatible disease reaction to compatible in *Rpg1*-containing cv. Morex in response to *Puccinia graminis* f. sp. *tritici* race MCCF. **A**, Total seedling leaf proteins were precipitated with either phosphoserine or phosphothreonine antibodies and the Western blot bands visualized with RPG1 specific antibody or **B** and **C**, proteins were precipitated with RPG1 specific antibody and the bands visualized with **B**, phosphoserine or phosphothreonine antibodies separately or **C**, RPG1-specific antibodies. **D**, Disease phenotype. PKI = protein kinase inhibitor. Lanes 1–9: no. PKI, A8, A7, H8, H7, Staurosporin, K252a, K252b, and Steptoe no PKI, respectively. Cv. Steptoe does not produce an RPG1 protein.

tions essential for the disease resistance response. These putative proteins remain to be identified.

Phosphorylation is one of the most widespread post-translational modifications, and multiple cellular processes are regulated by phosphorylation and dephosphorylation (Durek et al. 2010; Grimsrud et al. 2010; Nakagami et al. 2010). However, its role in *R*-gene activation or function has not been widely investigated to date. Rapid and transient protein phosphorylation in parsley and tomato cell suspension cultures has been reported (Dietrich et al. 1990; Felix et al. 1991). Protein kinase inhibitors such as K-252a and staurosporin block these elicitor-induced changes in phosphorylation and prevent the induction of plant defense responses. Protein phosphorylation was also required for early steps in CF9-dependent AVR9-activated defense systems and tobacco–*Tobacco mosaic virus-N* interaction. Known proteins phosphorylated include the MAPK (or orthologues) MPK3 and MPK6, WIPK and SIPK, and tobacco syntaxin NtSyp121 (Zhang and Klessig 1998a; Zhang et al. 1998; Heese et al. 2005). One of the most extensively investigated defense-signaling pathway in plants is the flagellin activated MAPK pathway in *Arabidopsis* (Asai et al. 2002). Flagellin, a highly conserved protein found in bacterial flagella, is recognized as a PAMP by FLS2 (LRR receptor kinase flagellin-sensitive 2) that activates a MAPK cascade conferring resistance to bacterial and fungal pathogens (Asai et al. 2002). FLS2 has also been shown to interact with BAK1 (BRI1-associated receptor kinase), an LRR kinase that regulates the brassinosteroid receptor kinase BRI1 (Nam and Li 2002; Chinchilla et al. 2007). The BAK1/FLS2 interaction is very rapid (within the first minutes of flagellin exposure) and results in enhanced pathogen resistance, presumably through innate immunity (Chinchilla et al. 2007). These observations are of interest to the understanding of the RPG1-mediated pathogen resistance, although the signaling we describe here seems to be very specific to the avirulent stem rust races and not due to PAMPs.

The majority of plant pathogen receptor genes cloned to date are of the NBS-LRR type (Jones and Dangl 2006); however, receptor-like kinases or proteins such as XA21 and CF9 are also known to recognize specific AVR-type molecules and confer resistance (Song et al. 1995; Hammond-Kosack and Jones 1997). The RPG1 protein has similarity to receptor-like protein kinases,

although it lacks any known receptor domain (Brueggeman et al. 2002). This suggests that RPG1 might interact with other receptor molecules at the cell surface; however, at this time, we do not have any evidence for or against that hypothesis.

To summarize, this is the first demonstration of a very rapid and specific R protein phosphorylation upon detection of a pathogenic fungus. The model we currently favor is that RPG1 acts as a receptor or in conjunction with a receptor, perceives the initial signal from the rust pathogen, and phosphorylates itself. This phosphorylation initiates the downstream signaling events, eventually resulting in the degradation or turn over of RPG1 by the proteosomal pathway. The relationship between the phosphorylation and degradation is not clear. It is possible that both function in concert to provide maximum disease resistance. Another possibility is that the degradation of RPG1 might be the end result rather than a prerequisite for the resistance reaction. It is possible that the degradation of RPG1 is to prevent runaway hypersensitive response, as has been suggested in the case of the *Arabidopsis* RPM1 R protein degradation (Boyes et al. 1998).

The mechanism by which the fungus is detected on the leaf surface is under investigation. Presumably, it is a compound which is found on or in the dormant spores and is activated by uptake of moisture or interaction with something on the leaf surface. At this point, we do not have enough information to speculate about this process.

MATERIALS AND METHODS

Plant materials.

Barley lines were grown in growth chambers in plastic pots containing potting mix with a day and night temperature of 21 ± 1 and $18 \pm 1^\circ\text{C}$, respectively, and a 16-h photoperiod provided by cool fluorescent tubes ($525 \mu\text{E}/\text{m}^2$). The cultivars or lines used are described in Table 1.

Stem rust inoculation.

Seven-day-old plants were inoculated with urediniospores of avirulent (MCCF, SCCL-C7a, or HKHJ) or virulent (QCCJ) *P. graminis* f. sp. *tritici* races or with virulent *P. graminis* f. sp. *secalis* isolate 92-MN-90. Controls were grown under the

Table 1. Description of barley cultivars and mutants used in RPG1 phosphorylation assays

Barley genotypes ^b	Stem rust phenotype ^c	RPG1 ^a		
		Protein ^d	Phosphorylation ^e	Degradation ^f
Morex	Resistant	+	+	+
Chevron	Highly resistant	+	+	+
Q21861	Highly resistant	+	+	+
Beacon	Moderately resistant	+	+	+
OSU6	Susceptible	+	+	+
<i>rpr1</i>	Susceptible	+	+	+
GP	Susceptible	NP	NP	NP
GP/ <i>Rpg1</i> -T1	Highly resistant	+	+	+
GP/ <i>Rpg1</i> -pK1	Susceptible	+	+	–
GP/ <i>Rpg1</i> -pK2	Susceptible	+	–	–
Stephoe	Susceptible	NP	NP	NP

^a NP = no protein.

^b Morex, Chevron, Q21861, Beacon, Golden Promise (GP), and Steptoe are barley cultivars and lines. OSU6 (PBI004-7-0-015 accession no. 8321) is a wild barley accession with a GTT insertion in the *Rpg1* gene resulting in an S-R amino acid substitution plus an F insertion (Mirlohi et al. 2008). *rpr1* is a deletion mutant of a gene required for *Rpg1* function (Zhang et al. 2006). GP/*Rpg1*T1 is a single-copy transgenic *Rpg1* line in GP genomic background (Horvath et al. 2003). GP/*Rpg1*pK1 is a pseudokinase domain 1 mutant transgene (KK152/153NQ) in GP background (Nirmala et al. 2006). GP/*Rpg1*pK2 is a kinase domain 2 mutant transgene (KK461/462NQ) in GP background (Nirmala et al. 2006).

^c Phenotypes have been previously determined (Steffenson 1992; Brueggeman et al. 2002; Horvath et al. 2003; Nirmala et al. 2006; Zhang et al. 2006) and were confirmed in this study.

^d Presence or absence of RPG1 protein has been previously determined (Nirmala et al. 2006) and was confirmed in this study. Cv. Steptoe has a defective *Rpg1* gene and does not produce detectable RPG1 protein. GP does not have the *Rpg1* gene and does not produce RPG1.

^e Phosphorylation of RPG1 protein was determined in this study.

^f Degradation of RPG1 protein has been previously determined (Nirmala et al. 2007) (J. Nirmala, unpublished).

same conditions but were mock inoculated only with talc. Plants to be assayed for rust infection were inoculated with the rust urediniospores at a rate of 0.25 mg/plant with talc as a carrier (Sigma-Aldrich, St. Louis) at a 1:20 ratio. After inoculation, the plants were misted and placed in the dark under humid conditions for 22 h. Thereafter, plants were exposed to light and misted periodically. After 4 h at this regime, the misting was stopped and the leaves were left to dry slowly. When the leaf surfaces were completely dry, plants were moved back to the growth chambers at 24°C and 100% relative humidity. Plants were phenotyped 14 days after rust inoculation.

For phosphorylation assays, the plants were inoculated with urediniospores or mock inoculated as above. After inoculation, the plants were lightly misted with water and immediately placed in a dark, humid chamber and timing started. This was done one plant at a time in order to make it as reproducible as possible. The elapsed time between inoculation, misting, and placing the plants in a moist chamber was approximately 30 s. Leaf samples were taken at the indicated time points and immediately frozen in liquid nitrogen. The frozen material in liquid nitrogen was brought to the laboratory and prepared for protein analysis as described below.

Stripe rust inoculation.

Seedlings were inoculated with barley stripe rust (*P. striiformis* f. sp. *hordei*), race PSH-72. Urediniospores were mixed with talc (Sigma-Aldrich) at a 1:20 ratio and spread on the leaves. The inoculated plants were kept in a dew chamber at 10°C in the dark for 24 h and then transferred to a growth chamber at a diurnal temperature cycle gradually changing from 4°C at 2:00 a.m. to 20°C at 2:00 p.m. with a daily 16-h photoperiod.

Spot blotch inoculation.

The second leaves of seedling were inoculated with spot blotch (*C. sativus*) isolate ND85F (100 µl of a suspension of 5,000 conidia/ml) and kept at 100% humidity overnight. The following day, plants were transferred to growth chambers and grown for 7 days prior to disease phenotyping and analysis.

Protein kinase inhibitor assays.

Plants of resistant cv. Morex were treated separately with 5-µM concentration of staurosporin, H7, A7, A8, or H8, or 2.5-µM concentrations of K252a or K252b, all from Sigma-Aldrich. 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 postinoculation and subjected to RPG1 phosphorylation assay or were scored for disease reaction on the 14th day after inoculation.

Phosphoimmunoprecipitation and Western blot analysis.

Approximately 200 µg of leaf tissue was ground in 500 µl of ice-cold extraction buffer (0.5 M sorbitol, 50 mM Tris HCl [pH 7.5], 10 mM MgCl₂, and 1 mM dithiothreitol [DTT]). Cell debris was removed by centrifugation at 15,300 × g for 10 min, and total protein remaining in the supernatant was quantified by a dye-binding assay according to the manufacturer's instructions (Bio-Rad, Hercules, CA, U.S.A.). For phosphoimmunoprecipitation, 500 µg of total protein was combined with 20 µl of either phosphothreonine-specific (mono-anti-phosphothreonine ascites, catalog no. PTHR 12-M) or phosphoserine-specific (mono anti-phosphoserine immunoglobulin G, catalog no. PSER12-M) antibodies (Alpha Diagnostics, Austin, TX, U.S.A.) separately in extraction buffer and 500 µl of 2× immunoprecipitation buffer (1 M KCl, 0.02 M EDTA, and 2 mM PMSF) and rotated end-over-end at 4°C for 12 h. Protein A-agarose (30 µl) (Invitrogen, La Jolla, CA, U.S.A.) was added and incubated on ice for 1 h to precipitate the immuno-

complexes, which were collected at 15,300 × g. Immunocomplexes were washed four times with 1 ml of ice-cold immunoprecipitation buffer, resuspended in 30 µl of Laemmli sample buffer, boiled at 95°C for 3 min, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted to polyvinylidene difluoride (PVDF) membranes and blocked in Tris-buffered saline-Tween (TBST) (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20 [pH 7.5]) containing 10% nonfat dry milk. The blots were reacted with RPG1-specific polyclonal antibodies (Nirmala et al. 2007) for 12 h at room temperature and then with horseradish peroxidase-conjugated secondary antibodies (Alpha Diagnostics). Bands were visualized with the Nu Glo chemiluminescent detection system according to the manufacturer's directions (Alpha Diagnostics).

RPG1 immunoprecipitation and Western blot analysis.

Approximately 200 µg of leaf tissue was ground in 500 µl of ice-cold extraction buffer (0.5 M sorbitol, 50 mM Tris HCl [pH 7.5], 10 mM MgCl₂, and 1 mM DTT). In total, 5 µl of the phosphatase inhibitor cocktail 3 (catalog no. P0044) from Sigma-Aldrich was included in the extraction buffer for every 200 µg of leaf tissue ground. Cell debris was removed by centrifugation at 15,300 × g for 10 min, and total protein remaining in the supernatant was quantified by a dye-binding assay according to the manufacturer's instruction (Bio-Rad). For RPG1 immunoprecipitation, 500 µg of total protein was combined with 30 µl of affinity-purified RPG1 antibody in extraction buffer and 500 µl of 2× immunoprecipitation buffer (1 M KCl, 0.02 M EDTA, and 2 mM PMSF) and rotated end-over-end at 4°C for 12 h. Protein A-agarose (30 µl) (Invitrogen) was added and incubated on ice for 1 h to precipitate the immunocomplexes, which were collected at 15,300 × g. Immunocomplexes were washed four times with 1 ml of ice-cold immunoprecipitation buffer, resuspended in 30 µl of Laemmli sample buffer, boiled at 95°C for 3 min, and analyzed by SDS-PAGE. Proteins were electroblotted to PVDF membranes and blocked in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20 [pH 7.5]) containing 10% nonfat dry milk. The blots were reacted with either phosphoserine-, phosphothreonine- or RPG1-specific antibodies for 12 h at room temperature and then with horseradish peroxidase-conjugated secondary antibodies (Alpha Diagnostics). Bands were visualized with the Nu Glo chemiluminescent detection system according to the manufacturer's directions (Alpha Diagnostics).

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